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***Apodemus* vs. *Eimeria*: Evolutionary factors
of speciation and genomic diversification
in host-parasite system**

PhD. Thesis

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Annotation

This thesis discusses and explains phylogenetic patterns observed in two different organisms: *Eimeria*, an unicellular parasite, and *Apodemus*, a rodent that often serves as a host for this parasitic species. The situation in rodents is intuitive, clearly reflecting their biogeographic history. Phylogenetic pattern in *A. agrarius* corresponds with its spread from the core locality of its distribution eastward. The lack of the genetic variability in European populations hints the recent origin of this population with the low number of founders. The phylogeny of *A. flavicollis*, a rodent inhabiting almost the whole Europe, reflects the situation during the last glacial maximum (i.e. speciation in several subpopulations that did not interbreed, but retained their independent nature).

The situation in *Eimeria* is more complex. Parasites always fight in “arm races”, trying to accommodate to their hosts as best they can, and to avoid their defense. This results in coevolutionary events such as cospeciation, host switches, duplications, and other events that form the genetic variability in parasites. The study of evolutionary relationships in *Eimeria* may be difficult due to lack of morphological and/or relevant molecular data. This thesis adds more information to this view.

Several other studies were also included in this thesis to provide a broader picture of the complexity of host-parasite systems.

Declaration:

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 26.3.2021

Anna Mácová

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List of papers and author's contribution

The thesis is based on the following papers:

Ms 1:

Máková A, Hoblíková A, Hypša V, Stanko M, Martinů J, Kvičarová J, 2018: Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations. Molecular Phylogenetics and Evolution 127: 179-189. doi: 10.1016/j.ympev.2018.05.009. IF: 4,412.

AM was responsible for study design, field sampling, microscopical examination of obtained faecal samples, morphological and morphometrical evaluation of coccidian oocysts, DNA extraction, and molecular techniques. AM also participated in data analyses, interpreting results, and in writing the manuscript. Author's contribution: 70 %.

Ms 2:

Trefančová A, Máková A, Kvičarová J, 2018: Isosporan oocysts in the faeces of bank voles (Myodes glareolus; Arvicolinae, Rodentia): Real parasites, or pseudoparasites? Protist 170: 104-120. doi: 10.1016/j.protis.2018.12.002. IF: 2,702.

AM provided part of the samples, helped with maintaining the rodent breeding facility, and participated in writing the manuscript. Author's contribution: 5 %.

Ms 3:

Holicová T, Sedláček F, Máková A, Vlček J, Robovský J, 2018: New record of Microtus mystacinus in eastern Kazakhstan: phylogeographic consideration. Zookeys 781: 67-80. doi: 10.3897/zookeys.781.25359. IF: 1,079.

AM collected the samples in the field, and performed the DNA extraction, and revised the manuscript. Author's contribution: 10 %.

Ms 4:

Myšková E, Brož M, Fuglei E, Kvičarová J, Mácová A, Sak B, Kváč M, Ditrich O, 2019: Gastrointestinal parasites of arctic foxes (Vulpes lagopus) and sibling voles (Microtus levis) in Spitzbergen, Svalbard. Parasitology Research 118: 3409-3418. doi: 10.1007/s00436-019-06502-8. IF: 1,641.

AM participated in the field sampling, and processed the samples from voles (dissection, microscopic examination), and revised the manuscript. Author's contribution: 5 %.

Ms 5:

Jarquín-Díaz VH, Ballard A, Mácová A, Jost J, Roth von Szepeséla T, Berktold K, Tank S, Kvičarová J, Heitlinger E, 2020: Generalist Eimeria species in rodents: Multilocus analyses indicate inadequate resolution of established markers. Ecology and Evolution 10: 1378-1389. doi: 10.1002/ece3.5992. IF: 2,392

AM participated in design of the project, DNA extraction of part of the samples, testing the primers, and revising the manuscript. Author's contribution: 25 %.

Draft 1:

Trefančová A, Kvičarová J, Mácová A, Stanko M, Hofmannová L, Hypša V, 2021: Switch, disperse, repeat: host specificity is highly flexible in rodent-associated Eimeria. Submitted.

AM participated in the field sampling, microscopical processing of the samples, DNA extraction, and PCR. AM also processed part of the sequences, and participated in writing the manuscript. Author's contribution: 10 %.

Draft 2:

Máková A, Hrdlička R, Kvičerová J: Phylogenetic relationship of coccidian parasites (Eimeriorina, Apicomplexa) infecting shrews: another piece to puzzle. Prepared for submission.

AM was responsible for the field work, processing of the samples (dissections, microscopical examination), molecular techniques (DNA extraction, PCR), data analyses, interpreting results, and writing the draft. Author's contribution: 75 %.

Declaration of author's contributions

I hereby declare that my author's contribution, which I state below (for Ms1, and Draft2), are true.

In České Budějovice, date: 26/3/2021


Anna Máčová

I hereby declare that I agree with the author's contribution of Anna Máčová in the manuscripts/drafts listed below.



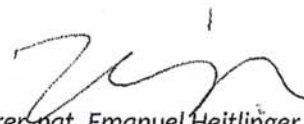
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1. Introduction

Parasites are important part of the biological tree, and form a very complex system full of relationships, dependencies, and adaptations. Hosts and their parasites coexist in various types of relationships; some of the parasites are „promiscuous“, infecting more host species (or even genera or families), while others may be strictly host-specific, limited only to a single host species. Parasites usually do not intend to kill their hosts; however, overparasitising of the host may lead to the loss of parasite’s nutrient source, the loss of site of the development or the entire environment, or even to the death of the host. At the first sight, it is a fragile system full of violence and exploitation, however, it keeps the balance, uses empty niches, and pushes the evolution forward (Thompson, 2005; Sorci and Garnier, 2019).

The main goal of the parasite is to feed on the host and to reproduce. The host responses via adaptations and modifications that help it to avoid from being parasitized (e.g. an animal host has a thicker skin to resist the biting insect). However, the parasite may also adapt to the new conditions, and may find the way how to still parasitize the host (e.g. to develop a sharper stylet to penetrate even the thicker host skin). Mechanisms have been developed to maintain these traits to a reasonable extent and to disadvantage further adaptations (e.g. making skin thick would be energy-intensive), so they do not lead to extremes (van Valen, 1973).

Various studies have been performed on host-parasite systems that point to the diversity of these relationships. For example, rodents (e.g. the genus *Apodemus* used in this thesis) can be infected with various parasites: from fungi as *Pneumocystis* (Demanche et al., 2015), through protozoan parasites as coccidia or *Cryptosporidium* (Nowell and Higgs, 1989; Higgs and Nowell, 1991; Hůrková et al., 2005, Čondlová et al. 2018), multicellular parasites as nematodes or cestodes (Prokopič, 1967; Callejón et al., 2010), to ectoparasites as lice or mites (Štefka and Hypša, 2008; Kaminskienė et al., 2020). From “accidental” infestation by ticks or mites to a very specific tight bond to a certain species, or even to a single genetic lineage of that species (Martinů et al., 2020).

The host-parasite relationships can be studied from the evolutionary point of view. Behind the observed phenomenon, there may be several different

scenarios that may explain the incongruences between the host and parasite phylogenies. When the cospeciation occurs, the phylogeny of host corresponds with the phylogeny of parasite, so, they speciate together. However, discrepancies in phylogenetic trees occur much more often. A new lineage of the currently existing parasite may appear on a single host (=duplication), or the host may speciate but the parasite remains on the former host lineage. This process is called “missing the boat”, and can be observed mainly in populations with less abundant parasites (=sorting event). Host switching means a situation when a parasite begins to colonize a new host species (“switches” to a new host species).

Several drivers of evolution have been described and are intensively studied – mutations, drift, gene flow, and selection. Mutations are the main source of changes and variation. They occur in every living creature, causing changes that can benefit, or disadvantage their bearers. Genetic drift is a random change in allele frequencies in following generations. It is connected with sexual reproduction, founder effect, or bottleneck. Gene flow very often corresponds to migration and allele exchange between populations. Selection favors one genotype over another (Fisher, 1958; Mayr, 1963; Hamilton, 1967; Kimura, 1983).

All these factors form the world as we know, and give the potential for the evolution of organisms into forms that we do not yet know. They help the organisms to adapt to new conditions, or to colonize new areas.

In my Ph.D. thesis, I focused on elucidation of the evolutionary history of intestinal coccidian parasites infecting small mammals, more specifically, eimerians parasitizing rodents of the genus *Apodemus*. These rodents are suitable model organisms - they are common, widely distributed, and easy to trap. Eimerians, obligate intracellular endoparasites infecting their gastrointestinal tracts, may represent a good marker to mirror the host evolution. I tried to obtain samples from different parts of Europe (and two Asian countries) to reveal the differences between populations inhabiting various localities.

2. Studied host-parasite system

2.1. *Apodemus*

Rodents of the genus *Apodemus* (Muridae: Murinae), common agricultural pests, are distributed across the whole Eurasia, and partly also in the northern Africa (Mitchell-Jones et al., 1999; Lalis et al., 2016). It is an old genus of murids (in comparison with e.g. *Mus* and *Rattus*) - its first members colonized Europe already in the Miocene (Martin Suárez and Mein, 2004; Suzuki et al., 2003).

The core area of the occurrence of this genus is in Asia (Serizawa et al., 2000; Suzuki et al., 2003). From there it spread to other parts of Palaearctic and Oriental regions. The genus *Apodemus* consists of two main subgenera: *Apodemus*, and *Sylvaemus*, with the unclear position of *A. argenteus*, *A. mystacinus*, and *A. gurkha*, which are also by some authors considered as subgenera (Musser et al., 1996; Serizawa et al., 2000; Filippucci et al., 2002). The *Apodemus/Sylvaemus/gurkha* group diverged 7-8 Mya (million years ago), during the time of variable climate where forests were replaced by open habitats. From this group, the *Apodemus* separated 5.4-6.0 Mya, and the *Sylvaemus* and *mystacinus* split 2.2-3.5 Mya (Michaux et al., 2002). This indicates that European species are younger than those from Asia, which supports the origin of this genus in the East (Serizawa et al., 2000). The whole genus comprises around 30 species, and its species diversity grows eastward (Orlov et al., 1996).

Three species are widely distributed in Europe: *A. agrarius* (belonging to the subgenus *Apodemus*), *A. flavicollis*, and *A. sylvaticus* (both the subgenus *Sylvaemus*). The western part of Europe is inhabited mainly by *A. sylvaticus*, in central Europe mixed with *A. flavicollis*, which prevails eastward, where is the meeting zone with *A. agrarius*.

A. flavicollis and *A. sylvaticus* often share the most of their area of occurrence. It is difficult to distinguish them in the field because they are morphologically very similar. The most often used morphological trait is the length of the hind leg, however, the ranges of values of both species may overlap (Anděra and Horáček, 2005; Jojić et al., 2014). Another morphological traits are cranial markers (Barčiová and Macholán, 2009). The possibility of hybridization of these two species has been also discussed – whether for or against (Engel et al., 1973), and has been confirmed only under laboratory conditions (Larina, 1961).

An overview of these studies was performed by Fillipucci et al. (2002), inferring that the hybridization is not very likely.

The genetic structure of these two *Apodemus* species reflects the geographical contours of Europe with their most important mountain ranges, which served as distribution barriers during the glacier maxima. During glacial times, rodent colonies were forced to move southward where the weather conditions were milder. They lived there in separate communities, which were separated by forests, mountains, or other natural barriers, and they allopatrically speciated there (Serizawa et al., 2000; Suzuki et al., 2003). As we see from its distribution, *A. sylvaticus* spent the last glacial maximum in two main refugia – in Hispania separated by the Pyrenees, and on Appenine peninsula behind the Alps (Michaux et al., 2003; Demanche et al., 2015). The Italian refugium was also used by *A. flavicollis*, whose main refugium was in the Balkans, and partly also in the Near East (Hewitt, 1999; Filippucci et al, 2002; Michaux et al., 2004). Two main lineages were described in *A. flavicollis* – the Euro-Russian, and the Near East+Turkey. The Euro-Russian lineage consists of three sublineages: 1) Balkans and northern Europe, 2) Balkans and southern Russia, and 3) western Palearctic (Michaux et al., 2003; 2004; 2005). Several studies suggest that the core refugium was formed by three sites in the Balkans from where *A. flavicollis* colonized Europe (Taberlet et al., 1998; Hewitt, 1999).

The genetic structure of *A. agrarius* is highly uniform in Europe due to its intriguing biogeography. The core locality of its occurrence is in the Far East, in China. However, the whole European population of this species is supposed to origin from only few immigrants coming from eastern Russian area in the last glacial era (Latinne et al., 2020). The Chinese origin of the European population is not very likely because of the barriers (Himalayas, Tibetan Plateau, Tian Shan, Altai). These populations became isolated due to the secondary gap in the Baikal area (caused by the change of the climate to more dry, cold and windier – Duan et al., 2009), so there was a restricted gene flow from the original population (Sakka et al., 2010). In the last glacial in Europe, *A. agrarius* widely spread even in areas from which it later disappeared due to changes of habitats, e.g. in France (Aguilar et al., 2008).

From the geographic point of view, the European distribution of these 3 species partially overlaps, however, they usually do not live in absolute sympatry due to different habitat preferences (Anděra and Horáček, 2005). Studies of genetic

differences within a species have revealed inner diversification, demonstrated by high values of F_{st} in *A. flavicollis* or *A. sylvaticus* in Europe (Filippucci et al., 2002). However, species in the subgenus *Sylvaemus* evince low genetic divergence, apparently as a result of rapid radiation (Filippucci et al., 2002; Bellinvia et al., 2004).

Studies on rodents are important because of their irreplaceable role in ecosystems. They are part of food chains, serving as food for birds of prey, owls, or carnivores. On the other hand, they also help with the distribution of seeds, and by digging them, they unintentionally help the seeds to germinate better. They also serve as reservoirs or vectors of diseases that limit their own populations or populations of other animals. They represent a suitable model for evolutionary studies due to their high mutation rates in some genera/species – rodents are the fastest developing group within mammals (Nabholz et al., 2008).

Two comprehensive studies concerning phylogeny of *A. agrarius* have been performed recently. One was done on *cyt b* and microsatellites (Latinne et al., 2020). The authors revealed that eastern populations are more heterogeneous than the western (which is in concordance with publications of Kartavtseva and Pavlenko, 2000; Atopkin et al., 2007; Sakka et al., 2010), which hints on the recent expansion to west Palearctic. It can be proved by Spitzenberger and Engelberger (2014) who documented the first occurrence of this species in Austria in 1996 and monitored its expansion in this country. The estimated effective size of the eastern population was three times higher than the western one, which also corresponds with the recent divergence and expansion in this area. The low nucleotide diversity, allelic richness, and heterozygosity in western Palearctic also prove this hypothesis (Latinne et al., 2020). The second study (Cerezo et al., 2020) used more informative approaches (ddRAD sequencing), which, however, also proved these findings.

2.2. *Eimeria*

Eimeria (Apicomplexa: Coccidia: Eimeriorina: Eimeriidae) are obligatory intracellular parasitic protists of digestive tract of various hosts. More than 1,500 species have been described (Dubey, 2019). This parasite can be found e.g. in birds (Upton et al., 1990), marsupials (Duszynski, 2016), mammals

(including rodents, lagomorphs, bats, carnivores, ungulates, and also humans) (Wilber et al., 1998; Duszynski, 2002; Duszynski and Couch, 2013), reptiles (Asmundson et al., 2006; Duszynski and Morrow, 2014), or amphibians (Duszynski et al, 2007).

These parasites can be “harmless”, causing almost no problems to their hosts, however, several species are able to heavily invade host tissues, leading to deaths and great economic losses (Beattie et al., 2001), e.g. in poultry industry. The hosts get infected by ingestion of sporulated (i.e. fully developed and matured) oocysts. In their digestive tract, the sporozoites are released from the oocysts, and invade the host tissues. First, several generations of merogony (asexual reproduction) occur intracellularly, which leads into more cells affected with coccidia. When merogony ends, gamogony (sexual reproduction) occurs producing unsporulated (i.e. immature) oocysts that leave the host with faeces, prepared to infect the new host individual.

The above mentioned stages of life cycle are different in different species. Although endogenous development has not yet been fully described in many species, we can see the differences. Different species develop in distinct parts of the digestive tract (e.g. duodenum, jejunum, ileum, caecum), there is a certain number of merogony generations, variability in the length of prepatent and patent periods, and of sporulation time (Joyner and Long, 1974; Soekardono et al, 1975; Oda and Nishida, 1991).

“Higher eimerians” (Eimeriorina) differ from “lower eimerians” (Adeleorina) in the absence of syzygy (pairing of two individuals forming a gametocyst) in their life cycle. Individual genera of Eimeriorina are described and recognized based on the number of sporocysts in the oocyst, and number of sporozoites in the sporocyst. On the species level, eimerians differ in morphological traits of sporulated oocysts, such as character of the oocyst wall (number of layers, colour, thickness), shape (e.g. subspherical, ovoidal, oval, ellipsoidal, pyriform) and size of the oocyst, and presence/absence of outer/inner structures (e.g. micropyle, granules, residua).

Another trait, considered as a marker for distinguishing the species of *Eimeria*, is the identity of the host species and the range of the host specificity. Eimerians have long been considered strictly host-specific, which means that certain species can be found only in certain hosts (Joyner and Long, 1974;

Joyner, 1982). Later, it turned out that this was not the rule, at least for some of them (de Vos, 1970; Hůrková et al., 2005; Ghimire, 2010; Kvičerová and Hypša, 2013; Hůrková Hofmannová et al., 2016; MS 1, MS 5, Draft 1).

Eimerians have been studied from various points of view. The very first studies dealt only with morphology and taxonomy (Pellérdy, 1974; Levine, 1982), then the veterinary science focused on their pathogenicity and life cycles (e.g. Kreier and Baker, 1987; Beattie et al., 2001; Pakandl et al., 2009), and the current science focuses on molecular traits, phylogenetic relationships, NGS sequencing, and population-genetics (e.g. Zhao and Duszynski, 2001a; Morrison et al., 2004; Vermeulen et al., 2016; Su et al., 2017; MS 1, MS 5, Draft 1). They are most studied in poultry, which is in concordance with their great pathogenic potential for these hosts; their importance is also indicated by the fact that for all 7 most pathogenic species the whole genomes are sequenced (Blake et al., 2020). From the phylogenetic point of view, eimerians form a complex group. The genus *Eimeria* is paraphyletic; other genera, such as *Cyclospora* or some *Isospora*, cluster inside it. Eimerians tend to form host-specific phylogenetic clusters. This was disproved recently, at least for rodents (MS 1, MS 2, Draft 1). The similar situation as in rodents is also in Eulipotyphla (Draft 2). In birds, eimerians form at least family-specific clusters (Ogedengbe et al., 2018). The situation in other groups and/or other taxa of coccidia is not very clear, due to little research interest and low amount of data.

3. Objectives

The main goal of this study was to elucidate phylogeny of the common coccidian parasite – *Eimeria*, infecting abundant rodent of the genus *Apodemus*, and to compare the observed patterns with the situation in different host groups. The research included field sampling of rodents in various European countries (and two countries in Asia), microscopical examination, DNA extraction, molecular techniques, and data processing by bioinformatic tools.

The specific objectives are as follows:

- To analyze evolutionary relationships between coccidia of the genus *Eimeria* infecting rodents of the genus *Apodemus* – characterization of populations, demographic history, variability of populations.
- To reveal the timing and separation of historic events (origin of lineages in different refugia) in comparison with recent events (speciation, hybridization, etc.).
- To characterize detected coccidian species at the morphological and molecular level.
- To obtain samples from different (including so far unsampled) localities in Europe, with the main focus on *A. agrarius* and parasites associated with this host species.
- To examine coccidia found in other host taxa during the sampling (Arvicolidae, Soricidae), to analyze their phylogenetic relationships and host specificity, and to compare the results with those revealed in *Apodemus*.

4. Methods

The author of this thesis participated in the majority of field sampling, which resulted in data analyzed in these manuscripts. The sampling was performed at a variety of locations in Europe, and also in two Asian countries. Detailed information on localities and/or maps can be found in the publications below.

Rodents were trapped using the Sherman small animal live traps, or standard wooden snap traps, under official permits (numbers KUJCK 11134/2010 OZZL/2/Ou, and 27873/ENV/11). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia and by the Ministry of the Environment of the Czech Republic (numbers PP 42/2006, 13841-11, and 22395/2014-MZE-17214). Insectivores were accidentally found dead on paths and roads in the course of our research. The animals were identified based on morphological features, however, their tissue samples were also collected for further molecular analyses. Faeces and part of the intestine were collected from each trapped animal and preserved individually in 4% potassium dichromate ($K_2Cr_2O_7$) solution for analyses of endoparasites. The samples were processed by the centrifugation-flotation concentration method with Sheather's sucrose solution (with a density of 1.30), and observed and measured using the light microscopy (Duszynski and Wilber, 1997; Zajac and Conboy, 2006).

DNA was extracted from the tissue samples using the Blood and Tissue Kit (Qiagen). DNA from the faecal samples was isolated using the FastDNA © Spin for Soil Kit (MP Biomedicals), or the NucleoSpin© Soil kit (MACHEREY-NAGEL).

In the majority of our publications, we used the Sanger sequencing with several different primer sets, according to the studied organisms. The studied genes were cytochrome *b* (for rodents), cytochrome *c* oxidase I and III, and nuclear gene 18S rDNA (for coccidia). Primer sequences for each respective gene can be found in the below mentioned manuscripts and drafts.

Another approach was the use of the Access Array system (MS 5). A combination of 48 primers allows for the deeper insight into the topic. In addition to the primers used for Sanger sequencing, we also used the primers for the plastid gene encoding the open reading frame ORF 470.

The sequences obtained by Sanger sequencing were processed in SequenceScanner, EditSeq, and SeqMan (DNASTAR). Alignments were created by ClustalW algorithm in the programs BioEdit, or MUSCLE in Geneious, and phylogenetic relationships were computed in MrBayes and PhymI.

5. Results and Discussion

The main focus of my PhD study was to investigate the interspecific and intraspecific phylogenetic relationships of eimerians infecting rodents of the genus *Apodemus*. Together with my colleagues from the laboratory, we collected data across 13 European countries during 2006-2014. Finally, we obtained 165 COI and 74 18S rDNA sequences of *Eimeria* spp. from 1,515 examined individuals of the genus *Apodemus*. The total prevalence of *Eimeria* was 33 %. Phylogenetic analyses of both studied genes, published in **MS 1**, showed that eimerians infecting this rodent genus formed lineages with various degrees of host specificity. We named the resulting lineages according to their oocyst morphology as *apionodes* (api), *alorani* (alor), *jerfinica* (jerf), *kaunensis* (kaun), and *uptoni* (upt). The *alorani* and *kaunensis* lineages/morphotypes were located inside the *apionodes* supercluster (MS 1, Fig. 2). *Jerfinica* and *kaunensis* were strictly *Apodemus*-specific; *jerfinica* formed two sublineages, one from the *A. flavicollis* (AF) and *A. sylvaticus* (AS) hosts, and the second contained samples only from *A. agrarius* (AA) hosts. The *apionodes* supercluster had an intriguing composition. The basal group (which we called *apionodes* I) was Arvicolinae-specific. *Kaunensis* and *apionodes* III possessed wide host specificity, infecting both arvicoline and *Apodemus* hosts. Two sublineages, *apionodes* II and *apionodes* IV, had narrower host range, infecting only the genus *Apodemus* (3 eimerians from bank voles, clustering in *apionodes* IV, later proved to be mistaken/misclassified). The most strict host specificity was detected in the *alorani* lineage, which infected only *A. agrarius* (MS 1, Figs. 2, 5). From the calculation of the relative age of these lineages we revealed that the more recent = the more host-specific group (MS 1, Fig. 4). This suggested the shift from broad to strict host specificity, and recent switches from AF/AS to AA. The GammaST data showed a great genetic difference of AA in comparison with AF and AS (MS 1, Fig. 4). Therefore, this change of host was not just a mere colonization of the new host, but a total switch with the abandonment of the former host. Due to intensive sampling, we recorded a low number of missing haplotypes (MS 1. Figs. 5, S4), so we can assume that our data cover most of the possible genetic diversity.

Later, we extended this study by adding more samples from Arvicolid rodents (**Draft 1**) to see the more complex picture of *Eimeria* spp. of European rodents. The results of analyses of COI and 18S rDNA were congruent with the pattern observed in MS 1. Besides the arvicolid-specific group api I, eimerians infecting exclusively voles also formed a separate lineage, morphologically congruent with *E. coahuiliensis*. Lineages containing eimerians infecting both voles and *Apodemus* (kaun and api III) evinced an

inner structure forming sublineages that separated eimerians infecting these distinct hosts (Draft 1, Fig. 1, 3). The division within the *kaunensis* lineage was interesting also from the morphological point of view; the sublineage containing samples from *A. flavicollis* and *A. sylvaticus* possessed oocyst residuum (OR), an important morphological trait of the sporulated oocyst, which was formerly considered taxonomically or even phylogenetically important feature (Reduker et al., 1987; Zhao and Duszynski, 2001b). However, the second sublineage, infecting exclusively arvicolid voles, was composed of very similar oocysts (morphologically corresponding to *E. kaunensis*), but lacking the OR (which is in discordance with the original description of this species by Arnastauskiene et al., 1978). We recorded a low genetic diversity inside the lineages within the *apionodes* supercluster. The most extreme example was api IV, which despite the high number of sequences included, consisted of a single haplotype. Low genetic diversity may indicate a recent origin of these lineages, which could be the result of rapid and repeated host switches.

The same pattern (i.e. the formation of the host-specific clusters) was observed in eimerians infecting some members of Eulipotyphla (**Draft 2**). We obtained eimerian samples from *Sorex* sp., *Crociodura* sp., *Neomys* sp., and *Suncus murinus*, from which we obtained around 20 DNA sequences, each for the COI, COIII and 18S rDNA genes, which were analyzed both separately and in a concatenated analysis (cytochrome genes). Majority of eimerians from *Crociodura* sp. formed two separate clusters (Croc1 and Croci2) with different position on phylogenetic tree. The cluster Croci1 contained also eimerians from *S. murinus*, member of the same subfamily, Crocidurinae. Eimerians from the subfamily Soricinae also formed host-specific clusters. An interesting finding was the presence of a cluster which we called “mix”, containing eimerians originated from all observed species (except of *Suncus*). This pattern was not an artifact, because this cluster was conserved in analyses of all studied genes. Two samples of *Isospora*, included in the analyses, grouped with isosporans from moles (Draft 2, Figs. 3, 6). We were able to obtain photomicrographs of the majority of samples, however, their assignment to the already described species was difficult due to the lacks in the descriptions of the extant species. Several clusters consisted of morphologically similar oocysts (e.g. the clusters Croci1 and Croci2, despite being placed on different branches in the phylogenetic tree, were similar in several traits) (Draft 2, Figs. 2-6). The main problem is the very low number of eimerians found in shrews so far, their insufficient description, and the absence of molecular data.

In the course of these studies, isosporans infecting voles whose phylogenetic position did not meet our expectations, were closely related to isosporans infecting birds (**MS 2**). We aimed to elucidate this phenomenon by more complex analyses, including morphology and phylogeny, complemented with experimental infections of voles. The history of the genus *Isospora* is intriguing; currently, it is considered polyphyletic, and divided into two genera: i) *Isospora* (Eimeriidae), possessing a Stieda body (SB), infecting birds and reptiles, and ii) *Cystoisospora* (Sarcocystidae), without SB, infecting mammals (Carreno et al., 1998; Franzen et al., 2000; Barta et al., 2005; Samarasinghe et al., 2008). The isosporans detected in our samples from voles possessed a SB, which indicated that they belonged to the genus *Isospora* (MS 2, Figs 1-5). Molecular analyses of three genes (COI, COIII, and 18S rRNA) revealed their close relationship with bird isosporans (which is in concordance of the usual host range of this species) (MS 2, Figs. 6-8). Experimental infection of several voles with these oocysts was not successful, the rodents did not produce new generations of parasites (i.e. did not discharge oocysts in faeces). Only the first few days, they excreted deformed or damaged oocysts – those that were used for inoculation but did not invade the intestinal cells. All three approaches – morphological, molecular, and experimental - proved that these isosporans were not real parasites of voles, but pseudoparasites acquired from food. We can assume that among other isosporans described from rodents, some will also be pseudoparasites; unfortunately, these isosporans were described only morphologically, therefore, with the lack of molecular data, we cannot make any certain conclusion.

One of the pleasures of science is the discovery of something “by chance”, as a by-product of other research. During our trip to Kazakhstan, where we collected samples for different project, we also collected samples from *Microtus mystacinus* (syn. *M. levis*). Later, it turned out that this rodent species is a new record from the locality where it had not been found before (**MS 3**). The former area of the occurrence of this species in Kazakhstan was the western and northwestern part of the country. Our samples originated from Sekisovka (almost the easternmost part), and Dzhambul/Taraz (southern part of the country). Phylogenetic analyses based on cyt *b* evinced a genetic difference of these new samples compared to other sequences of this species in the GenBank database (MS 3, Fig. 1). This may suggest the natural introduction into new localities. The proposed colonization route from the glacial refugia was probably from the Balkans, or from the Black or Caspian Sea region. Our finding provides the first genotyping of *M. mystacinus* from the eastern part of its

distribution. It is just a piece of a complex mosaic that is worth further, more complex and targeted research.

Sometimes it happens that we are looking for something where it is not present. It was the case of **MS 4**, where we were looking for the gastrointestinal parasites of polar foxes and sibling voles on Svalbard. While we found parasites in foxes (nematodes, coccidia, and microsporidia), surprisingly, parasites were absent in voles. A single parasite, found in one individual of *M. mystacinus*, was the new genotype of *Cryptosporidium* (which was the first record in voles on Svalbard, but was not present in foxes). It is normal that the top predators are infected with parasites that they obtain from prey. On Svalbard, it is then obvious that the voles are not the source of infection. The main part of a fox's diet is carcasses of reindeer, ptarmigans, or other birds and their eggs, while voles are only marginal. In the past, a really interesting phenomenon was the presence of *Echinococcus multilocularis* both in foxes and in voles, which is in contrast with our findings. This can be explained by different localities of sampling or by seasonality.

To obtain more complex data on phylogeny of the studied organism/s, it is worth to use the next generation sequencing (NGS), or its combination with the Sanger sequencing (Metzker, 2009). In **MS 5**, we analyzed eimerian parasites infecting *Mus musculus* and *Apodemus* spp. using the Sanger sequencing of three genes (18S rRNA, COI, and ORF 470), and compared the results with a multilocus approach using the Access Array. This platform allows sequencing of 48 samples against 48 primers, which generates a large amount of data for a complex picture of phylogenetic position and identity of the samples (Anderson et al., 2010). Phylogenetic analyses of sequences of the three genes produced more or less congruent topologies (MS 5, Figs. 2 - 4); the sequences of eimerians from *M. musculus* formed three main clusters, corresponding to the species *E. falciformis*, *E. ferrisi*, and *E. vermiformis*. Sequences from other host species from various families, however, clustered also inside these three clusters (MS 5, Figs. 2 - 4). A haplotype network composed of 161 COI sequences formed 20 different haplotypes (MS 5, Fig. 5), indicating low genetic diversity. Multilocus analyses performed on a small subset of 31 individuals showed a similar pattern. The sequences formed three main clusters: i) *E. falciformis*, ii) *E. vermiformis*, and iii) eimerians from *A. agrarius* (MS 5, Figs. 6, 7).

We compared the results obtained from each of the studied genes together with the results of multilocus analysis. We proved the traditional finding that the 18S rRNA gene is good for inferring phylogeny in deeper nodes, but it is not informative enough

for higher resolution (Zhao and Duszynski, 2001a; Kvičerová et al., 2008; Ogedengbe et al., 2018). More surprising, however, was the finding that a similar lack of information was also detected in the COI gene, which was commonly used as the gene of first choice; several eimerians from different host species had identical sequences/haplotypes (e.g. haplotype II infecting *A. flavicollis*, *A. sylvaticus*, *C. glareolus*, and *M. musculus*), indicating an insufficient resolution of this gene. Finally, it turned out that the most informative gene was ORF 470, which has been recommended in the literature before (Zhao and Duszynski, 2001b; Ogedengbe et al., 2015). However, the majority of studies have been performed based on COI, therefore, there are not many ORF 470 sequences of coccidia in the GenBank database. The sequences obtained in the course of our research partially filled this gap, and we highly recommend to follow this approach and to use the apicoplast genes for studies on eimerians and other coccidia.

6. Summary

The dissertation summarizes the outputs of several articles and drafts that were authored or co-authored by Anna Mácová and that focused on coccidian species infecting various rodent hosts, their interspecific and intraspecific phylogenetic relationships, and host specificity. At the parasite level, we found that rodent eimerians are not so strictly host-specific as it was previously believed. Based on analyses of 18S rDNA and COI, they form clusters ranging from the oldest groups with wider host specificity to the youngest branches of eimerians with strict specificity (represented e.g. by *E. alorani* infecting only *A. agrarius*). The gammaST values, and the calculated age of this lineage indicate a recent host switch. Therefore, we can consider eimerians as flexible parasites, capable of using new hosts that colonize new areas. This pattern, i.e. the formation of host-specific clusters, can be observed also in eimerians infecting the subfamily Arvicolinae, but also in Soricidae that belong to different order, Eulipotyphla.

The Stockholm paradigm is a concept that aims to explain the discrepancies in host specificity, when some species are strictly host-specific, however, their sister species may infect a wide variety of hosts. This concept works with four ecological theories: ecological fitting, the geographic mosaic theory of coevolution, taxon pulses, and the oscillation hypothesis. The basic idea is that many of pathogens and/or parasites have the potential to infect new hosts, and the only reason they do not do it is because they have not encountered them yet. Parasites/pathogens have variety of predispositions to infect new hosts; it provides a basis for a future explosion of

diseases when climate change and formerly geographically separated organisms begin to share the same area of occurrence (Hoberg and Brooks, 2015). It seems, however, that often it is not a new colonization of the host, or a new host-switch, but a return to a previously abandoned host (Janz et al., 2006). This may also be the case of our results. Considering the Stockholm paradigm with the oscillation hypothesis, we must look at the host specificity from a completely different point of view. In addition, such a complex host-parasite picture may be also affected/distorted by the fact that not every infection is “real”; sometimes we may detect the pseudoinfections that need to be carefully distinguished from the real infections.

In view of the above mentioned facts and data, the host specificity cannot be used to determine the parasite species, as was previously used (Joyner, 1982; Levine and Ivens, 1988; Paterson and Gray, 1997). Majority of coccidia species were described and identified based on the oocyst morphology. Many species descriptions are, however, incomplete, based only on simple drawings (sometimes even of an unsporulated oocyst), without photomicrographs or measurements, and without the knowledge of endogenous development. The advent of molecular techniques has been a major step towards a deeper knowledge of species and their relationships, not only in coccidia. Some markers, such as 18S rDNA or COI, have become the “gold standard” for the identification of many groups of eukaryotes. However, as is obvious from our results, we cannot rely only on the traditional markers. Sometimes it is better to use the gene that is less abundant in the GenBank database (e.g. ORF 470) than commonly used genes, such as COI, which in the end are not as effective as was believed (MS 5).

7. Literature

Aguilar JP, Thierry P, Sigé B, Michaux J, 2008: Occurrence of the stripe field mouse lineage (*Apodemus agrarius* Pallas 1771; Rodentia; Mammalia) in the late Pleistocene of southwestern France. *Comptes Rendus Palevol* 7: 217-225.

Anděra M, Horáček I, 2005: *Poznáváme naše savce*. 2. vydání. Sobotáles, Praha, 327 pp.

Anderson M, Chen P, Wang J, Kaper F, Lin M, Pieprzyk M, Jones R, 2010: High-throughput microfluidic preparation of targeted resequencing libraries using the access array system. *J Biomol Tech* S26.

Arnastauskiene T, Kazlauskas J, Maldziunaite S, 1978: On the natural groupings of the intestinal parasites of mouse rodents of the preserve of Kamsa and their dependence

on host biotope, species and its population structure. Acta Zool Litu 16: 15–32. (In Russian).

Asmundsson IM, Duszynski DW, Campbell JA, 2006: Seven new species of *Eimeria* Schneider, 1875 (Apicomplexa: Eimeriidae) from colubrid snakes of Guatemala and a discussion of what to call ellipsoid tetrasporocystic, dizoic coccidian of reptiles. Syst Parasitol 63: 91-103.

Atopkin DM, Bogdanov AS, Chelomina GN, 2007: Genetic variation and differentiation in striped field mouse *Apodemus agrarius* inferred from RAPD-PCR analysis. Russ J Genet 43: 665–676.

Barčiová L, Macholán M, 2009: Morphometric key for the discrimination of two wood mice species, *Apodemus sylvaticus* and *A. flavicollis*. Acta Zool Academ Sci Hung 55: 31-38.

Barta JR, Schrenzel MD, Carreno RA, Rideout BA, 2005: The genus *Atoxoplasma* (Garnham 1950) as a junior objective synonym of the genus *Isospora* (Schneider 1881) species infecting birds and resurrection of *Cystoisospora* (Frenkel 1977) as the correct genus for *Isospora* species infecting mammals. J Parasitol 91: 726-727.

Beattie SE, Fernando MA, Barta JR, 2001: A comparison of sporozoite transport after homologous and heterogenous challenge in chickens immunized with the Guelph strain or the Florida strain of *Eimeria maxima*. Parasitol Res 87: 116-121.

Bellinvia E, 2004: A phylogenetic study of the genus *Apodemus* by sequencing the mitochondrial DNA control region. J Zool Syst Evol Research 42: 289-297.

Blake DP, Worthing K, Jenkins MC, 2020: Exploring *Eimeria* genomes to understand population biology: Recent progress and future opportunities. Genes 11: 1103.

Callejón R, de Rojas M, Nieberding C, Foronda P, Feliú C, Guevara D, Cutillas C, 2010: Molecular evolution of *Trichuris muris* isolated from different Muridae hosts in Europe. Parasitol Res 107: 631-641.

Carreno RA, Schnitzler BE, Jeffries AC, Tenter AM, Johnson AM, Barta JR, 1998: Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora*. J Eukaryot Microbiol 45: 184-188.

Cerezo MLM, Kucka M, Zub K, Yingguang FC, Bryk J, 2020: Population structure of *Apodemus flavicollis* and comparison to *Apodemus sylvaticus* in northern Poland based on RAD-seq. BMC Genomics 21: 241.

Čondlová Š, Horčíčková M, Sak B, Květoňová D, Hlásková L, Konečný R, Stanko M, McEvoy J, Kváč M, 2018: *Cryptosporidium apodemi* sp. n. and *Cryptosporidium ditrichi* sp. n. (Apicomplexa: Cryptosporidiidae) in *Apodemus* spp. Eur J Protistol 63: 1–12.

de Vos AJ, 1970: Studies on the host range of *Eimeria chinchillae* De Vos & Van der Westhuizen, 1968. Onderstepoort J Vet Res 37: 29-36.

Demanche C, Deville M, Michaux J, Barriel V, Pinçon C, Aliouat-Denis CM, Pottier M, Noël C, Viscogliosi E, Aliouat EM, Dei-Cas E, Morand S, Guillot J, 2015: What do *Pneumocystis* organisms tell us about the phylogeography of their hosts? The case of the woodmouse *Apodemus sylvaticus* in continental Europe and western Mediterranean islands. PLoS One10: e0120839.

Duan Z, Zhao K, Peng Z, Li J, Diogo R, Zhao X, He S, 2009: Comparative phylogeography of the Yellow River schizothoracine fishes (Cyprinidae): vicariance, expansion, and recent coalescence in response to the Quaternary environmental upheaval in the Tibetan Plateau. Mol Phyl Evol 53: 1025–1031.

Dubey JP, 2019: Coccidiosis in Livestock, Poultry, Companion Animals, and Humans; CRC Press, Taylor & Francis Group: Boca Raton, FL, USA, 394 pp.

Duszynski DW, 2002: Coccidia (Apicomplexa: Eimeriidae) of the Mammalian Order Chiroptera. Special Publication of the Museum of Southwestern Biology, No. 5, First Impressions, Inc., Albuquerque, New Mexico, 42 pp.

Duszynski DW, 2016: The Biology and Identification of the Coccidia (Apicomplexa) of Marsupials of the World. Academic Press, London, 241 pp.

Duszynski DW, Couch L, 2013: The Biology and Identification of the Coccidia (Apicomplexa) of Rabbits of the World. Academic Press, London, 340 pp.

Duszynski DW, Morrow JJ, 2014: The Biology and Identification of the Coccidia (Apicomplexa) of Turtles of the World. Academic Press, London, 210 pp.

Duszynski DW, Upton SJ, Bolek M, 2007: Coccidia (Apicomplexa: Eimeriidae) of the amphibians of the world. Zootaxa (Magnolia Press) 1667: 1–77.

Duszynski DW, Wilber PG, 1997: A guideline for the preparation of the species descriptions in the Eimeriidae. J Parasitol 83: 333-336.

Engel W, Vogel W, Voiculescu I, Ropers HH, Zenzes MT, Bender K, 1973: Cytogenetic and biochemical differences between *Apodemus sylvaticus* and *Apodemus flavicollis*,

possibly responsible for the failure to interbreed. *Comp Biochem Physiol B* 44: 1165-1173.

Filippucci MG, Macholán M, Michaux JR, 2002: Genetic variation and evolution in the genus *Apodemus* (Muridae: Rodentia). *Biol J Linn Soc* 75: 395-419.

Fisher RA, 1958: *The Genetical Theory of Natural Selection*. New York: Dover Publications, 360 pp.

Franzen C, Müller A, Bialek R, Diehl V, Salzberger B, Fätkenheuer G, 2000: Taxonomic position of the human intestinal protozoan parasite *Isospora belli* as based on ribosomal RNA sequences. *Parasitol Res* 86: 669-676.

Ghimire TR, 2010: Redescription of genera of family Eimeriidae Minchin, 1903. *Int J Life Sci* 4: 26-47.

Hamilton WD, 1967: The Genetic Evolution of Social Behavior (Part I and II). *J Theor Biol* 7: 1–52.

Hewitt GM, 1999: Post-glacial recolonization of European biota. *Biol J Linn Soc Lond* 68: 87-112.

Higgs S, Nowell F, 1991: A review of the species of *Eimeria* infecting hosts in the genus *Apodemus*. *Syst Parasitol* 20: 203-209.

Hoberg EP, Brooks DR, 2015: Evolution in action: climate change, biodiversity dynamics and emerging infectious disease. *Philosophical Transactions of the Royal Society B* 370: 20130553.

Hůrková L, Baker A, Jirků M, Modrý D, 2005: Two new species of *Eimeria* Schneider 1875 (Apicomplexa: Eimeriidae) from the broad-toothed field mouse, *Apodemus mystacinus* Danford and Alston 1877 (Rodentia: Muridae) from Jordan. *Parasitol Res* 97: 33-40.

Hůrková Hofmanová L, Romeo C, Štohanzlová L, Jirsová D, Mazzamuto MV, Wauters LA, Ferrari N, Modrý D, 2016: Diversity and host specificity of coccidia (Apicomplexa: Eimeriidae) in native and introduced squirrel species. *Eur J Protistol* 56: 1-14.

Janz N, Nylin S, Wahlberg N, 2006: Diversity begets diversity: host expansions and the diversification of plant-feeding insects. *BMC Evol Biol* 6: 4.

Jojić V, Bugarski-Stanojević V, Blagojević J, Vujošević M, 2014: Discrimination of the sibling species *Apodemus flavicollis* and *A. sylvaticus* (Rodentia, Muridae). *Zool Anz* 253: 261–269.

Joyner LP, Long PL, 1974: The specific characters of the *Eimeria*, with special reference to the Coccidia of the fowl. Avian Pathol 3: 145-157.

Joyner LP, 1982: Host and site specificity. In: Long PL (Ed.): The biology of the coccidia. University Park Press, Baltimore, pp. 35-62.

Kaminskienė E, Radzijeuskaja J, Stanko M, Balčiauskas L, Paulauskas A, 2020: Associations between different Laelapidae (Mesostigmata: Dermanyssoidea) mites and small rodents from Lithuania. Exp Appl Acarol 81: 149-162.

Kartavtseva IV, Pavlenko MV, 2000: Chromosomal variability of the field mouse *Apodemus agrarius* (Rodentia, Muridae). Genetika 36: 223–236.

Kimura M, 1983: The Neutral Theory of Molecular Evolution. Cambridge: Cambridge University Press, 367 pp.

Kreier JP, Baker JR, 1987: Parasitic protozoa. Allen and Unwin, London, 241 pp.

Kvičerová J, Hypša V, 2013: Host-parasite incongruences in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. PLoS One 8: e63601.

Kvičerová J, Pakandl M, Hypša V, 2008: Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: Evolutionary significance of biological and morphological features. Parasitology 135: 443-452.

Lalis A, Leblois R, Liefried S, Ouarour A, Reddy Beeravolu C, Michaux J, Hamani A, Denys C, Nicolas V, 2016: New molecular data favour an anthropogenic introduction of the wood mouse (*Apodemus sylvaticus*) in North Africa. J Zool Syst Evol Res 54: 1–12.

Larina NI, 1961: Some data on interspecific relationships. (Crosses between wood and yellow-necked mouse under laboratory conditions). Nauchnye Dokl Vyss Shkoly 4: 37-42.

Latinne A, Navascués M, Pavlenko M, Kartavtseva I, Ulrich RG, Tiouchichine M-L, Catteau G, Sakka H, Quéré J-P, Chelomina G, Bogdanov A, Stanko M, Hang L, Neumann K, Henttonen H, Michaux J, 2020: Phylogeography of the striped field mouse, *Apodemus agrarius* (Rodentia: Muridae), throughout its distribution range in the Palaearctic region. Mamm Biol 100: 19-31.

Levine ND, 1982: Taxonomy and life cycles of coccidia. In: Long PL (Ed.): The biology of the coccidia. University park press, Baltimore, pp. 1-33.

Levine ND, Ivens V, 1988: Cross-transmission of *Eimeria* spp. (Protozoa, Apicomplexa) of rodents – a review. J Protozool 35: 434-437.

Martín Suárez E, Mein P, 2004: The late Pliocene locality of saint-Vallier (Drôme, France). Eleven micromammals. Geobios 37: S115-S125.

Martinů J, Štefka J, Poosakkannu A, Hypša V, 2020: “Parasite turnover zone” at secondary contact: a new pattern in host-parasite population genetics. Mol Ecol 29: 4653-4664.

Mayr E, 1963: Animal Species and Evolution. The Belknap Press of Harvard University Press, Cambridge, MA, 797 pp.

Metzker ML, 2009: Sequencing technologies - the next generation. Nat Rev Genet 11: 31–46.

Michaux JR, Bellinva E, Lymberakis P, 2005: Taxonomy, evolutionary history and biogeography of the broad-toothed field mouse (*Apodemus mystacinus*) in the eastern Mediterranean area based on mitochondrial and nuclear genes. Biol J Linn Soc Lond 85: 53-63.

Michaux JR, Chevret P, Filippucci MG, Macholán M, 2002: Phylogeny of the genus *Apodemus* with a special emphasis on the subgenus *Sylvaemus* using the nuclear IRBP gene and two mitochondrial markers: cytochrome b and 12S rRNA. Mol Phylogenet Evol 23: 123-136.

Michaux JR, Libois R, Paradis E, Filippucci MG, 2004: Phylogeographic history of the yellow-necked fieldmouse (*Apodemus flavicollis*) in Europe and in the Near and Middle East. Mol Phylogenet Evol 32: 788-798.

Michaux JR, Magnanou E, Paradis E, Nieberding C, Libois R, 2003: Mitochondrial phylogeography of the woodmouse (*Apodemus sylvaticus*) in the Western Palearctic region. Mol Ecol 12: 685-697.

Mitchell-Jones AJ, Amori G, Bogdanowicz W, Kryštufek B, Reijnders PJH, Spitzenberger F, Stubbe M, Thissen JBM, Vohralík V, Zima J, 1999: The atlas of European mammals. London: Academic Press, 496 pp.

Morrison DA, Bornstein S, Thebo P, Wernery U, Kinne J, Mattsson JG, 2004: The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). Int J Parasitol 34: 501-514.

- Musser GG, Brothers EM, Carleton MD, Hutterer R, 1996: Taxonomy and distributional records of Oriental and European *Apodemus*, with a review of the *Apodemus-Sylvaemus* problem. *Bonner Zool Beitr* 46: 143-190.
- Nabholz B, Glemin S, Galtier N, 2008: Strong variations of mitochondrial mutation rate across mammals—the longevity hypothesis. *Mol Biol Evol* 25: 120–130.
- Nowell F, Higgs S, 1989: *Eimeria* species infecting wood mice (genus *Apodemus*) and the transfer of two species to *Mus musculus*. *Parasitology* 3: 329-336.
- Oda K, Nishida Y, 1991: Prepatent and patent periods, and production and sporulation of oocysts of *Eimeria subspherica* isolated in Japan. *J Vet Med Sci* 53: 615-619.
- Ogedengbe JD, Ogedengbe ME, Hafeez MA, Barta JR, 2015: Molecular phylogenetics of eimeriid coccidia (Eimeriidae, Eimeriorina, Apicomplexa, Alveolata): A preliminary multi-gene and multi-genome approach. *Parasitol Res* 114: 4149-4160.
- Ogedengbe ME, El-Sherry S, Ogedengbe JD, Chapman HD, Barta JR, 2018: Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). *Int J Parasitol* 48: 59–69.
- Orlov VN, Bulatova NS, Nadjafova RS, Kozlovsky AI, 1996: Evolutionary classification of European wood mice of the subgenus *Sylvaemus* based on allozyme and chromosome data. *Bonner Zool Beitr* 46: 191-202.
- Pakandl M, 2009: Coccidia of rabbit: a review. *Folia Parasitol* 56: 153-166.
- Paterson AM, Gray RD, 1997: Host-parasite co-speciation, host switching and missing the boat. In: Clayton DH, Moore J (Eds.): *Host-Parasite Evolution: General Principles and Avian Models*. Oxford: Oxford University Press, pp. 236–250.
- Pellérdy L, 1974: *Coccidia and coccidiosis*. Akadémiai Kiadó, Budapest, 959 pp.
- Prokopič J, 1967: *Hymenolepis muris-sylvatici* (Rudolphi, 1819) = *Variolepis crenata*. *Folia Parasitol* 14: 365-369.
- Reduker DW, Duszynski DW, Yates TL, 1987: Evolutionary relationships among *Eimeria* spp. (Apicomplexa) infecting cricetid rodents. *Can J Zool* 65: 722-735.
- Sakka H, Quéré JP, Kartavtseva I, Pavlenko M, Chelomina G, Atopkin D, Bogdanov A, Michaux J, 2010: Comparative phylogeography of four *Apodemus* species (Mammalia: Rodentia) in the Asian Far East: evidence of Quaternary climatic change in their genetic structure. *Biol J Linn Soc* 100: 797-821.

Samarasinghe B, Johnson J, Ryan U, 2008: Phylogenetic analysis of *Cystoisospora* species at the rRNA ITS1 locus and development of a PCR-RFLP assay. *Exp Parasitol* 118: 592-595.

Serizawa K, Suzuki H, Tsuchiya K, 2000: A phylogenetic view on species radiation in *Apodemus* inferred from variation of nuclear and mitochondrial genes. *Biochem Genet* 38: 27-40.

Soekardono S, Ernst JV, Benz GW, 1975: The prepatent and patent periods of *Eimeria alabamensis* and further description of the exogenous stages. *Vet Parasitol* 1: 19-33.

Sorci G, Garnier S, 2019: Evolutionary ecology: Evolution of parasitism. In: Fath B (Ed.): *Encyclopedia of Ecology*, 2nd edition, vol. 2., pp. 304-309.

Spitzenberger F, Engelberger S, 2014: A new look at the dynamic western distribution order of *Apodemus agrarius* in Central Europe (Rodentia: Muridae). *Lynx* 45: 69-79.

Su S, Hou Z, Liu D, Jia C, Wang L, Xu J, Tao J, 2017: Comparative transcriptome analysis of second- and third-generation merozoites of *Eimeria necatrix*. *Parasit Vectors* 10: 388.

Suzuki Y, Sato JJ, Tsuchiya K, Luo J, Zhang YP, Wang YX, Jiang XL, 2003: Molecular phylogeny of wood mice (*Apodemus*, Muridae) in East Asia. *Biol Linn Soc* 80: 469-481.

Štefka J, Hypša V, 2008: Host specificity and genealogy of the louse *Polyplax serrata* on field mice, *Apodemus* species: A case of parasite duplication or colonisation? *Int J Parasitol* 38: 731-741.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF, 1998: Comparative phylogeography and postglacial colonization routes in Europe. *Mol Ecol* 7: 453-464.

Thompson JN, 2005: *The geographic mosaic of evolution*. University of Chicago Press, Chicago, IL.

Upton SJ, Campbell TW, Weigel M, McKown RD, 1990: The Eimeriidae (Apicomplexa) of raptors: review of the literature and description of new species of the genera *Caryospora* and *Eimeria*. *Can J Zool* 68: 1256-1265.

Van Valen L, 1973: A new evolutionary law. *Evol Theory* 1: 1–30.

Vermeulen ET, Lott MJ, Eldridge MDB, Power ML, 2016: Evaluation of next generation sequencing for the analysis of *Eimeria* communities in wildlife. *J Microbiol Methods* 124: 1-9.

Wilber PG, Duszynski DW, Upton SJ, Seville RS, Corliss JO, 1998: A revision of the taxonomy and nomenclature of the *Eimeria* spp. (Apicomplexa: Eimeriidae) from rodents in the tribe Marmotineae (Sciuridae). Syst Parasitol 39: 113-135.

Zajac AM, Conboy GA, 2006: Veterinary Clinical Parasitology. Blackwell Publishing, Iowa, USA, pp. 3-8.

Zhao X, Duszynski DW, 2001a: Phylogenetic relationship among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. Int J Parasitol 31: 715-719.

Zhao X, Duszynski DW, 2001b: Molecular phylogenies suggest the oocyst residuum can be used to distinguish two independent lineages of *Eimeria* spp. in rodents. Parasitol Res 87: 638-643.

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Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations



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ABSTRACT

Recent studies show that host switching is much more frequent than originally believed and constitutes an important driver in evolution of host-parasite associations. However, its frequency and ecological mechanisms at the population level have been rarely investigated. We address this issue by analyzing phylogeny and population genetics of an extensive sample, from a broad geographic area, for commonly occurring parasites of the genus *Eimeria* within the abundant rodent genera *Apodemus*, *Microtus* and *Myodes*, using two molecular markers. At the most basal level, we demonstrate polyphyletic arrangement, i.e. multiple origin, of the rodent-specific clusters within the *Eimeria* phylogeny, and strong genetic/phylogenetic structure within these lineages determined at least partially by specificities to different host groups. However, a novel and the most important observation is a repeated occurrence of host switches among closely related genetic lineages which may become rapidly fixed. Within the studied model, this phenomenon applies particularly to the switches between the eimerians from *Apodemus flavicollis*/*Apodemus sylvaticus* and *Apodemus agrarius* groups. We show that genetic differentiation and isolation between *A. flavicollis*/*A. sylvaticus* and *A. agrarius* faunas is a secondary recent event and does not reflect host-parasite coevolutionary history. Rather, it provides an example of rapid ecology-based differentiation in the parasite population.

1. Introduction

The long-held view of host-parasite coevolution as being a process mainly determined by co-speciation events has dramatically changed in recent years, mainly due to the frequent incongruencies detected between host and parasite phylogenies (Paterson and Banks, 2001; Meinilä et al., 2004; Ricklefs et al., 2004; Hoberg and Brooks, 2008; Agosta et al., 2010; Araujo et al., 2015). This change has led to a recognition that parasites are not just passive companions of their hosts but rather organisms with their own biology and many host-independent traits. Consequently, well-established questions in this field, such as how parasites maintain their host spectra, how generalists become specialists, and vice versa, or what are the mechanisms, pre-conditions and frequency of host switching, are now seen in an entirely new light (Agosta et al., 2010).

Since genealogy constraint (manifested as a nearly strict co-speciation history) has been rejected as a predominant driver of the parasite speciation and distribution among the hosts, alternative

hypotheses have had to be developed. Ecological fitting, a well-established hypothesis in the general ecological framework (Agosta and Klemens, 2008), has recently been adopted as a putative mechanism for new host colonization by a parasite (Agosta et al., 2010; Araujo et al., 2015; Messenger et al., 2015). Since parasites are long recognized as organisms strongly adapted to the environment provided by the specific host(s), their capability to survive in different environment should in theory be very limited. Frequent host switches observed in many parasite groups and often followed by speciation events thus pose an interesting question. The ecological fitting theory predicts that in some cases the adaptations evolved in particular environment (the host in the parasitological framework) and may allow the organism to survive under different conditions and successfully colonize the new environment (the new host). Malcicka et al. (2015) suggest that invasive species of parasites provide typical examples of such events. Since the ecological fitting mechanism is likely to work at the ecological level, i.e. on the short-term scale, it should result in a considerable variability in host spectra ranges among various parasite lineages/populations. Such

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a complex picture has indeed been detected in several host-parasite systems studied at the population level, e.g. *Pneumocystis*-primates, *Austrognathodes*-penguins, *Polyplax*-*Apodemus*, or *Ligula*-fish hosts (Demanche et al., 2001; Banks et al., 2006; Štefka and Hypša, 2008; Štefka et al., 2009). These differences in host spectra (i.e. the number and taxonomy of the host species) between the parasite species/populations could, in turn, affect the genetic structure of the species/populations. According to Nadler's hypothesis (Nadler, 1995), in multihost parasites, additional hosts increase the opportunities for dispersal, and thus reduce the parasites' population structure. Recently, Falk and Perkins (2013) supported this prediction with an empirical population study of two pinworm species, parasitizing in various reptiles in the Caribbean. Considering this recent development, it is clear that the elucidation of host switches/colonization in parasite populations is a key factor in understanding population genetics and evolution of parasites. Several such studies have been published on various parasitic associations, however, the majority of them were focused on the plants-phytophages, perhaps due to their better accessibility (Agosta, 2006; Habermannová et al., 2013; Nylin et al., 2014).

Here we propose the parasites of the genus *Eimeria*, associated with the rodent genus *Apodemus*, as a suitable model for such a coevolutionary study. Both counterparts, the hosts and the parasites, are well-studied organisms and thus provide a reliable background for the analyses. Mice of the genus *Apodemus* are the most common rodents in the Palearctic region. The geographic distribution and habitats of some species (e.g. *A. flavicollis* and *A. sylvaticus*) overlap, so they live in sympatry, competing for food resources (Michaux et al., 2005; Sakka et al., 2010). They have been recorded from a variety of habitats, both natural and urban (Nowak, 1999; Anděra and Beneš, 2002; Wilson and Reeder, 2005), and have served as model species in several genetic/evolutionary studies (e.g. Nieberding et al., 2004, 2005; Meyer-Lucht and Sommer, 2005; Štefka and Hypša, 2008; Sakka et al., 2010; Demanche et al., 2015). Coccidia of the genus *Eimeria* are frequent parasites of this rodent genus (Lewis and Ball, 1983; Levine and Ivens, 1990; Higgs and Nowell, 2000). So far, morphological and molecular studies have indicated a complex relationship between *Eimeria* and their hosts, with some *Eimeria* species being able to infect several species of *Apodemus*, while others have so far been described only from a single host species (Lewis and Ball, 1983; Higgs and Nowell, 1991; Hůrková et al., 2005; Kvičarová and Hypša, 2013).

In our previous phylogenetic work, we showed that *Eimeria* samples collected from the genus *Apodemus* branched at different positions across the phylogenetic tree (Kvičarová and Hypša, 2013). This indicates that during eimerian evolution, the colonization of *Apodemus* by *Eimeria* occurred multiple times. Such a situation provides good opportunity to investigate host switches and evolution of host specificity at population level within a complex system. In this study we thus use an extensive sampling to analyze the polyphyletic distribution of *Eimeria* within the genus *Apodemus* (Muridae) from the genealogical and population genetics perspective. To provide a suitable ecological background, we further add the eimerian parasites collected from two additional and abundant rodent genera, *Myodes* and *Microtus* (Arvicolinae). We show that within this host spectrum, colonizations and complete host switches have occurred several times within the recent, i.e. species/population time-scale. We also determine several independent cases of recent host switches from a broader host spectrum (represented by several species or even genera) to a strictly specific association with *A. agrarius*, as possible examples of the ecology-based differentiation caused by immigration of a new host species.

This new insight into the evolution of host specificity within and among parasite populations may have important implications from both theoretical and practical perspective. From the theoretical point of view, it indicates that to achieve a reliable coevolutionary reconstruction, the studies and methodologies should take into account much broader spectrum of possibilities. For example, it demonstrates that there is no general probability of host switching vs. duplication, which

could be established for a parasite species based merely on its biological features. Similarly, within the applied fields, such as epidemiology or disease control, the complex patterns observed in this study indicate that to establish a proper epidemiological models and control scheme may in many cases require a more detailed study based on extensive sample at population level. Particularly, possible occurrence of different genetic lineages and/or cryptic species of the pathogen, with different epidemiological characteristics, has to be examined.

Considering these circumstances, we specifically address in this study the following questions: (1) do the *Apodemus*-associated eimerians, scattered across the *Eimeria* tree, present consistently *Apodemus*-specific branches or just individual random infections? (2) considering close phylogenetic relationships and sympatric occurrence of the three *Apodemus* species, together with other rodent genera, do the *Eimeria* display any degree of host specificity? (3) if yes, how is such specificity reflected in their population genetic structure?

2. Material and methods

2.1. Sampling

Field studies were carried out in the course of 2006–2014, under official permits provided by the Czech Republic/European Union or collaborating institutions (Permit Numbers KUJCK 11134/2010 OZZL/2/Ou and 27873/ENV/11); the protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia and by the Ministry of the Environment of the Czech Republic (Permit Numbers 13841-11 and 22395/2014-MZE-17214). A list of localities and collected species is provided in Tables S1 and S2. Rodents were trapped using the classic wooden snap traps for mice. The traps were deployed in the late evening, left in the field overnight and picked up in the early morning. The faecal samples were collected directly from the gut of each individual animal. Faeces from each individual animal were kept in 4% potassium dichromate ($K_2Cr_2O_7$) solution. Host tissues (a small piece of ear or tail) were preserved in absolute ethanol for molecular determination of the host species.

2.2. Coprological examination and oocyst morphology

The presence of parasites in collected faeces was examined microscopically by flotation in Sheather's sucrose solution with a density of 1.30 (Duszynski and Wilber, 1997; Zajac and Conboy, 2006). Determination of the coccidian species/morphotypes was based on the morphology and morphometry of the sporulated oocysts, according to guidelines published by Duszynski and Wilber (1997) and Berto et al. (2014). An Olympus BX53 light microscope equipped with DP-73-1-51 high resolution image cooled digital camera Olympus and Olympus cellSens Standard 1.13 imaging software were used.

2.3. DNA extraction, PCR amplification of selected genes, sequencing

Eimerian DNA from positive faecal samples was isolated with FastDNA[®] SPIN for Soil Kit (MP Biomedicals, LLC, Santa Ana, California, USA) according to the manufacturer's instructions. For amplification, a mitochondrial gene for cytochrome c oxidase subunit I (COI) and a gene for the small subunit (SSU) of 18S rRNA, were selected. Both of these genes provide some methodological advantages but also suffer from certain shortcomings. While the COI sequences are known as good markers for intraspecific and interspecific studies, they are poorly represented for eimerians in the GenBank database. On the other hand, the 18S rRNA gene is the best represented eimerian gene in the GenBank, allowing for taxonomically broad analyses, but within *Eimeria* its variability is relatively low. A combination of these two genes thus provides an optimal means for extracting the available phylogenetic information on *Eimeria*.

Specific primers for amplification of ~800 bp of eimerian COI and

~1 400 bp of eimerian 18S rDNA were adopted from Schwarz et al. (2009) and Kvičerová et al. (2008), respectively. HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) was used for all PCR reactions. PCR products were enzymatically purified and directly sequenced; five independent PCR products were sequenced for each sample. Consensus of the sequences were used for the subsequent analyses. Sequencing was carried out by Macrogen, Inc. (Amsterdam, the Netherlands) on an automatic 3730XL DNA analyzer.

2.4. Determination of the host species

Since some host species (namely *Apodemus flavicollis*/*A. sylvaticus*, *Microtus arvalis*/*M. agrestis*) have overlapping morphometries, and almost indiscernible juveniles and subadults, it was not able in several cases to determine them unequivocally to the species in the field. In such cases, we used the methods of molecular biology. Host DNA was extracted from the host tissue using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Species-specific primers were used for the PCR identification or verification of the host species, amplifying the mitochondrial DNA control region (in the case of *Apodemus* spp.; Bellinva, 2004), or mitochondrial cytochrome *b* oxidase (in Arvicolinae; Jaarola and Searle, 2002). PCR products were enzymatically purified and directly sequenced by Macrogen, Inc. (Amsterdam, the Netherlands) on an automatic 3730XL DNA analyzer.

2.5. Sequence assembling, alignments, and phylogenetic analyses

The obtained sequences of *Eimeria* were identified by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), assembled using the Sequence Scanner v.1.0 (Applied Biosystems), EditSeq 5.05 and SeqMan 5.05 (DNASTAR, Inc., Madison, Wisconsin, USA) programs, and deposited in the NCBI GenBank database under the Accession numbers provided in Tables S2 and S3. The samples were tentatively assigned to the so far described *Eimeria* species based on their morphological examination (see Table S4) and similarity to the reference sequences available in the GenBank database. Alignments were created in BioEdit v.7.2.5 (Hall, 1999) and in MAFFT v.7 (Katoh et al., 2002; Katoh and Standley, 2013), and adjusted manually. 18S rDNA sequences were aligned in the nucleotide mode, COI sequences were aligned in the amino acid mode, then switched to nucleotide mode and used for the analyses. Using jModeltest (Posada, 2008, 2009), we selected GTR + Γ + I as the best fitting model to be used in the subsequent phylogenetic analyses. In addition, we performed Bayesian analysis of concatenated matrices under the above described model, and Bayesian analysis under the codon-based model for the COI matrix. Phylogenetic relationships were analyzed by Bayesian inference (BI) and maximum likelihood (ML) methods. BI was performed in MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001) for 10 million generations; the trees were summarized after removing 25% burn-in. Average standard deviations of split frequencies were 0.033860 for the COI-derived tree, and 0.030280 for the 18S rDNA tree. ML was carried out in PHYML v.3.1 (Guindon and Gascuel, 2003), with bootstrap values calculated by 1000 replicates. Final trees were visualized and exported using TreeView v.1.6.6 program (Page, 1996).

Statistical significance of the proposed host specificities was formally tested for several lineages. More specifically, we tested *alorani* and offshoot of *apionodes* II for which we suggested secondarily established strict host specificity after switching to a new host, *A. agrarius* (see Results), and four additional lineages (*apionodes* I, *apionodes* II, *jerfinica*, and *uptoni*) for which the data within our sample indicate specificity either to *Apodemus* spp. or the subfamily Arvicolinae. In all cases, we used a statistical model to test whether the host species remains significant predictor of the parasite lineage even when possible effect of sampling localities is taken into account. The test was done using generalized linear mixed-effect models with binomial response in

R platform (R Development Core Team, 2014) using the package lme4 (Bates et al., 2015), with host and locality as explanatory variables and the presence of parasite lineage as a response.

2.6. Population genetics analyses

Since we obtained COI sequences of different lengths, we used the following procedure to build an optimal set of haplotypes, i.e. the set of sufficient sequence lengths on one hand and reasonable taxonomical representation on the other. In the first step, we determined a maximum length limit under which all lineages delimited by the preceding phylogenetic analyses (Figs. 2–4) were represented by several sequences. We then removed all sequences shorter than the limit and trimmed the remaining sequences to the limit length. With this procedure, we obtained a matrix of 177 sequences 547 bp long. This matrix was used for the reconstruction of haplotype networks in programs TCS v.1.21 (Clement et al., 2000), PopART v.1.7 (<http://popart.otago.ac.nz>; Bandelt et al., 1999), and the calculation of diversity indices in DnaSP v.5 (Librado and Rozas, 2009). For the *Apodemus*-associated lineages, we estimated within-lineage differentiation due to host specificity by calculating GammaST, a measure of genetic distance between populations (Nei, 1982), among the samples from different hosts using the DnaSP program. For this comparison, we only considered the groups containing at least three samples collected from a single host species.

Finally, we used the BEAST v.1.8.2 program package (Bouckaert et al., 2014) to estimate the relative ages of the lineages. For the time calibration, we used a relative scale with the “age” of the root set arbitrary to 10. This procedure allowed for the results interpretation in both relative scale and a putative absolute scale. Within the relative framework, we could compare relative “ages” of all lineages to the obviously young *alorani* offshoot, without assuming specific ages. In the putative absolute framework, we used the estimate of *A. agrarius* age (4.5 mya; Sakka et al., 2010) as upper bound for the age of the *alorani* lineage. We based this putative calibration on the evolutionary scenario we derived from our phylogenetic analysis and the host specificities of the lineages (details in Results and Discussion). Briefly, we conclude that the *alorani* lineage originated by switching from *A. flavicollis*/*sylvaticus* to *A. agrarius*, after the latter host species spread into Europe from the Far East, and cannot therefore be older than *A. agrarius* itself. The analysis was done under the GTR + Γ + I model with the molecular clock set at the lognormal relaxed mode. By checking for convergence in the Tracer v.1.6.0 (<http://beast.bio.ed.ac.uk>), we eventually ran the analysis for 35 mil. generations. We then discarded 25% of the trees and created a consensus of the remaining samples. We prepared graphical representation of the tree in the FigTree program v.1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) and applied the node relative ages from the BEAST results.

3. Results

3.1. Sampling

In total, 1 515 individuals of *Apodemus* spp., 200 of *Microtus* spp., and 364 of *Myodes glareolus* were collected within the period of 2006–2014 from an area covering 13 European countries and the west of Russia (Figs. 1, S1, S2a–c, Table S5). Of these samples, 680 (32.7%) were *Eimeria*-positive. Hosts of the positive samples were determined using both morphological and molecular methods. For the *Eimeria* samples from *Apodemus*, we obtained 165 sequences of the COI gene and 74 sequences of the SSU gene (see Table S2). For the *Eimeria* samples from Arvicolinae, we obtained 36 sequences of the COI gene and 37 sequences of the SSU gene (see Table S2). Furthermore, we obtained several *Eimeria* sequences from other small mammals (*Crocidura* sp., *Mus* sp., *Neomys* sp., and *Sorex araneus*). These sequences were included in the analyses to improve the sample background.

3.2. Phylogeny

The COI analyses yielded well-resolved trees with a strongly supported inner structure (Fig. 2). The same topologies were obtained under the GTR + Γ + I model and the codon-based model. The trees obtained via the SSU analyses were compatible regarding their main features with the COI trees, but they were less resolved (Fig. 3). Concatenation of both matrices yielded a tree similar to the topology based on 18S rDNA, however, the *kaunensis* lineage remained preserved there (Fig. S1). From the evolutionary point of view, the most notable feature of the trees was the taxa clustering being strongly influenced, but not entirely determined, by the taxonomic position of the host organisms. Eimerians collected from the two rodent groups investigated here, i.e. Muridae (represented by the genus *Apodemus*) and Arvicolinae (represented by the genera *Microtus* and *Myodes*), clustered in several well-formed and supported lineages. For the clarity of the following description and discussion, we delimited three dominant monophyletic lineages of these eimerians (Fig. 2). Since this delimitation was in close agreement with the established morphology-based taxa (species), we designated these lineages by the species names, i.e. *jerfinica*, *uptoni* and *apionodes*. In *apionodes* lineage, we designated individual sublineages as *apionodes* I–IV, as well as morphospecies *alorani* and *kaunensis*, paraphyletic in respect to *apionodes* (Fig. 2).

Of these lineages, two strictly *Apodemus*-specific lineages created monophyletic phylogenetically distinct groups, corresponding to *jerfinica* and *uptoni*. The rest of the lineages, encompassing the majority of the *Eimeria* samples, clustered as a monophyletic group with the posterior probability 1, and its sister group composed of three closely related sequences of *Eimeria* from *Mus* and *Heliophobius*. The host specificities of these lineages varied considerably. Interestingly, a tendency to a switch from Arvicolinae- to *Apodemus*-specificity could be seen within this cluster of lineages (Fig. 2). The most basal lineage *apionodes* I was entirely Arvicolinae-specific. Of the other lineages, the *kaunensis*, *apionodes* III, and *apionodes* IV were formed by a mix of samples from various host groups (i.e. Muridae, Arvicolinae, and a single sample from *Cavia porcellus*), with the more recent lineage (*apionodes* IV) prevailed by the *Apodemus* samples. Finally, two derived offshoots (*apionodes* II and *alorani*) were only *Apodemus*-specific, and the *alorani* lineage/species was even specific to a single *Apodemus* species, *A. agrarius* (Fig. 2, Table S2; for statistical test see Table S6).

This latest pattern strongly suggests a recent host switch from the *A. flavicollis/A. sylvaticus* (Af/As) to *A. agrarius* (Aa). In the tree derived via BEAST during calculations of relative ages, the arrangement of the lineages corresponded to that in the BI tree, except the switch between lineages *apionodes* II and *kaunensis* (Fig. 4). The COI-derived topology described above was compatible with the 18S rDNA based trees, except for six samples (designated AF 15_CZ7, AF 47_CZ9, AS20_IT63, AF 92D_DE47, AA B2A4_BG71, and AA 21655_SK36) showing conflicting positions; in all cases, these samples originated from mixed infections (as established by microscopical examination) and phylogenetic discrepancies were thus most likely due to amplification of different species/lineage for each genetic marker.

The sampling presented here covered a large part of Europe, from western France to eastern Bulgaria, and from southern Finland to southern Italy (Figs. 1, S2, S3a–c and Table S1). Within this area, the eimerian lineages varied slightly in their distribution, but showed considerable overlaps (Figs. 1 and S3a–c). Due to these many overlaps, all sampled areas were inhabited by multiple parasite lineages. Often the samples with the same host specificity, but of different phylogenetic position, were collected sympatrically, even at the same localities. An interesting geographic pattern was found for the eimerian lineages with the host switch from Af/As to Aa hosts. While in the less diversified lineage *apionodes* II the Aa samples were distributed inside the area of the Af/As samples, in the more diversified lineage *apionodes* IV the Af/As and its sister *alorani* Aa samples were collected from mutually disjunct localities (Fig. 5).

Apart from these lineages of the main interest (i.e. the *Apodemus*/Arvicolinae-associated samples), our data set also included samples from other rodent and non-rodent hosts. With one exception, all of these samples clustered outside the *Apodemus*/Arvicolinae-specific clusters. Some of them formed independent clusters based on their host characteristics, such as poultry-specific cluster, rabbit-specific cluster, or bird *Isospora*-specific cluster. However, few of the samples were unique by their origin (i.e. no other samples from the same host were available), and their phylogenetic clustering is thus difficult to interpret. The most peculiar case was *E. caviae*, the only sample which did not originate from the *Apodemus*/Arvicolinae hosts but clustered within one of the *Apodemus*-specific cluster (Fig. 2). Since the molecular data on eimerians from the same or closely related host are not available, we do not make at this point any evolutionary speculation and focus on the well-formed and supported *Apodemus*/Arvicolinae-specific clusters.

3.3. Population genetics

When collapsed into haplotypes, the set of the above mentioned 177 COI sequences (see chapter 2.6. in Material and Methods) yielded 45 unique haplotypes (Table S2) which split into two uncoupled networks and several isolated haplotypes when evaluated under the criteria of statistical parsimony as implemented in the TCS program (Fig. S4). The larger and substantially more complex network corresponded to the node A designated in the Fig. 4. The cohesion of this network, i.e. the low number of missing haplotypes, suggested that the sampling effectively covered relatively recent continuous diversification. This allowed for a reliable inference supporting the two independent switches to *A. agrarius* from the Af/As lineages (Figs. 2, 4 and 5). Their recent occurrence was further supported by a comparison of the relative ages of the lineages; e.g., the estimated relative age of the *alorani* lineage (3.5) was roughly one third of the whole tree depth (10) (Fig. 4). Apart from these topology-based arguments, a strong genetic barrier rising during the switch between Af/As on the one hand, and Aa on the other, was clearly reflected by the GammaST estimates within *jerfinica*, the only lineage with Aa samples considerably intermingled with Af/As. Of the three interspecific comparisons, the genetic differentiation expressed by GammaST is considerably lower between Af and As than differentiation between either of these species and Aa (Fig. 4).

4. Discussion

The phylogenetic trees and population networks show that *Eimeria* from several rodent genera form a remarkably complex system. The gross picture reveals three notable tendencies: (1) a strong genetic/phylogenetic structure in which well supported clusters are determined by specificities to different host groups, (2) polyphyletic arrangement, i.e. multiple origin, of the rodent-specific clusters within the *Eimeria* phylogeny, and (3) repeated host switches within the rodent-specific clusters (i.e. among closely related haplotypes) which may become rapidly fixed. The two former features are in line with several recent studies (e.g. Štefka and Hypša, 2008; Power et al., 2009; Štefka et al., 2009; Kvičerová and Hypša, 2013; Pineda-Catalan et al., 2013; Kvičerová et al., 2014; Ogedengbe et al., 2018) contradicting the traditional view of host specificity as a conserved and phylogenetically important parameter. The third feature is derived from the population-level analyses. In our opinion, it provides the most important contribution of the presented data to the conception of host-parasite coevolution.

Apart from these genetic features, it is interesting to note that compared to many other groups of parasites, the eimerian groups studied here display a surprising concordance between their taxonomy based on morphological features and the phylogenetic relationships inferred from molecular data. This is documented in the Table S4 that lists distinct sets of oocyst morphological features, corresponding to some of the previously described *Eimeria* species, which can be

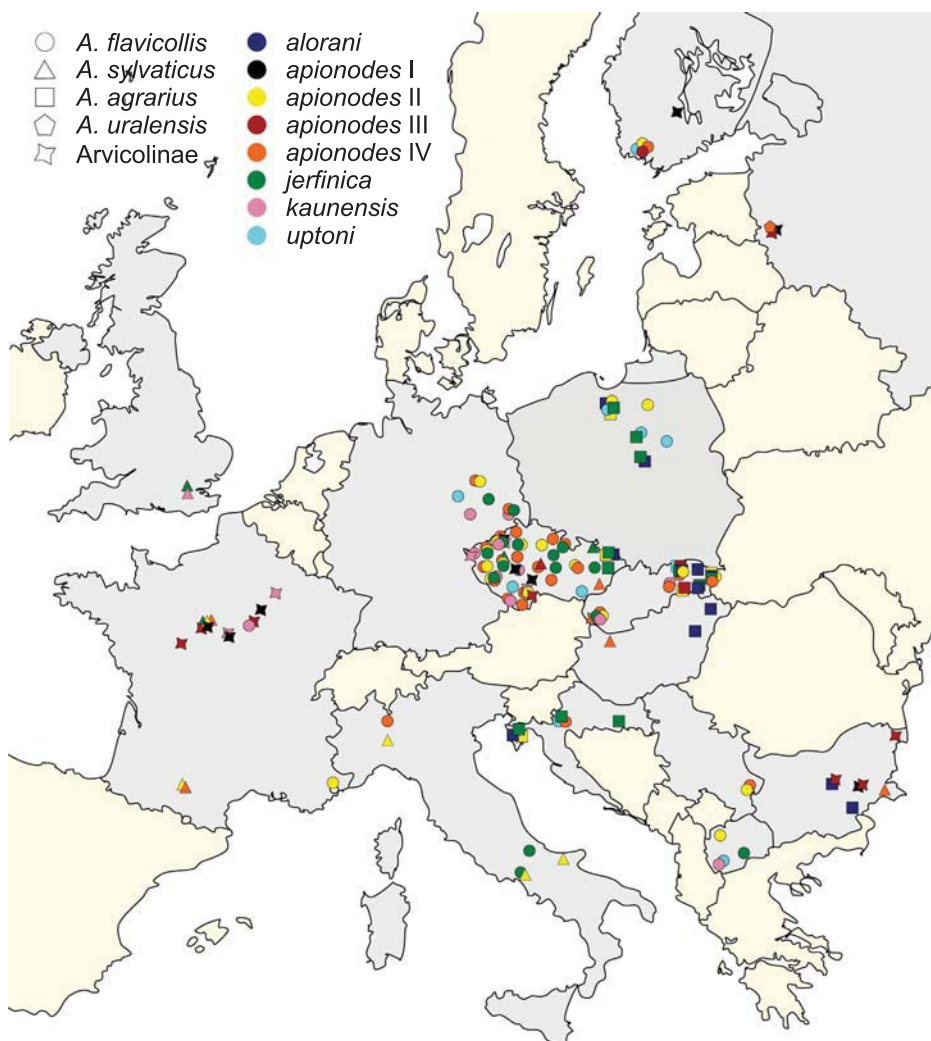


Fig. 1. Distribution of individual *Eimeria* lineages across the sampled localities. Symbols are attributed to the hosts, colours are attributed to the parasites.

attributed to the genetically-delimited lineages. However, apart from this general fit, the distribution of morphological traits across the tree also shows several peculiarities. For example, coccidia from the lineage corresponding to the *kaunensis* morphotype possess oocyst residuum (OR) but cluster within the group B, typical by its absence (Fig. 4). The other two rodent-specific *Eimeria* possessing OR (i.e. *E. cahirinensis* and *E. callospermophili*) cluster at a distant position within the tree (Fig. 2). This finding, rejecting the hypothesis of two distinct rodent *Eimeria* lineages based on the presence/absence of OR (Zhao and Duszynski, 2001a,b)), further demonstrates the effect of sampling effort, and hence the representativeness of the sampled material, on the phylogenetic/evolutionary inferences. Below, we discuss in more details the observed patterns and their possible consequences for the host-parasite coevolutionary concept.

4.1. Host specificity at the phylogenetic level

The non-monophyletic nature of the samples confirms and further extends our previous observation based solely on eleven samples of the *Apodemus*-associated *Eimeria* (Kvičerová and Hypša, 2013). However, due to the considerably larger number of samples (182 *Apodemus*-associated samples + 56 Arvicolinae-associated samples) a consistent picture of the parasites' distribution across their hosts and geographic ranges can now be drawn from the data. Thus, unlike our previous study, none of the samples presented here forms a single-sequence "orphan" lineage. All of the clusters containing eimerian parasites from *Apodemus* and/or Arvicolinae hosts form monophyletic groups composed of at least 8 sequences. This shows that each of the *Apodemus*-associated samples represents a specific branch, not just a random non-specific infection.

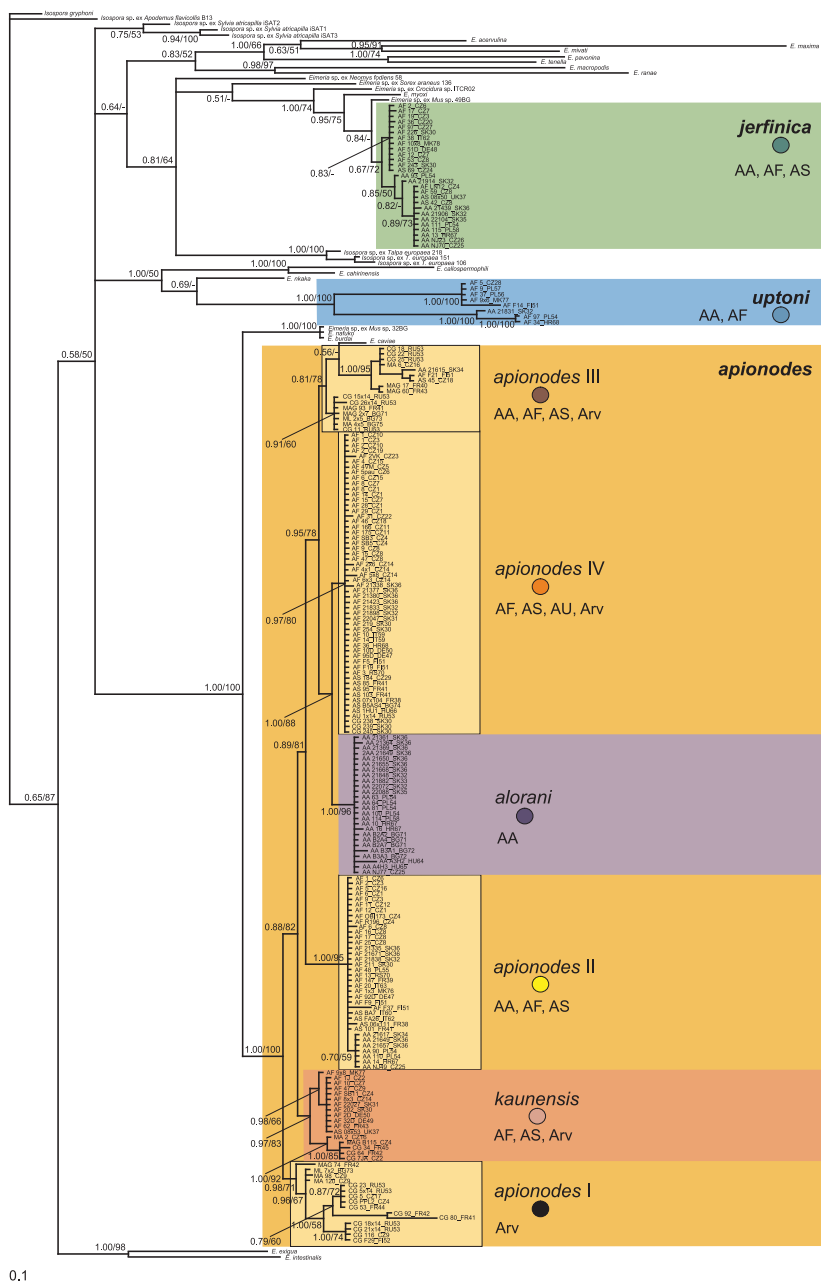


Fig. 2. Phylogenetic relationships of the eimerians inferred by the BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities under the BI analysis/bootstraps values derived from ML analysis. Posterior probabilities and bootstraps supports lower than 0.50 or 50%, respectively, are marked with dash (–). Each original sample code consists of the abbreviation of the host species, specific code of the sample, country code, and the map reference. AA, *Apodemus agrarius*; AF, *Apodemus flavicollis*; AS, *Apodemus sylvaticus*; AU, *Apodemus uralensis*; Arv, *Arvicola*.

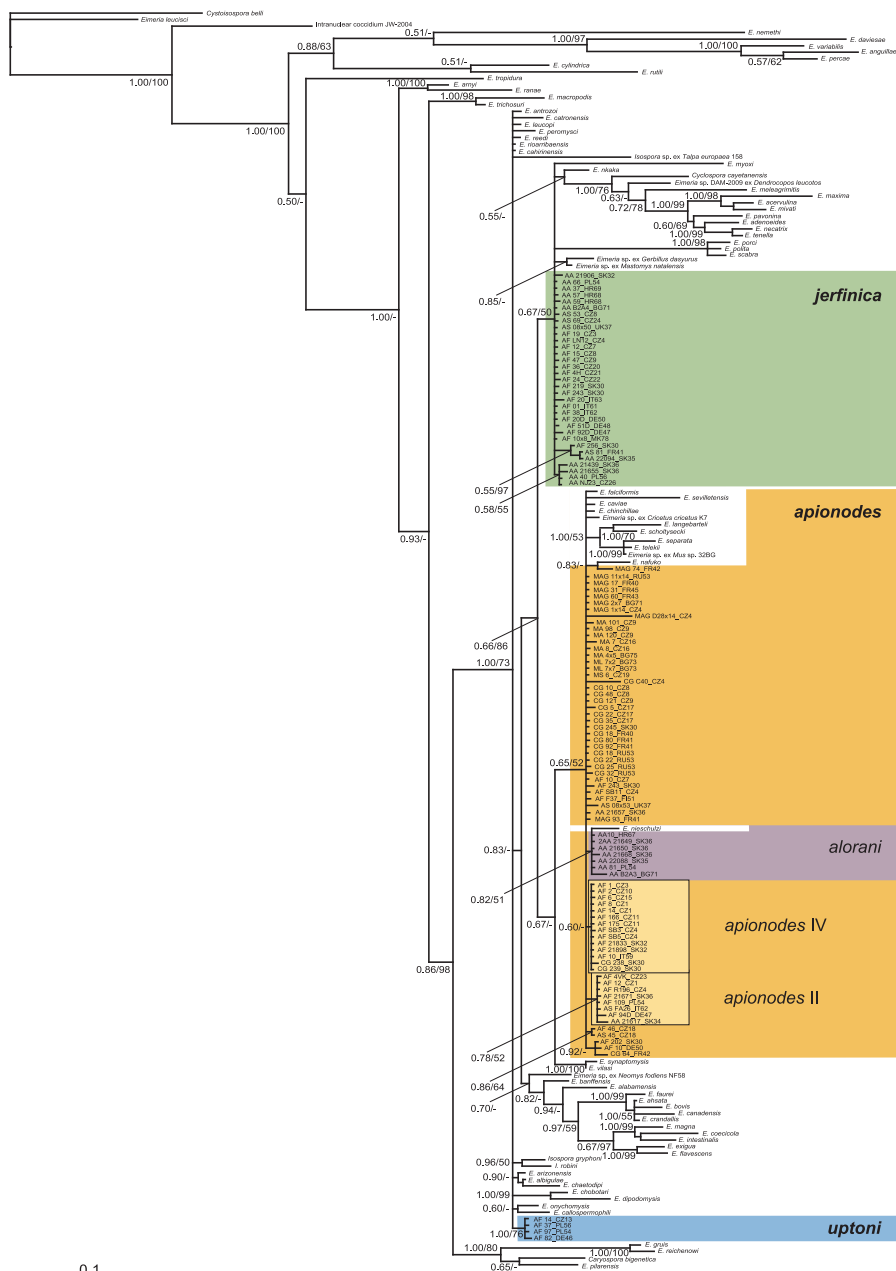


Fig. 3. Phylogenetic relationships of the eimerians inferred by the BI analysis of the 18S rDNA sequences. Numbers at the nodes show posterior probabilities under the BI analysis/bootstraps values derived from ML analysis. Posterior probabilities and bootstrap supports lower than 0.50 or 50%, respectively, are marked with dash (–). Each original sample code consists of the abbreviation of the host species, specific code of the sample, country code, and the map reference. AA, *Apodemus agrarius*; AF, *Apodemus flavicollis*; AS, *Apodemus sylvaticus*; AU, *Apodemus uralensis*; Arv, Arvicolinae.

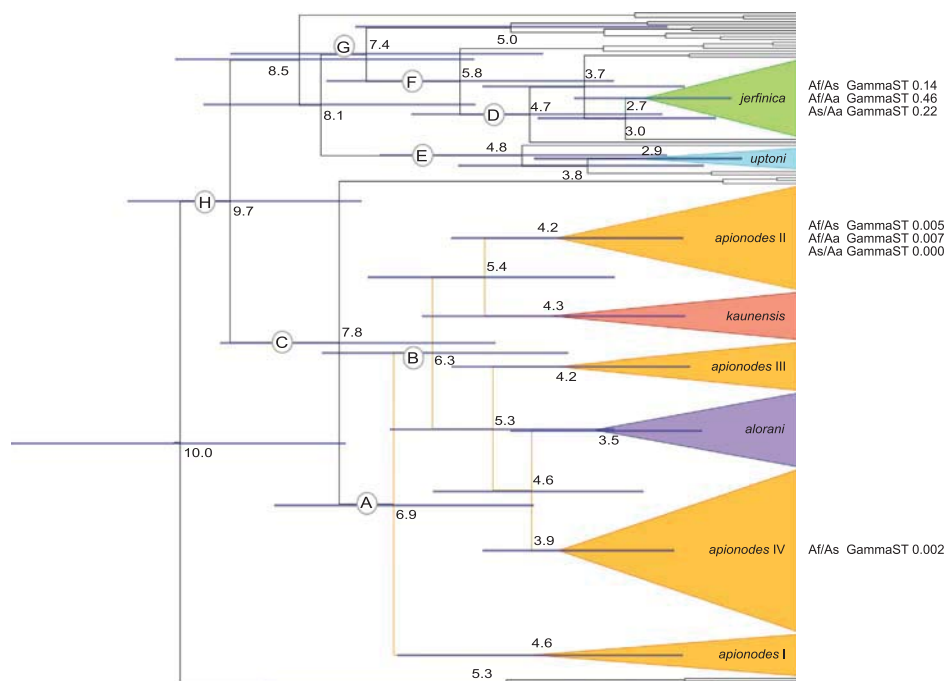


Fig. 4. Divergence times and genetic differentiations calculated by the BEAST package and DnaSP. The numbers at the nodes show relative “ages” of the lineages (see Material and Methods), the bars at the nodes represent posterior probabilities values and 95% credibility intervals.

Although the *Apodemus*/Arvicolinae-associated lineages branch in a non-monophyletic manner among the eimerians from other hosts, they all retain affinity to these rodent groups. An interesting example of the relationship between the host specificity and genetic structure is provided by the *Eimeria* associated with Af/As, and the Aa. Within the *apionodes* group, the Aa samples form two well-defined and strictly specific clusters, contrasting to the entirely intermixed sequences from Af and As. For these two clusters, the observed restriction to a single host proved statistically significant (i.e. not determined by the locality; see Table S6) for the *alorani* lineage composed of 26 samples, while it was nonsignificant for the less numerous offshoot of the *apionodes* II ($n = 7$). This arrangement, at least in the *alorani* lineage, is likely to reflect different evolutionary histories of the three *Apodemus* species. Two of them, Af and As, are closely related species of the subgenus *Sylvaemus* (Martin et al., 2000; Michaux et al., 2002) which separated around 4 million years ago (Michaux and Pasquier, 1974), and after Quaternary climatic oscillations recolonized the Europe from their southern refugia (Michaux et al., 2005). Currently, they co-occur in sympatry or even in syntopy throughout the majority of their European distribution (Michaux et al., 2005). In contrast, the distribution of phylogenetically distant Aa (subgenus *Apodemus*) overlaps with Af/As only in the eastern part of their geographical distribution (Suzuki et al., 2008). An analysis by Sakka et al. (2010) shows that populations of *A. agrarius* create a very complicated system with several main foci, the probable Quaternary refugia. China, Russian Far East, and Korea represent important centers of diversification for this species. Its eastern population covers southern parts of the Russian Far East, China, Korea, and Taiwan. The western population, isolated from the eastern one by several biogeographic barriers, is distributed across Kazakhstan, Kyrgyzstan, the Caucasus, and the Balkans, and reaches central Europe.

Unlike the demographically stabilized eastern group, the western population shows clear signatures of a recent expansion (Sakka et al., 2010). Based on the dating, both Suzuki et al. (2008) and Sakka et al. (2010) suggest that colonization of the central Palaearct may have taken place around 175 000–190 000 years ago. The hypothesis, that only a single population lineage has penetrated into Europe, is supported by the genetic and molecular analyses (Filippucci et al., 2002; Suzuki et al., 2008).

It is reasonable to suppose that the Aa-specific lineages, e.g. the *alorani* branch, did not originate before the first waves of *A. agrarius* reached Europe. Therefore, considering the Sakka et al. (2010) dating of the *A. agrarius* origin in the Far East region (4.5 mya; see Material and Methods) and the delay of its expansion to Europe and hypothetical origin of the *alorani* group (shown in Fig. 4), the strictly *Apodemus*-specific branches described here seem of relatively recent origin in comparison to the suggested ages of the Af/As taxa (Michaux et al., 2005; Suzuki et al., 2008). The current patterns of phylogeny/specificity shown here thus seem to reflect recent evolutionary events (switches, adaptations), rather than stable long-term coevolution. These events resulted in origin of several lineages with different degree of host specificity, ranging from a single host species to several host genera. This makes the system of European *Eimeria* in rodents a promising model for investigating the diversification/speciation processes on the ecological scale.

4.2. Host switches and specificity at the population level

The pattern of genetic differentiation between Af/As and Aa samples is further strengthened by the situation in the *jerfinica* group. Here, although the samples from *A. flavicollis*, *A. sylvaticus* and *A. agrarius* are

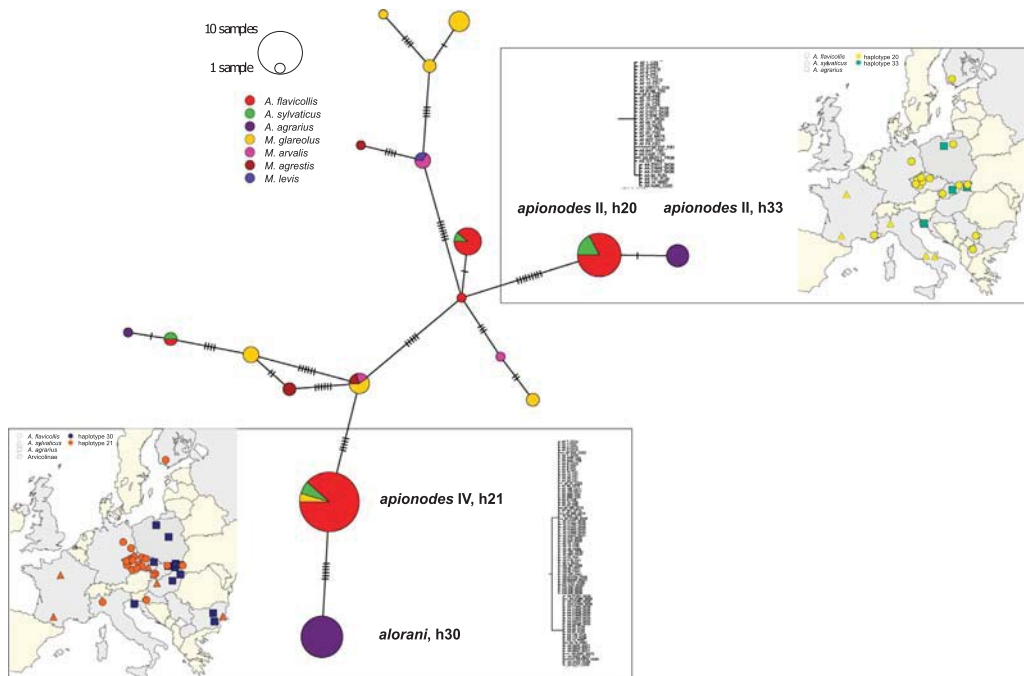


Fig. 5. Haplotype network of the lineage A (as defined in Fig. 4) constructed in PopART. Spatial relationship between the Af/As and Aa haplotypes are shown in the maps.

mixed, rather than forming distinct lineages, the differentiation test reveals barriers among the species. Of the three pairs of differentiation, the two involving *A. agrarius* (GammaST 0.46 for Af vs. Aa, and GammaST 0.22 for As vs. Aa) are considerably stronger than the Af/As differentiation (GammaST 0.14). Repeated genetic differentiation between the Af/As and Aa samples poses an interesting question about the underlying mechanism. No simple answer would fully explain the observed patterns. Since the Aa-specific samples clearly represent host switches, phylogenetic/genealogic constraint can not be responsible for this barrier. Neither can simple ecological parameters provide an explanation, as some of the Aa samples were obtained from the same localities as the Af and As samples, even during a single collection. In theory, a sampling bias, e.g. insufficient sampling effort, could result in an erroneous inference of seemingly host-specific haplotypes or lineages, such as the two Aa-specific offshoots. However, as shown in the Figs. 1 and 5, the Aa-specific samples were collected from a broad geographic range, shared with the Af/As samples. This provides evidence of long-maintained genetic separation of these groups in sympatry and shows that the Aa clusters are not artifacts of sampling just a local temporary subpopulation. It should be noted that the switch in specificity towards *A. agrarius* is not mere colonization, i.e. extension of the host spectrum, but a complete switch involving the entire abandonment of the original host taxa. In the *alorani* group, this process apparently gave rise to a morphologically distinguished lineage fully adapted to the new host. Thus, it seems likely that some more complex circumstances play a key role in this process, possibly, for example, differences in the hosts' physiologies and/or more subtle ecological differences. The patterns discussed above indicate that while the majority of coevolutionary studies address the question of host switches from the phylogenetic perspective, investigations of the early genetic differentiation may be the more pertinent approach. In our results, the

well-resolved and supported part of the tree (*apionodes* + *alorani* + *kaunensis*) shows an interesting variation in degree of the host specificity among the lineages. While some of the lineages were found in several species of different families, others are specific to a single family or even a single species (Fig. 2; Table S6 with statistical tests). It is difficult with such unique events, even by applying rigorous parsimony rules, to establish direction of the evolutionary changes (i.e. narrowing the host spectrum by stronger specialization/adaptation vs. broadening the spectrum by colonization of new host species). However, several of the observed patterns may reflect a putative extension from the single-family specificity (e.g. the lineages *apionodes* I, *apionodes* II, *alorani*, *jerfinica*, and *uptoni*) to the mixture of two host families (e.g. lineages *apionodes* III, *apionodes* IV, and *kaunensis*), and can be perceived as possible instances of ecological fitting. In contrast, the sudden disruption between strict Af/As specificity and Aa specificity is more difficult to attribute to an established ecological mechanism.

These findings have also broader relevance within the established concepts of population structure and diversity in parasites. For example, the Nadler's hypothesis (Nadler, 1995) postulates direct relationship between the host range and genetic diversity, predicting that parasites (i.e. populations, species) with broader host spectrum display weaker population structure due to their better dispersal opportunity. Our results suggest that when analyzing such relationships, genetic diversity and host specificity should be evaluated within a common framework with genealogy and host switches dynamics. This can for example be illustrated on the lineages *apionodes* II and *apionodes* IV + *alorani* (Fig. 5). Within these groups, seemingly capable of dissemination via three or four host species living in sympatry, the parasites associated with *A. agrarius* do not contribute to the overall gene flow due to their strict host specificity. Similarly, such a monophyletic recent offshoot with narrower host specificity will likely

display lower genetic structure than the paraphyletic ancestral population with a broader host spectrum.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ympev.2018.05.009>.

References

- Agosta, S.J., 2006. On ecological fitting, plant-insect associations, herbivore host shifts, and host plant selection. *Oikos*. Wiley Online Library 114, 556–565.
- Agosta, S.J., Klemens, J.A., 2008. Ecological fitting by phenotypically flexible genotypes: implications for species associations, community assembly and evolution. *Ecol. Lett.* 11, 1123–1134.
- Agosta, S.J., Janz, N., Brooks, D.R., 2010. How specialists can be generalists: resolving the “parasite paradox” and implications for emerging infectious disease. *Zoologia* 27, 151–162.
- Anděra, M., Beneš, B., 2002. Atlas rozšíření savců v České republice. Předěžná verze IV. Hlodavci (Rodentia) – část 2. Myšovití (Muridae), myšivkovití (Zapodidae). Národní muzeum; Praha, Czech Republic (in Czech).
- Araujo, S.B.L., Braga, M.P., Brooks, D.R., Agosta, S.J., Hoberg, E.P., von Hartenthal, F.W., Boeger, W.A., 2015. Understanding host-switching by ecological fitting. *PLoS One* 10, 0139225.
- Bandelt, H., Forster, P., Röhl, A., 1999. Median-joining networks for inferring in-transpecific phylogenies. *Mol. Biol. Evol.* 16, 37–48.
- Banks, J.C., Palma, R.L., Paterson, A.M., 2006. Cophylogenetic relationships between penguins and their chewing lice. *J. Evol. Biol.* 19, 156–166.
- Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67, 1–48.
- Bellinva, E., 2004. A phylogenetic study of the genus *Apodemus* by sequencing the mitochondrial DNA control region. *J. Zool. Syst. Evol. Res.* 42, 289–297.
- Berto, B.P., McIntosh, D., Lopes, C.W.G., 2014. Studies on coccidian oocysts (Apicomplexa: Eucoelidiaria). *Rev. Bras. Parasitol. Vet.* 23, 1–15.
- Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput. Biol.* 10, e1003537.
- Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. *Mol. Ecol.* 9, 1657–1659.
- Demanche, C., Berthelemy, M., Petit, T., Polack, B., Wakefield, A.E., Dei-Cas, E., Guillot, J., 2001. Phylogeny of *Pneumocystis carinii* from 18 primate species confirms host specificity and suggests coevolution. *J. Clin. Microbiol.* 39, 2126–2133.
- Demanche, C., Deville, M., Michaux, J., Barriel, V., Pinçon, C., Aliouat-Denis, C.M., Pottier, M., Noël, C., Viscigiosi, E., Aliouat, E., Dei-Cas, E., Morand, S., Guillot, J., 2015. What do *Pneumocystis* organisms tell us about the phylogeography of their hosts? The case of the woodmouse *Apodemus sylvaticus* in Continental Europe and western Mediterranean islands. *PLoS One* 10, e0120839.
- Duszynski, D.W., Wilber, P.G., 1997. A guideline for the preparation of species descriptions in the Eimeriidae. *J. Parasitol.* 83, 333–336.
- Falk, B.G., Perkins, S.L., 2013. Host specificity shapes population structure of pinworm parasites in Caribbean reptiles. *Mol. Ecol.* 22, 4576–4590.
- Filippucci, M.G., Macholán, M., Michaux, J.R., 2002. Genetic variation and evolution in the genus *Apodemus* (Muridae: Rodentia). *Biol. J. Linn. Soc.* 75, 395–419.
- Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696–704.
- Habermannová, J., Bogusch, P., Straka, J., 2013. Flexible host choice and common host switches in the evolution of generalist and specialist cuckoo bees (Anthophila: Sphecidae). *PLoS One* 8, e64537.
- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95–98.
- Higgs, S., Nowell, F., 1991. A review of the species of *Eimeria* infecting hosts in the genus *Apodemus*. *Syst. Parasitol.* 20, 203–209.
- Higgs, S., Nowell, F., 2000. Population biology of *Eimeria* (Protozoa: Apicomplexa) in *Apodemus sylvaticus*: a capture/recapture study. *Parasitology* 120, 355–363.
- Hoberg, E.P., Brooks, D.R., 2008. Structuring the biosphere: episodic host-switching, geographic colonization, and diversification in complex host-parasite systems. *J. Biogeogr.* 35, 1533–1550.
- Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755.
- Hůrková, L., Baker, M.A., Jirků, M., Modrý, D., 2005. Two new species of *Eimeria* Schneider 1875 (Apicomplexa: Eimeriidae) from the broad-toothed field mouse, *Apodemus mystacinus* Danford and Alston 1877 (Rodentia: Muridae) from Jordan. *Parasitol. Res.* 97, 33–40.
- Jaarola, M., Searle, J.B., 2002. Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. *Mol. Ecol.* 11, 2613–2621.
- Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucl. Acids Res.* 30, 3059–3066.
- Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780.
- Kvičerová, J., Hypša, V., 2013. Host-parasite incongruities in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. *PLoS One* 8, e63601.
- Kvičerová, J., Hypša, V., Dvořáková, N., Mikulíček, P., Jandzik, D., Gardner, M.G., Javanbakht, H., Tiar, G., Široký, P., 2014. *Hemolivia* and *Hepatozoon*: haemogregarines with tangled evolutionary relationships. *Protist* 165, 688–700.
- Kvičerová, J., Pakandl, M., Hypša, V., 2008. Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. *Parasitology* 135, 443–452.
- Levine, N.D., Ivens, V., 1990. The Coccidian Parasites of Rodents. CRC Press, Boca Raton, FL, USA.
- Lewis, D.C., Ball, S.J., 1983. Species of *Eimeria* of small wild rodents from the British Isles, with descriptions of two new species. *Syst. Parasitol.* 5, 259–270.
- Librado, P., Rozas, J., 2009. DnaSP v. 5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.
- Malcicka, M., Agosta, S.J., Harvey, J.A., 2015. Multi level ecological fitting: indirect life cycles are not a barrier to host switching and invasion. *Glob. Chang. Biol.* 21, 3210–3218.
- Martin, Y., Gerlach, G., Schlötterer, C., Meyer, A., 2000. Molecular phylogeny of European murid rodents based on complete cytochrome b sequences. *Mol. Phylogenet. Evol.* 16, 37–47.
- Meinilä, M., Kuusela, J., Zietara, M.S., Lumme, J., 2004. Initial steps of speciation by geographic isolation and host switch in salmonid pathogen *Gyrodactylus salaris* (Monogenea: Gyrodactylidae). *Int. J. Parasitol.* 34, 515–526.
- Messenger, L.A., Garcia, L., Vanhove, M., Huaranca, C., Bustamante, M., Torrico, M., Torrico, F., Miles, M.A., Llewellyn, M.S., 2015. Ecological host fitting of *Trypanosoma cruzi* TcI in Bolivia: mosaic population structure, hybridization and a role for humans in Andean parasite dispersal. *Mol. Ecol.* 24, 2406–2422.
- Meyer-Lucht, Y., Sommer, S., 2005. MHC diversity and the association to nematode parasitism in the yellow-necked mouse (*Apodemus flavicollis*). *Mol. Ecol.* 14, 2233–2243.
- Michaux, J.R., Chevret, P., Filippucci, M.G., Macholan, M., 2002. Phylogeny of the genus *Apodemus* with a special emphasis on the subgenus *Sylvaemus* using the nuclear IRBP gene and two mitochondrial markers: cytochrome b and 12S rRNA. *Mol. Phylogenet. Evol.* 23, 123–136.
- Michaux, J.R., Libois, R., Filippucci, M.G., 2005. So close and so different: comparative phylogeography of two small mammal species, the yellow-necked fieldmouse (*Apodemus flavicollis*) and the woodmouse (*Apodemus sylvaticus*) in the western Palearctic region. *Heredity* 94, 52–63.
- Michaux, J., Pasquier, L., 1974. Dynamique des populations de mulots (Rodentia, *Apodemus*) en Europe durant le Quaternaire. Premières données. *Bull. Soc. Géol. France* 7, 431–439 (in French).
- Nadler, S.A., 1995. Microevolution and the genetic structure of parasite populations. *J. Parasitol.* 81, 395–403.
- Nei, M., 1982. Evolution of human races at the gene level. In: Bonne-Tamir, B., Cohen, T., Goodman, R.M. (Eds.), *Human Genetics. Part A. The Unfolding Genome*. Alan R. Liss, New York, pp. 167–181.
- Nieberding, C., Libois, R., Douady, C.J., Morand, S., Michaux, J.R., 2005. Phylogeography of a nematode (*Heligmosomoides polygus*) in the western Palearctic region: persistence of northern cryptic populations during ice ages? *Mol. Ecol.* 14, 765–779.
- Nieberding, C., Morand, S., Libois, R., Michaux, J.R., 2004. A parasite reveals cryptic phylogeographic history of its host. *Proc. Biol. Sci.* 271, 2559–2568.
- Nowak, R.M., 1999. *Walker's Mammals of the World*, sixth ed. The Johns Hopkins University Press, Baltimore and London.
- Nylin, S., Slove, J., Janz, N., 2014. Host plant utilization, host range oscillations and diversification in nymphalid butterflies: a phylogenetic investigation. *Evolution* 68, 105–124.
- Ogedengbe, M.E., El-Sherry, S., Ogedengbe, J.D., Chapman, H.D., Barta, J.R., 2018. Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). *Int. J.*

- Parasitol 48, 59–69.
- Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Applic. Biosci.* 12, 357–358.
- Paterson, A.M., Banks, J., 2001. Analytical approaches to measuring cospeciation of host and parasites: through a glass, darkly. *Int. J. Parasitol.* 31, 1012–1022.
- Pineda-Catalan, O., Perkins, S.L., Peirce, M.A., Engstrand, R., Garcia-Davila, C., Pinedo-Vasquez, M., Aguirre, A.A., 2013. Revision of hemoproteid genera and description and redescription of two species of chelonian hemoproteid parasites. *J. Parasitol.* 99, 1089–1098.
- Posada, D., 2008. jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.* 25, 1253–1256.
- Posada, D., 2009. Selection of models of DNA evolution with jModelTest. *Methods Mol. Biol.* 537, 93–112.
- Power, M.L., Richter, C., Emery, S., Hufschmid, J., Gillings, M.R., 2009. *Eimeria trichosuri*: phylogenetic position of a marsupial coccidium, based on 18S rDNA sequences. *Exp. Parasitol.* 122, 165–168.
- Ricklefs, R.E., Fallon, S.M., Bermingham, E., 2004. Evolutionary relationships, co-speciation, and host switching in avian malaria parasites. *Syst. Biol.* 53, 111–119.
- Sakka, H., Quéré, J.P., Kartavtseva, I., Pavlenko, M., Chelomina, G., Atopkin, D., Bogdanov, A., Michaux, J., 2010. Comparative phylogeography of four *Apodemus* species (Mammalia: Rodentia) in the Asian Far East: evidence of Quaternary climatic changes in their genetic structure. *Biol. J. Linn. Soc.* 100, 797–821.
- Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009. Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *J. Parasitol.* 95, 871–880.
- Štefka, J., Hypša, V., 2008. Host specificity and genealogy of the louse *Polyplax serrata* on field mice, *Apodemus* species: a case of parasite duplication or colonisation? *Int. J. Parasitol.* 38, 731–741.
- Štefka, J., Hypša, V., Scholz, T., 2009. Interplay of host specificity and biogeography in the population structure of a cosmopolitan endoparasite: microsatellite study of *Ligula intestinalis* (Cestoda). *Mol. Ecol.* 18, 1187–1206.
- Suzuki, H., Filippucci, M.G., Chelomina, G.N., Sato, J.J., Serizawa, K., Nevo, E., 2008. A biogeographic view of *Apodemus* in Asia and Europe inferred from nuclear and mitochondrial gene sequences. *Biochem. Genet.* 46, 329–346.
- Wilson, D.E., Reeder, D.M., 2005. *Mammal Species of the World: A Taxonomic and Geographic Reference*, third ed., vol. 2. The Johns Hopkins University Press, Baltimore.
- Zajac, A.M., Conboy, G.A., 2006. *Veterinary Clinical Parasitology*. Blackwell Publishing, Iowa, USA.
- Zhao, X., Duszynski, D.W., 2001a. Molecular phylogenies suggest the oocyst residuum can be used to distinguish two independent lineages of *Eimeria* spp. in rodents. *Parasitol. Res.* 87, 638–643.
- Zhao, X., Duszynski, D.W., 2001b. Phylogenetic relationships among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. *Int. J. Parasitol.* 31, 715–719.

Ms 2:

Trefančová A, Máčová A, Kvičerová J, 2018: Isosporan oocysts in the faeces of bank voles (Myodes glareolus; Arvicolinae, Rodentia): Real parasites, or pseudoparasites? Protist 170: 104-120.

ORIGINAL PAPER

Isosporan Oocysts in the Faeces of Bank Voles (*Myodes glareolus*; Arvicolinae, Rodentia): Real Parasites, or Pseudoparasites?



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Coccidia of the genus *Isospora*, their origin, taxonomy, and host specificity have been discussed for many years. The crucial point in question being the division of the genus, based on distinct evolutionary history and the presence/absence of the Stieda body, into the genera *Isospora* (Eimeriidae) parasitizing mainly birds and reptiles, and *Cystoisospora* (Sarcocystidae) parasitizing mammals. The description of the majority of *Isospora* species from rodents is based solely on the oocysts found in their faeces. Some of them have been described with the presence of the Stieda body, some without it, and, simultaneously, for all the described species the molecular data are entirely lacking. This study reveals the origin of isosporan oocysts found in faeces of bank voles based on morphological analyses, phylogenetic analyses, and experimental infections. Morphological analyses showed the presence of the Stieda body complex on sporocysts. Phylogenetic analyses demonstrated close phylogenetic relationships between *Isospora* from bank voles and avian isosporans. Experimental inoculations of bank voles with sporulated oocysts of *Isospora* did not result in the production of unsporulated oocysts. Hence, these organisms should be considered pseudoparasites of the bank voles/rodents (probably originating from avian *Isospora* species).

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Key words: *Isospora*; *Cystoisospora*; coccidian; rodent; vole; pseudoparasite.

Introduction

The genus *Isospora* (Apicomplexa: Coccidia: Eimeriorina) was discovered and described by Schneider in 1881, being then most often detected in the faeces of birds, dogs, cats, and also rodents. However, the first described species, *Isospora rara*

(Schneider 1881), was found in an invertebrate host, the gastropod *Limax* sp. (Duszynski and Upton 2001; Ghimire 2010; Levine 1982; Pellérdy 1974). From the 1970s through to the 1990s, there was a significant boom in describing new species of coccidia (particularly eimerians), the majority of descriptions being based solely on the oocysts found in the faeces of a presumed host. Moreover, in some cases, identical species were described under different names (assuming their high host

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specificity). However, the majority of these early described species have not been further confirmed by neither experimental nor molecular methods. Today, it is supposed that in some cases they may represent pseudoparasites (Ghimire 2010), i.e. parasites transferred via the paratenic/transport hosts, or accidentally passing through the gastrointestinal tract of nonspecific hosts. Pseudoparasitism may concern even the type species *I. rara*, because Schneider (1881) did not clearly prove the origin of infection in the gastropod (Ghimire 2010; Pellérdy 1974). Moreover, since then, no other *Isospora* spp. have been detected/described from the invertebrates (Ghimire 2010; Levine 1988).

Oocysts of *Isospora*-type (i.e. with two sporocysts, each containing four sporozoites), routinely found in the faeces of mammals, have for decades presented questions and conundrums (e.g. Barnard et al. 1974; Barta et al. 2005; Carreno and Barta 1999; Ernst et al. 1969; Ghimire 2010; Levine and Ivens 1990; Levine and Mohan 1960; Morrison et al. 2004; Prasad 1961; Smith 1981; Streitel and Dubey 1976). Since the discovery that isosporans infecting mammals are phylogenetically related to Sarcocystidae and that their sporocysts always lack Stieda bodies (SB), whereas isosporans infecting birds are related to Eimeriidae and possess SB (Barta et al. 2005; Carreno et al. 1998; Franzen et al. 2000; Samarasinghe et al. 2008), the situation has become even more complicated. Thus, the SB, not only regarding its presence or absence, but also its shape and size, has come to provide the key morphological trait. At present, all known *Isospora* species from mammals that lack the SB have been reassigned to the genus *Cystoisospora*, as initially proposed by Frenkel (1977). Moreover, members of the *Cystoisospora* (i.e. without SB) often have a heteroxenous life

cycle with secondary intermediate/paratenic hosts (e.g. rodents) that harbour asexual, monozytic tissue cyst stages typically found in mesenteric lymph nodes, some of them also possessing extraintestinal stages occurring in various organs and tissues (Dubey 1975, 1978a, 1978b; Dubey and Frenkel 1972; Smith 1981). The sporulated oocysts of *Cystoisospora* spp. can infect both intermediate as well as definitive host.

For the majority of *Isospora* species (i.e. with SB) described from mammals, there is no knowledge on life cycles, no cross-transmission studies, nor any detailed studies on their endogenous developmental stages. The only exception is *Isospora masoni* described from the hispid cotton rat (*Sigmodon hispidus*; Rodentia: Cricetidae) by Upton et al. (1985) who carried out an experimental transmission study as well as a detailed study on its endogenous development. This species was described possessing the SB complex. Nonetheless, there are currently no molecular data verifying its classification into the genus *Isospora*. Thus, oocysts of *Isospora* found in the faeces of mammals are considered to be spurious findings from prey items – usually birds – merely passing through the mammal's gut (Ghimire 2010; Prasad 1961; Streitel and Dubey 1976). However, there are no complex studies verifying this hypothesis. A similar phenomenon was observed for example in adeleid coccidia (Adeleorina) parasitizing invertebrates, and their ability to pass through the gastrointestinal tract of vertebrates (Berto et al. 2008, 2010; Teixeira et al. 2003). This study aims to reveal the origin of *Isospora*-type oocysts found in the faeces of bank voles (*Myodes glareolus*; Rodentia: Arvicolinae) based on three different approaches – morphological analyses, experimental infections, and molecular methods.

Table 1. List of sampled localities and numbers of trapped bank voles (*M. glareolus*) in the course of 2015–2017.

Locality	2015 (no. of <i>M. glareolus</i>)	2016 (no. of <i>M. glareolus</i>)	2017 (no. of <i>M. glareolus</i>)	Total number of trapped animals
České Budějovice (South Bohemian Region)	1	10	8	19
Kláštevec nad Ohří (Ústí nad Labem Region)	–	–	6	6
Litvínov (Ústí nad Labem Region)	–	8	202	210
Lužnice (South Bohemian Region)	30	20	13	63
Stružná (Karlovy Vary Region)	–	8	–	8
Třísov (South Bohemian Region)	9	–	–	9
Týmákov (Pilsen Region)	1	2	20	23
Total	41	48	249	338

Results

Sampling and Coprological Examination

Faeces of 338 voles from snap traps were collected from 2015 to 2017 in the Czech Republic (Table 1). Only 17/338 (5%) vole faecal sam-

ples were *Isospora*-positive, while 93/338 (27.5%) were *Eimeria*-positive. Most of the *Isospora*-positive samples were obtained in the surroundings of Týmákov (Pilsen Region), where the prevalence in 2017 surprisingly reached 34.8% (Table 2).

Table 2. List of *Isospora*-positive *M. glareolus* trapped using the classic wooden snap traps, their origin, period of collection, and intensity of infection.

Sample code	Sex	Locality	Period of collection	Intensity of infection
50_MG_PLE	♀	Plešnice (Pilsen Region)	September 2015	I++
49_MG_TYM	♂	Týmákov (Pilsen Region)	March 2016	I+++
57_MG_TYM	♂	Týmákov (Pilsen Region)	March 2016	I+++
60_MG_TYM	♀	Týmákov (Pilsen Region)	March 2016	I++
89_MG_TYM	♂	Týmákov (Pilsen Region)	May 2016	I+++
6_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2016	I+
7_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2016	I+++
P14_MG_LIT	♀	Litvínov (Ústí nad Labem Region)	October 2016	I+
9_MG_LUZ	♀	Lužnice (South Bohemian Region)	June 2017	I+++
2_MG_TYM	♀	Týmákov (Pilsen Region)	August 2017	I+++
7_MG_TYM	♂	Týmákov (Pilsen Region)	September 2017	I++
13_MG_TYM	♀	Týmákov (Pilsen Region)	September 2017	I+
18_MG_TYM	♀	Týmákov (Pilsen Region)	September 2017	I+

I+ slight infection; I++ moderate infection; I+++ heavy infection.

Table 3. List of the live-trapped *Isospora*-negative *M. glareolus* used for the experimental infections, their origin, and period of collection.

Sample code	Sex	Locality	Period of collection
11_MG_LUZ	♂	Lužnice (South Bohemian Region)	June 2017
1_MG_TYM	♀	Týmákov (Pilsen Region)	August 2017
12_MG_TYM	♂	Týmákov (Pilsen Region)	September 2017
15_MG_TYM	♀	Týmákov (Pilsen Region)	September 2017

Table 4. Coprological examination of bank voles used for the experimental infections, and schedule of the experimental infections.

	11MG_LUZ_17	1MG_TYM_17	12MG_TYM_17	15MG_TYM_17
Microscopy before the treatment with Baycox®	Negative	Coccidia-negative, eggs of <i>Capillaria</i> ++	Coccidia-negative, eggs of <i>Trichuris</i> +	Coccidia-negative, eggs of <i>Trichuris</i> +++
Baycox® 1st application	19/09/2017	19/09/2017	19/09/2017	19/09/2017
Baycox® 2nd application	22/09/2017	22/09/2017	22/09/2017	22/09/2017
Microscopy after the treatment with Baycox®	Negative	Coccidia-negative, <i>Capillaria</i> ++	Coccidia-negative, <i>Trichuris</i> +	Coccidia-negative, <i>Trichuris</i> +++
Experimental infection	10/10/2017 isolate 9MGLUZ/17	10/10/2017 isolate 2MGTYM/17	10/10/2017 isolate 9MGLUZ/17	10/10/2017 isolate 2MGTYM/17

Table 5. Isosporan species described from rodent hosts. M, micropyle; OR, oocyst residuum; OW, oocyst wall; SB, Stieda body; highlighted are those species that were described from rodents of the subfamily Arvicolinae.

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
<i>Isospora anatolicum</i> Sayin, Dincer & Meric, 1977	<i>Spalax leucodon</i> (Spalacidae)	Asia	Spherical; 9–11 × 8–9	Ovoid; 6–9 × 4–6	No	No OR or M; smooth pale greenish yellow OW (0.8)
<i>Isospora arvalis</i> Mikeladze, 1973	<i>Microtus arvalis</i> (Muridae)	Russia	Subspherical; 10–12 × 8–12	Ovoid; 6–8 × 4–6	No	No OR or M; smooth colorless OW (2)
<i>Isospora assensis</i> Svanbaev, 1979	<i>Spermophilus fulvus</i> (Sciuridae)	Russia	Ovoid – spherical; 18–19 × 18–24	Ellipsoidal – spherical; 8–13 × 7–11	No	OR present; no M; smooth, double-contoured OW (1.5–2)
<i>Isospora aurangabadensis</i> Kshirsagar, 1980	<i>Rattus rattus</i> (Muridae)	India	Spherical or subspherical; 32–44 × 32–40	Ovoid, globose or elongate	Yes	No OR or M; yellowish brown OW (1.5–2)
<i>Isospora batabatica</i> Musaev & Veisov, 1960	<i>Arvicola amphibius</i> (Muridae)	Russia	Almost spherical or ovoid; 20–24 × 19–21	Ovoid; 9–14 × 6–9	Yes	No OR or M; smooth colorless double-contoured OW (1)
<i>Isospora californica</i> Davis, 1967	<i>Peromyscus californicus</i> (Muridae)	North America	Spherical to ellipsoidal or ovoid; 18–32 × 18–27	Ovoid to lemon-shaped; 13–20 × 8–13	Yes	No OR or M; smooth grey-green to light brown OW (1)
<i>Isospora calomyscus</i> Musaev & Veisov, 1965	<i>Calomyscus bairdardi</i> (Muridae)	Russia	Ellipsoidal; 20–23 × 16–20	Ovoid; 14–17 × 10–15	Yes	No OR or M; smooth, yellow-brown OW (1.5)
<i>Isospora citelli</i> Levine, Ivens & Krudener, 1957	<i>Spermophilus variegatus</i> (Sciuridae)	North America, Russia	Subspherical; 22–23 × 21–22	Broadly lemon-shaped; about 15 × 10	Yes	No OR or M; smooth brownish OW (1)
<i>Isospora clethrionomydis</i> Golemanski & Yankova, 1973	<i>Myodes glareolus</i> (Muridae)	Europe (Bulgaria)	Spherical; 23–27 × 23–27	Ovoid; 21–23 × 11–12	Yes	No OR or M; thin colorless or light yellowish OW
<i>Isospora clethrionomys</i> Arnautskiene & Maldzhuniyte, 1981	<i>M. glareolus</i> (Muridae)	Eastern Europe (Lithuania)	9–11 × 7–10	Ovoid to spindle-shaped	No record	No record

Table 5 (Continued)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
<i>Isoospora dawadimiensis</i> Kasim & Al Shawa, 1985	<i>Jaculus jaculus</i> (Dipodidae)	Saudi Arabia	Ovoid or almost spherical; 22–26.5 × 20.5–22	Ellipsoidal; 12–16.5 × 9–10.5	No	No OR or M; smooth, light brown or pale green OW about (1)
<i>Isoospora dryomidis</i> Glebezdin, 1974	<i>Dryomys nitedula</i> (Myoxidae)	Russia	Ellipsoidal; 23–26 × 20–23	Ellipsoidal; 12–15 × 9–12	No	No OR or M; smooth, colorless OW
<i>Isoospora egypti</i> Prasad, 1960	<i>Meriones shawi</i> (Muridae)	Africa	Subspherical; 20–22 × 16–20	Ovoid; 10–12 × 6–8	Yes	No OR or M; smooth light brown OW
<i>Isoospora erythraurica</i> Veisov, 1964	<i>Meriones libycus</i> (Muridae)	Russia	Spherical or subspherical; about	Ellipsoidal or spherical; 12–18 × 10–16	Probably not	No OR or M; smooth colorless – yellow–brown OW about (1.5–2.5)
<i>Isoospora freundi</i> Yakimoff & Gousseff, 1935	<i>Cricetus cricetus</i> (Muridae)	Russia	24–30 × 24–30 Spherical or subspherical; 13–27 × 17–24	14 × 8–9	No	No OR or M; smooth double-contoured OW
<i>Isoospora golemanskii</i> Levine, 1982	<i>Apodemus flavicollis</i> (Muridae)	Europe	Spherical or subspherical; 23–28 × 20–28	Ovoid; 12–16 × 8–11	No record	No OR or M
<i>Isoospora hammondi</i> Barnard, Ernst & Stevens, 1971	<i>Oryzomys palustris</i> (Muridae)	North America	Ovoid; 24–30 × 16–21	Ellipsoidal; 13–18 × 11–15	Yes	No OR or M; smooth colorless to pinkish OW about (1)
<i>Isoospora hastingsi</i> Davis, 1967	<i>Peromyscus truei</i> (Muridae)	North America	Ovoid; 29–33 × 22–24	Lemon-shaped	Yes	No OR or M; smooth tan or yellow-tan OW about (1.2)
<i>Isoospora krishnamurthyi</i> Kshirsagar, 1980	<i>R. rattus</i> (Muridae)	India	Spherical or slightly ovoid; 36–48 × 35–40	Ovoid; 21–26 × 17–22	Yes	No OR or M; pale yellowish OW (1.8)
<i>Isoospora laguri</i> Iwanoff-Gobzem, 1934	<i>Lagurus lagurus</i> (Muridae)	Russia	Ovoid; 24–32 × 16–22	16–21 × 8–13	No record	OR present; no M; thick OW
<i>Isoospora * masoni</i> Upton, Lindsay, Current & Ernst, 1985	<i>Sigmodon hispidus</i> (Muridae)	North America	Ellipsoidal	Ovoid; 7–9 × 5–6	Yes	No OR or M; smooth, thin membrane-like OW

Table 5 (Continued)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
<i>Isospora mcdowellii</i> Saxe, Levine & Ivens, 1960	<i>Microtus pennsylvanicus</i> (Muridae)	North America	Spherical to subspherical; 9–11 × 8–10	Ellipsoidal; 6–8 × 4–5	No record	No OR or M; thin OW
<i>Isospora meriones</i> Musaev & Vinogradovi Veisov, 1965	<i>Meriones vinogradovi</i> (Muridae)	Russia	Ellipsoidal; 18–28 × 14–24	Piriform; 10–16 × 6–10	Yes	No OR or M; smooth colorless – yellow-brown OW about (1.5–2) No OR or M; smooth OW (1.5)
<i>Isospora mexicanusubsimi</i> Vance & Duszynski, 1985	<i>Microtus mexicanus subsimus</i> (Muridae)	Mexico	Spherical; 21–26 × 21–26	Ovoid; 12–16 × 10–12	Yes	No OR or M; smooth colorless OW (1.5)
<i>Isospora ordubadica</i> Musaev & Veisov, 1960	<i>Meriones persicus</i> (Muridae)	Russia	Ovoid or subspherical; 18–20 × 14–18	Ovoid to ellipsoidal; 10–12 × 8–10	No	No OR or M; smooth colorless OW (1)
<i>Isospora peromysci</i> Davis, 1967	<i>Peromyscus maniculatus</i> (Muridae)	North America	Elongate or ellipsoidal; 25–43 × 14–28	Broadly ovoid; 13–21 × 11–15	No	No OR or M; smooth, pale green, tan, or light brown OW (0.8)
<i>Isospora ratti</i> Levine & Ivens, 1965	<i>Rattus norvegicus</i> (Muridae)	North America	Subspherical; 22–24 × 20–21	Symmetrical, broadly ovoid; about 16 × 11	Yes	No OR or M; smooth, pale tan to tan OW (1)
<i>Isospora samsensis</i> Svanbaev, 1979	<i>Spermophilus fulvus</i> (Sciuridae)	Russia	Ovoid; 25–32 × 22–26	Ellipsoidal; 11–13 × 8–9	No	No OR or M; smooth greenish OW (1.2–1.5)
<i>Isospora</i> sp.* Barnard, Ernst & Dixon, 1974	<i>S. hispidus</i> (Muridae)	North America (Alabama)	Subspherical to ovoid; 21–28 × 20–25	Ovoid; 14–18 × 9–12	Yes	No OR or M; smooth, reddish to light brown OW (1.3)
<i>Isospora</i> sp. Nukerbaeva & Svanbaev, 1973	<i>Myocastor coypus</i> (Myocastoridae)	Russia	Short-oval to spherical; 25–28 × 25–28	About 14 × 11	No record	OR present; no M; OW (1.5)
<i>Isospora</i> sp. Stout & Duszynski, 1983	<i>Dipodomys agilis</i> (Heteromyidae)	North America	Spherical or subspherical; 21–28 × 20–28	Broadly ovoid; 12–19 × 9–13	Yes	No OR or M; smooth pale yellow OW (1.6)

Table 5 (Continued)

Species	Type host	Type locality	Shape and size of oocysts	Shape and size of sporocysts	Presence of SB	Other
<i>Isospora</i> sp. * Svanbaev, 1963	<i>Marmota menzbieri</i> (Scuridae)	Russia	Short-ovoid to spherical; 20–22 × 19–20	Ovoid; 14–15 × 7–8	No record	No OR or M; smooth, greenish; double-contoured OW (1)
<i>Isospora spermophili</i> Levine, 1984	<i>Spermophilus maximus</i> (Scuridae)	Russia	Ellipsoidal, subspherical or spherical; 18–29 × 18–24	Ellipsoidal or spherical; 8–13 × 7–11	No record	OR present; no M; smooth yellow-green or brown OW (1.5–2)
<i>Isospora tamariscini</i> Levine, 1985	<i>Meriones tamariscinus</i> (Muridae)	Russia	Ellipsoidal or subspherical; 21–30 × 20–26	Ellipsoidal or ovoid; 12–14 × 7–10	No record	OR present; no M; smooth yellow-green OW (1.5–1.9)
<i>Isospora teres</i> Iwanoff-Gobzem, 1934	<i>L. lagurus</i> (Muridae)	Russia	Spherical; 24–36 × 24–36	16–21 × 8–13	No record	No OR or M
<i>Isospora uralicae</i> Svanbaev, 1956	<i>Apodemus sylvaticus</i> (Muridae)	Russia	Ovoid; about 26 × 22.5	Ovoid; about 14 × 9	No	No OR or M; smooth greenish double-contoured OW (1.6)
<i>Isospora vanadica</i> Musaev & Veisov, 1965	<i>Meriones persicus</i> (Muridae)	Russia	Ellipsoidal or ovoid; 20–28 × 14–24	Ovoid; 10–16 × 6–12	No	OR present; no M; yellowish to dark brown OW (1.5–2.5)
<i>Isospora vinogradovi</i> Musaev & Veisov, 1965	<i>M. vinogradovi</i> (Muridae)	Russia	Ellipsoidal, rarely ovoid; 22–28 × 18–24	Piriform; 12–16 × 8–10	Yes	OR present; no M; smooth colorless OW (2–2.5)

Using the Sherman live traps, 11 bank voles were trapped in the South Bohemian Region (Lužnice) and Pilsen Region (Tymákov). Four of them were used for the experimental infections (Table 3). All live, repeatedly examined individuals predetermined for the experimental infections, were coccidia-negative. Three of them were repeatedly positive for eggs of intestinal nematodes (*Capillaria* sp. and *Trichuris* sp.) (Table 4).

Morphology and Morphometry

Morphological analyses showed that all sporocysts in *Isospora*-type oocysts found in the faeces of bank voles possessed SB and substieda bodies (sSB). This feature reinforces their presumed phylogenetic affinity to bird isosporans (family Eimeriidae). The samples used for the morphological and morphometrical analyses are described below (MG, *Myodes glareolus*; TYM, locality Tymákov; LUZ, locality Lužnice). Out of them, 2_MG_TYM and

9_MG_LUZ were used for the experimental infections. All given measurements (including those in Table 5) are in micrometers (μm), with the means given in parentheses following the ranges.

2_MG_TYM (Fig. 1)

Oocysts were ovoidal to ellipsoidal, $24.0\text{--}29.0 \times 19.0\text{--}24.0$ (25.8×21.5) with a smooth, bi-layered oocyst wall (OW) approximately 1.2–1.4 thick. The colour of the wall was pale yellow–green. Neither micropyle (M) nor oocyst residuum (OR) were present. The oocysts possessed at least 3 distinct polar granules (PGs) of irregular shape. Sporocysts were ovoidal to ellipsoidal, $14.0\text{--}17.2 \times 9.0\text{--}11.0$ (15.0×10.1) with a thin, colorless wall. The sporocysts possessed a conspicuous nipple-like SB (1.2×1.0), and wide-rounded sSB. The parastieda body (pSB) was absent. The sporocyst residuum (SR) was present as a relatively compact structure composed of

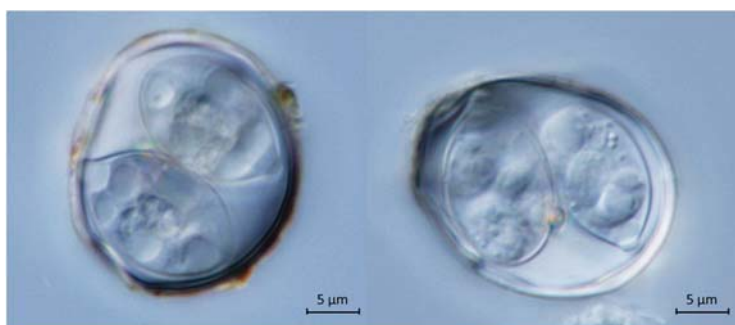


Figure 1. Sporulated oocysts obtained from *M. glareolus* isolate 2_MG_TYM (Tymákov, Pilsen Region, CZ).

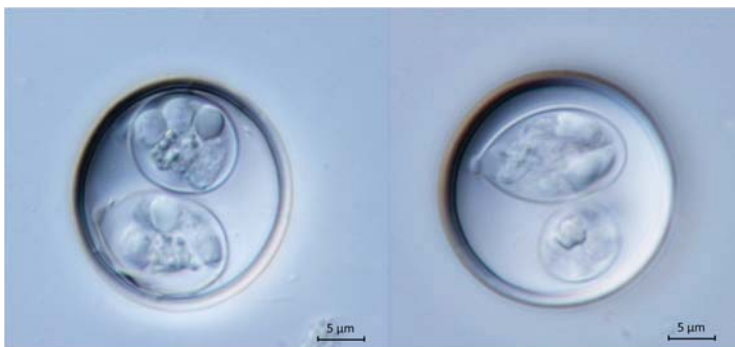


Figure 2. Sporulated oocysts obtained from *M. glareolus* isolate 49_MG_TYM (Tymákov, Pilsen Region, CZ).

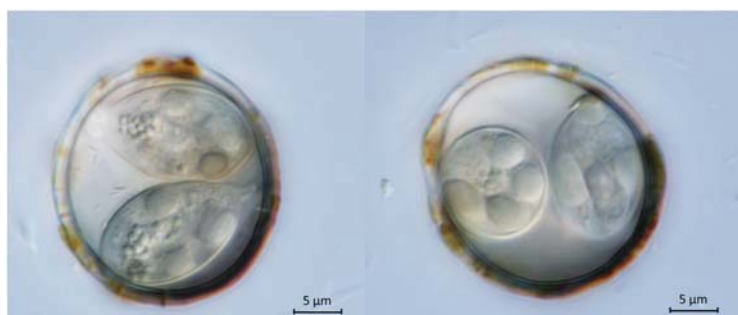


Figure 3. Sporulated oocysts obtained from *M. glareolus* isolate 57_MG_TYM (Týmákov, Pilsen Region, CZ).

large globules. Sporozoites were elongate, with globular to oval posterior and anterior refractile bodies, and the nucleus located between them (in the middle).

49_MG_TYM (Fig. 2)

Oocysts were spherical to subspherical, $23.5\text{--}28.3 \times 22.6\text{--}27.8$ (25.8×24.1) with a smooth, bi-layered and relatively thick OW (approximately 1.6). The colour of the wall was pale brown. Oocysts were without M, with a small poorly apparent compact OR. The oocysts possessed 2–4 splinter-like PGs. Sporocysts were ellipsoidal to bottle-shaped, $14.3\text{--}19.0 \times 9.8\text{--}11.4$ (15.9×10.7) with a thin, colorless wall. The sporocysts possessed nipple-like SB 1.1×0.7 , and indistinct sSB. The pSB was absent. The SR was present as globules of various sizes scattered among SPs. SPs were elongate, with posterior and anterior globular refractile bodies, and the nucleus located between them (in the middle).

57_MG_TYM (Fig. 3)

Oocysts were spherical to subspherical, $25.8\text{--}29.3 \times 24.1\text{--}27.0$ (27.7×25.7) with a smooth, bi-layered OW approximately 1.8 thick. The colour of the wall was yellowish to brownish. Neither M nor OR were present. The oocysts possessed 2–3 PGs of irregular shape and various size. Sporocysts were ellipsoidal to bottle-shaped, $13.7\text{--}18.7 \times 9.4\text{--}12.1$ (16.4×10.7) with a thin, colorless wall. The sporocysts possessed knob-like SB 1.3×1.0 , with rounded to conical sSB. The pSB body was absent. The SR comprised of large globules that were dispersed among SPs. SPs were elongate, with large posterior and small

anterior refractile bodies of globular shape, and the nucleus located between them (in the middle).

7_MG_LUZ (Fig. 4)

Oocysts were subspherical, $25.0\text{--}30.0 \times 23.0\text{--}24.0$ (28.3×23.7) with a smooth, bi-layered and relatively thick OW (approximately 1.8). The colour of the wall was pale yellow–brown. The oocysts lacked M, and possessed a poorly apparent compact OR. A single globular PG was present. Sporocysts were ellipsoidal, $15.7\text{--}18.9 \times 10.5\text{--}12.0$ (17.3×11.3) with a thin, colorless wall. The sporocysts possessed a knob-like SB 1.2×1.0 , and wide-rounded sSB. The pSB was absent. The SR was present as a compact structure composed of small globules. SPs were elongate, with globular to bean-shaped



Figure 4. Sporulated oocysts obtained from *M. glareolus* isolate 7_MG_LUZ (Lužnice, South Bohemian Region, CZ).



Figure 5. Sporulated oocysts obtained from *M. glareolus* isolate 9_MG_LUZ (Lužnice, South Bohemian Region, CZ).

posterior and anterior refractile bodies, and the nucleus located between them (in the middle).

9_MG_LUZ (Fig. 5)

Oocysts were subspherical to ellipsoidal, $24.0\text{--}28.4 \times 19.0\text{--}25.0$ (26.1×22.4) with a smooth, bi-layered OW approximately 1.4 thick. The colour of the wall was pale yellow-green. Neither M nor OR were present. The oocysts possessed at least 1 distinct PG of globular shape. Sporocysts were ellipsoidal, $14.0\text{--}18.6 \times 9.2\text{--}12.0$ (16.0×10.3) with a thin, colorless wall. The sporocysts possessed a conspicuous SB of triangular shape 1.2×1.1 , and sSB of rounded shape. The pSB was absent. The SR was present in a form of compact small globules apparently surrounded by a thin membrane. SPs were elongate, with posterior and anterior refractile bodies of globular shape, and the nucleus located between them (in the middle).

Phylogenetic Analyses

Phylogenetic analyses of all 3 genes showed that *Isospora* spp. obtained from arvicoline rodents (mainly bank voles) are closely related to the isosporans of birds (family Eimeriidae).

The sequences of *Eimeria* spp., obtained mostly from bank voles trapped in the Czech Republic, were also included in the phylogenetic analyses. All phylograms showed that these eimerians formed phylogenetically distinct lineages separated from isosporans obtained from arvicoline rodents/voles/bank voles.

Furthermore, this study shows that the genus *Isospora* (Eimeriidae) is not a monophyletic, as proposed by Barta et al. (2005), but a paraphyletic

taxon. These findings are best seen in the phylogenetic tree of 18S rDNA (Fig. 6), where the isosporans of reptiles (mostly from lizards) cluster almost on the basal position of the eimeriid coccidia, while isosporans of birds cluster within the family Eimeriidae, surprisingly close to the eimerians of rabbits. The paraphyly of the genus *Isospora* has also been recently proposed; based on these findings, establishing a number of new genera of eimeriid coccidia based on molecular phylogeny (monophyletic clades) has been discussed (Ogedengbe et al. 2018).

Experimental Infections

The experimental infections also supported our hypothesis of pseudoparasitism. Infections did not develop in any of the bank voles administered with the fully sporulated *Isospora*-like oocysts. It was, however, observed that for the first 4 days after the inoculation the bank voles shed more or less deformed/damaged oocysts passing through their gastrointestinal tract (Fig. 9). Based on these findings we can assume that it is not a real infection, but an accidental passage through the gastrointestinal tract of these rodents.

Discussion

The main problem of *Isospora* classification, species description, and host specificity determination is currently posed by the samples found in the faeces of mammals. Based on the relatively recent phylogenetic studies, a reclassification of the genus *Isospora* was carried out. The studies showed that isosporans infecting mammals are phylogenetically related to Sarcocystidae, while isosporans infecting

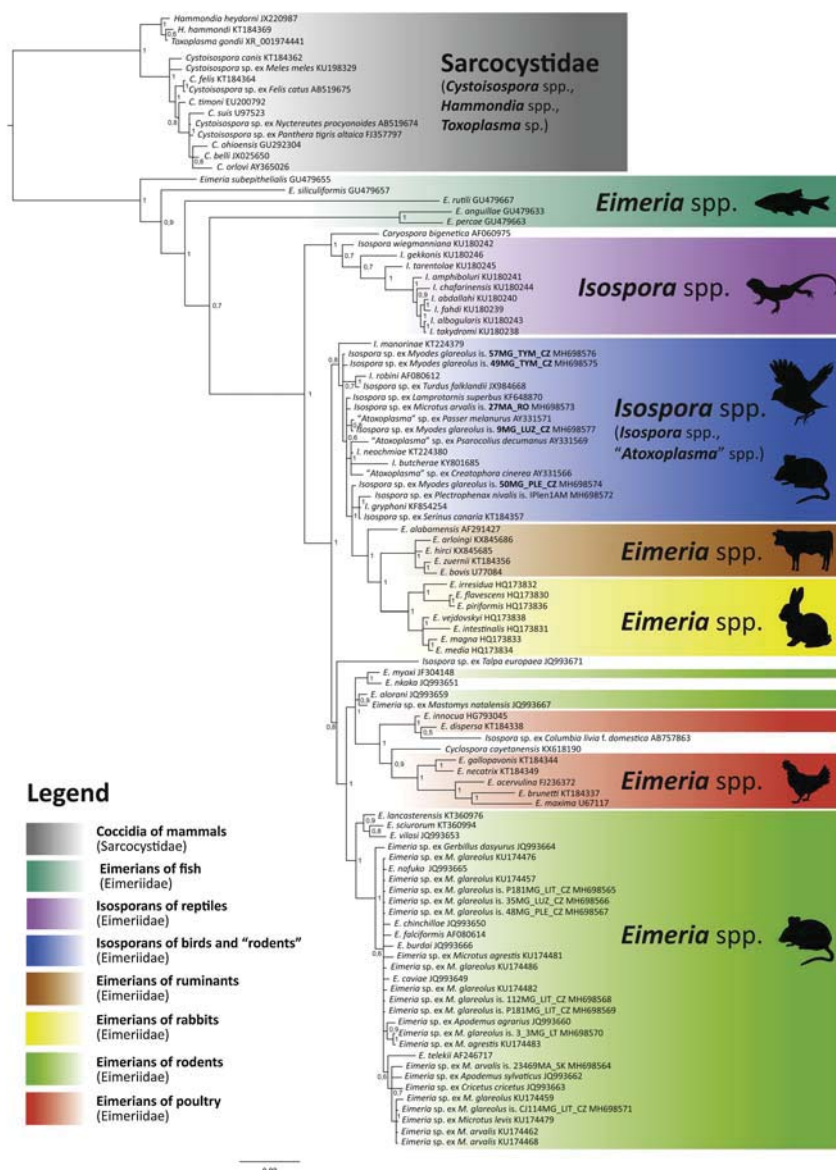


Figure 6. Phylogenetic relationships inferred by the BI analysis of the 18S rRNA sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. The family Sarcocystidae is used as an outgroup.

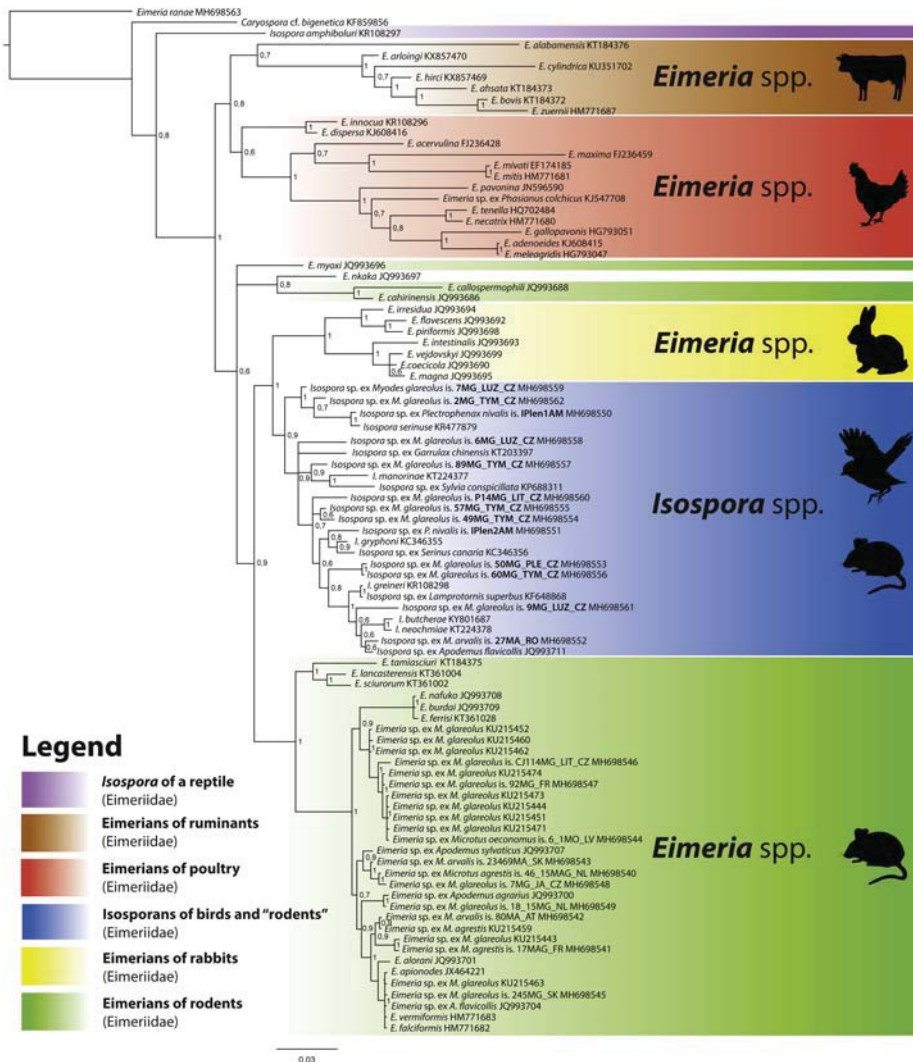


Figure 7. Phylogenetic relationships inferred by the BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Eimeria ranae* is used as an outgroup.

mainly birds are related to Eimeriidae, and the presence/absence of the SB complex was established as the key morphological trait (Barta et al. 2005; Carreno et al. 1998; Franzen et al. 2000; Jirků et al. 2002).

To date, the descriptions of coccidian species were mainly based on the morphology of oocysts occurring in the faeces of various hosts. In total, 38 *Isospora* spp. have been described from rodents till now. Out of these, 6 *Isospora* species have

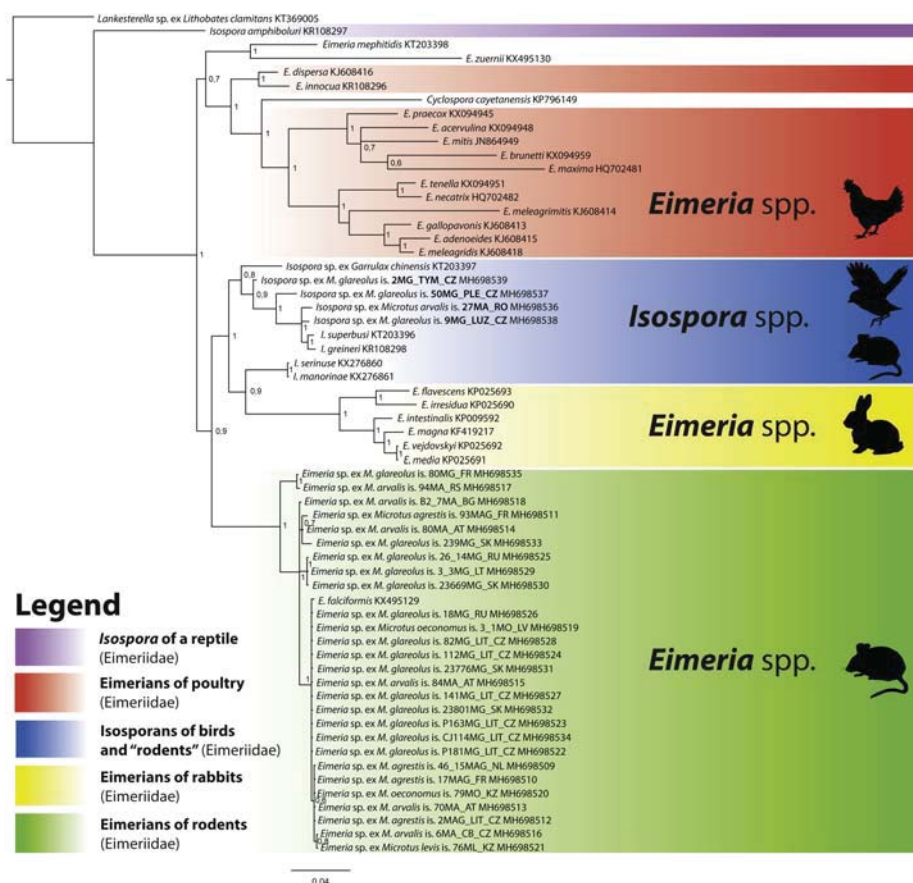


Figure 8. Phylogenetic relationships inferred by the BI analysis of the COIII sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Lankesterella* sp. is used as an outgroup.

been described from voles, specifically 2 of them from bank voles. Regarding their morphology, 17 *Isospora* spp. possessing the SB complex, 12 *Isospora* spp. lacking the SB, and 9 *Isospora* spp., for which the presence of the SB complex was not recorded, have been described (Levine and Ivens 1990; Table 5). At present, all those without the SB complex have been assigned to the genus *Cystoisospora* (Carreno and Barta 1999; Carreno et al. 1998).

However, species of *Isospora* possessing the SB complex and conspicuously resembling avian representatives of the genus have been described.

For instance, Levine and Mohan (1960) described an *Isospora* from cattle the oocysts of which were almost identical with the oocysts of *Isospora lacazei* originally described from an English sparrow (*Passer domesticus*; Aves: Passeriformes). Ernst et al. (1969) then described *Isospora* sp. from an opossum (*Didelphis marsupialis*; Mammalia: Didelphimorphia: Didelphidae) the oocysts of which also substantially resembled those of *I. lacazei*. The oocysts of *Isospora* sp. found in the hispid cotton rat (*S. hispidus*) by Barnard et al. (1974) also resembled isosporan oocysts from birds, furthermore, in that case, experimental infections were repeat-



Figure 9. Deformed oocysts that passed through the gastrointestinal tract of bank voles after the administration of isolate of sporulated *Isospora*-type oocysts. OW, oocyst wall; SB, Stieda body; SP, sporocyst.

edly carried out, but with unsuccessful results. The authors assumed that the above mentioned isosporans were pseudoparasites originated from birds.

In discrepancy with the study of Carreno and Barta (1999) is the study of Upton et al. (1985), which describes the morphology and endogenous development of *Isospora* possessing the SB in detail; *I. masoni* Upton, Lindsay, Current and Ernst, 1985 was described from the hispid cotton rat based on morphology and experimental transmission. Oocysts had a thin wall, and each sporocyst possessed the SB and sSB complex. For this species, the endogenous developmental stages were identified and localized mostly in enterocytes of ileum. Extraintestinal stages were not found, hence this species was considered to be a coccidium with a monoxenic direct life cycle. The prepatent period was 4–7 days and the patent period lasted for more than 40 days. Sporulation was endogenous, the sporocysts were shed directly in the faeces. Subsequently, successful experimental transmission to the hispid cotton rat via both sporocysts and sporulated oocysts was carried out. Its endogenous sporulation, thin OW, and relatively small sporocyst size ($7\text{--}9 \times 5\text{--}6$; Table 5) led Upton et al. (1985) to emphasize the uncertainty as to where this organism indeed belongs. Nonetheless, they placed this species in the genus *Isospora* based on the presence of SB and sSB, and also based on the apparent monoxenic life cycle. According to morphological and biological features, this coccidium cannot be unequivocally classified as coccidia either of the family Sarcocystidae or Eimeriidae. Unfortunately, there are no molecular data available for this species. Similarly, no molecular data exist on any *Isospora* spp.

described from rodents. Considering all the species given in Table 5, it has not been so far determined which is a *Cystoisospora* related to coccidia of the family Sarcocystidae, and which a real *Isospora* belonging to Eimeriidae, thus, demonstrating that a resolution of the origin of *Isospora* spp. is much more complicated.

It was previously reported that unsporulated and sporulated oocysts can pass unchanged and unharmed through the intestinal tract of the mammal host (Prasad 1961; Streitel and Dubey 1976). However, the oocysts can become deformed by multiple passage. Thus, the isosporan oocysts (i.e. with SB) were probably transmitted to bank voles (and also probably to other rodents) via the consumption of food contaminated by avian droppings. There remains the question of whether the oocysts of *Isospora* spp. discharged in this way (i.e. passed through the gastrointestinal tract of rodents) are subsequently able to infect the original avian host. Hypothetically, if sporocysts are intact the sporozoites inside should remain infective. The problem lies in the fact that we do not know from which bird species these isosporans originate.

Conclusion

To conclude, the hypothesis of pseudoinfection has been demonstrated based on morphological analyses, phylogenetic analyses, and experimental infection. Morphological analyses showed the presence of the SB complex on sporocysts. Phylogenetic analyses showed a very close phylogenetic relationship between *Isospora* spp. obtained from bank voles and avian isosporans. Experimental inoculations of the bank voles with sporulated oocysts of *Isospora* sp. did not result in the production of unsporulated oocysts. Hence, these organisms should be considered pseudoparasites of the bank vole/rodents. Furthermore, sporulated oocysts of some species of *Isospora* were found in the faeces of 2 live-trapped bank voles. Some of them were already partially deformed. During the further coprological examination, these rodents were coccidia-negative. The bank voles apparently ate contaminated food, and the oocysts merely passed through their gastrointestinal tracts.

Methods

Origin of the hosts and parasites: Bank voles (*M. glareolus*) were trapped in the course of 2015–2017 using classic wooden snap traps and Sherman live traps. The rodents were trapped across the Czech Republic in the South Bohemian Region

(České Budějovice, Lužnice, Třisov), Ústí nad Labem Region (Kláštrec nad Ohří, Litvínov), Karlovy Vary Region (Stružná), and in the Pilsen Region (Tymákov) (Table 1).

The faeces of each individual snap-trapped vole were collected and preserved in 4% (w/v) aqueous potassium dichromate ($K_2Cr_2O_7$) solution. Live-trapped animals were placed in plastic boxes (Velaz type T II, Velaz, Prague, Czech Republic). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia, and also by the Ministry of the Environment of the Czech Republic (Permit Numbers 27873/ENV/11 and 22395/2014-MZE-17214).

Coprology and morphological studies: Faecal samples collected in the field were examined for the presence of coccidian oocysts using density-gradient flotation with Sheather's sucrose solution (specific gravity 1.30) (Duszynski and Wilber 1997; Sheather 1923; Zajac and Conboy 2006). Coccidia-positive samples were allowed to sporulate on air at room temperature. The live-trapped voles were examined at least five times to ensure that they were really coccidia/*Isospora*-negative. For the determination of isosporan oocysts, an Olympus BX53 light microscope equipped with a digital camera and Olympus cellSens Standard 1.13 imaging software was used. The determination was based on the morphological and morphometrical analyses of sporulated oocysts (Berto et al. 2014; Duszynski and Wilber 1997).

DNA isolation, PCR amplification, and sequencing: Genomic DNA was extracted from the *Isospora*-positive faecal samples using the FastDNA[®] SPIN for Soil Kit (MP Biomedicals, LLC, Santa Ana, California, USA) following the manufacturer's protocol. PCR amplification was performed with coccidia-specific primers amplifying the gene encoding the small subunit of 18S rRNA, and mitochondrial genes for cytochrome c oxidase subunit I (COI) and III (COIII). Primers for 18S rDNA and COI were adopted from Schwarz et al. (2009) and Kvičerová et al. (2008), respectively. Sequences of primers amplifying the COIII region were provided by John R. Barta (University of Guelph, Ontario, Canada). HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) was used for all PCR reactions. PCR products were purified with alkaline phosphatase and exonuclease I enzymes (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and sequenced via the Sanger sequencing method in SEQme, s.r.o. (Dobříš, Czech Republic).

Sequence processing and phylogenetic analyses: The obtained sequences of *Isospora* spp. were verified by the BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were further processed using the Sequence Scanner v2.0 (Applied Biosystems), EditSeq 5.05, and SeqMan 5.05 (DNASTAR, Inc., Madison, Wisconsin, USA) programs. Coccidian sequences of 18S rRNA, COI, and COIII genes obtained from the NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) together with the newly obtained sequences of our samples were used in phylogenetic analyses. The accession numbers of all sequences used in the analyses (including the newly obtained sequences) are indicated on resultant phylogenetic trees (Figs 6–8). Alignments were created in Geneious v9.1.3 (<http://www.geneious.com>, Kearse et al. 2012) using the MAFFT v1.3.6 algorithm with default parameters (Katoh and Standley 2013), and manually adjusted. 18S rDNA sequences were aligned in the nucleotide mode; COI and COIII sequences were aligned in the amino acid mode, then switched to nucleotide mode, and used for the analyses. Phylogenetic relationships were reconstructed using the Bayesian inference (BI) in the program MrBayes v3.2.6 (Huelsenbeck and Ronquist 2001). The best fitting evolutionary models

were selected by the SMS: Smart Model Selection software (<http://www.atgc-montpellier.fr/sms/>, Lefort et al. 2017). BI analysis was performed using the GTR+ Γ +I evolutionary model for 10 million generations for all analyses, and the trees were summarized after removing 25% burn-in. Phylogenetic trees were visualized and exported by FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/>).

Experimental infections: For the experimental inoculations, isolates of fully sporulated oocysts of *Isospora* spp., originating from the faeces of voles trapped into the snap traps in the field samplings, were purified on the sucrose gradient.

Coccidia-negative live-trapped voles were kept individually in the plastic boxes (separated from each other), and their faeces were collected and examined daily for the presence of coccidia for 14 days; all animals were coproscopically negative by light microscopy, and were thus considered to be negative prior to the experimental infection. Moreover, before the experimental inoculation, the rodents were twice preventively treated with Baycox[®] (2.5% toltrazuril; Bayer Animal Health GmbH, Leverkusen, Germany) in the form of peroral suspension (46 ml/100 kg). More specifically, the first dose was administered 3 weeks before the experimental inoculation, and the second 3 days after the first dose.

Before the experimental inoculation, each animal was slightly anesthetized with diethylether (Penta s.r.o., Prague, Czech Republic). Sporulated oocysts of *Isospora* spp. were inoculated via syringe with an olive-tipped needle into the oesophagus of the anesthetized animal. After inoculation, the faeces of each animal were daily collected and examined by flotation technique with Sheather's sucrose solution until 23 days postinoculation (DPI). To obtain the faeces, the animals were individually placed into clean disinfected empty Velaz boxes (separated from each other) and left there for at least 4 h.

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References

- Barnard WP, Ernst JV, Dixon CF (1974) Coccidia of the cotton rat, *Sigmodon hispidus*, from Alabama. *J Parasitol* **60**:406–414
- Barta JR, Schrenzel MD, Carreno RA, Rideout BA (2005) The genus *Atoxoplasma* (Garnham 1950) as a junior objective synonym of the genus *Isoospora* (Schneider 1881) species infecting birds and resurrection of *Cystoisospora* (Frenkel 1977) as the correct genus for *Isoospora* species infecting mammals. *J Parasitol* **91**:726–727
- Berto BP, McIntosh D, Lopez CWG (2014) Studies on coccidian oocysts (Apicomplexa: Eucoccidiorida). *Braz J Vet Parasitol* **23**:1–15
- Berto BP, Lopes BDB, Flausino W, Teixeira Filho WL, Lopes CWG (2008) Contribution on the study of *Isoospora hemidactyli* Carini, 1936 and a report of an adeleid pseudoparasite of the house gecko *Hemidactylus mabouia*, from the Rio de Janeiro Metropolitan Region, Brazil. *Braz J Vet Parasitol* **17**:150–154
- Berto BP, Lopes BDB, Teixeira Filho WL, Flausino W, Lopes CWG (2010) Coccidios de vertebrados associados ao hábito alimentar de vertebrados: uma revisão breve dos gêneros Adelea, Adelina e Barroussia. *Braz J Vet Med* **32**:33–41
- BLAST search tool; Retrieved from: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>; Last accessed on the 8th May 2018.
- Carreno RA, Barta JR (1999) An eimeriid origin of isosporoid coccidia with stieda bodies as shown by phylogenetic analysis of small subunit ribosomal RNA gene sequences. *J Parasitol* **85**:77–83
- Carreno RA, Schnitzler BE, Jeffries AC, Tenter AM, Johnson AM, Barta JR (1998) Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isoospora* is most closely related to *Toxoplasma* and *Neospora*. *J Eukaryot Microbiol* **45**:184–188
- Dubey JP (1975) Experimental *Isoospora canis* and *Isoospora felis* infection in mice, cats, and dogs. *J Protozool* **22**:416–417
- Dubey JP (1978a) Life cycle of *Isoospora ohioensis* in dogs. *Parasitology* **77**:1–11
- Dubey JP (1978b) Pathogenicity of *Isoospora ohioensis* infection in dogs. *J Am Vet Med Ass* **173**:192–197
- Dubey JP, Frenkel JK (1972) Extra-intestinal stages of *Isoospora felis* and *I. rivolta* (Protozoa: Eimeriidae) in cats. *J Protozool* **19**:89–92
- Duszynski DW, Upton SJ (2001) *Cyclospora*, *Eimeria*, *Isoospora* and *Cryptosporidium* spp. . In Samuel WM, Pybus MJ, Kocan AA (eds) *Parasitic Diseases of Wild Mammals*. Iowa State University Press, IA, USA, pp 416–459
- Duszynski DW, Wilber PG (1997) A guideline for the preparation of species descriptions in the Eimeriidae. *J Parasitol* **83**:333–336
- Ernst JV, Cooper C Jr, Chobotar B (1969) *Isoospora boughtoni* Volk, 1938 and *Isoospora* sp. (Protozoa: Eimeriidae) from an opossum *Didelphis marsupialis*. *J Wildlife Dis* **5**:406–409
- FigTree v1.4.2 graphics data visualisation software; Retrieved from: <http://tree.bio.ed.ac.uk/>; Last accessed on the 30th July 2018.
- Franzen C, Müller A, Bialek R, Diehl V, Salzberger B, Fätkenheuer G (2000) Taxonomic position of the human intestinal protozoan parasite *Isoospora belli* as based on ribosomal RNA sequences. *Parasitol Res* **86**:669–676
- Frenkel JK (1977) *Besnoitia wallacei* of cats and rodents: with a reclassification of other cyst-forming isosporoid coccidia. *J Parasitol* **63**:611–628
- Geneious v9.1.3 bioinformatics software; Retrieved from: <http://www.geneious.com>; Last accessed on the 2nd July 2018.
- Ghimire TR (2010) Redescription of genera of family Eimeriidae Minchin, 1903. *Int J Life Sci* **4**:26–47
- Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**:754–755
- Jirků M, Modrý D, Šlapeta JR, Koudela B, Lukeš J (2002) The phylogeny of *Goussia* and *Choleoeimeria* (Apicomplexa; Eimeriorina) and the evolution of excystation structures in coccidia. *Protist* **153**:379–390
- Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* **30**:772–780
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**:1647–1649
- Kvičerová J, Pakandl M, Hypša V (2008) Phylogenetic relationships among *Eimeria* spp. (Apicomplexa Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. *Parasitology* **135**:443–452
- Lefort V, Longueville JE, Gascuel O (2017) SMS: Smart Model Selection in PhyML. *Mol Biol Evol* **34**:2422–2424
- Levine ND (1982) Taxonomy and Life Cycles of Coccidia. In Long PL (ed) *The Biology of the Coccidia*. University Park Press, Baltimore, USA, pp 1–33
- Levine ND (1988) The Protozoan Phylum Apicomplexa Vol. 2, CRC Press, Boca Raton, FL, USA, 154 p
- Levine ND, Ivens V (1990) The Coccidian Parasites of Rodents. CRC Press, Boca Raton, FL, USA, pp 5–183
- Levine ND, Mohan RN (1960) *Isoospora* sp. (Protozoa: Eimeriidae) from cattle and its relationship to *I. lacazei* of the English sparrow. *J Parasitol* **46**:733–741
- Morrison DA, Bornstein S, Thebo P, Wernery U, Kinne J, Mattson JG (2004) The current status of the small subunit rRNA phylogeny of coccidia (Sporozoa). *Int J Parasitol* **34**:501–514
- NCBI GenBank online database; Retrieved from: <https://www.ncbi.nlm.nih.gov/genbank/>; Last accessed on the 28th June 2018.
- Ogedengbe ME, El-Sherry S, Ogedengbe JD, Chapman HD, Barta JR (2018) Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). *Int J Parasitol* **48**:59–69
- Pellérdy L (1974) Coccidia and Coccidiosis. *Akadémiai Kiadó, Budapest, Hungary*, 65 p

Prasad H (1961) A new species of *Isospora* from the fennec fox *Fennecus zerda* Zimmerman. *Z Parasitenkd* **21**:130–135

Samarasinghe B, Johnson J, Ryan U (2008) Phylogenetic analysis of *Cystoisospora* species at the rRNA ITS1 locus and development of a PCR-RFLP assay. *Exp Parasitol* **118**:592–595

Schneider A (1881) Sur les psorospermies oviformes ou coccidies. *Especies nouvelles ou peu connues. Arch Zool Exp Gén* **9**:387–404 (In French)

Schwarz RS, Jenkins MC, Klopp S, Miska KB (2009) Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *J Parasitol* **95**:871–880

Sheather AL (1923) The detection of intestinal protozoa and mange parasites by a flotation technique. *J Comp Pathol* **36**:266–275

Smith DD (1981) The Sarcocystidae: Sarcocystis, Frenkelia, Toxoplasma, Besnoitia, Hammondia, and Cystoisospora. *J Protozool* **28**:262–266

SMS: Smart Model Selection online bioinformatics software; Retrieved from: <http://www.atgc-montpellier.fr/sms/>; Last accessed on the 20th September 2018.

Streitel RH, Dubey JP (1976) Prevalence of *Sarcocystis* infection and other intestinal parasitisms in dogs from a humane shelter in Ohio. *J Am Med Ass* **168**:423–424

Teixeira M, Albuquerque GR, Lopes CW, Florentino MV (2003) An adeleid coccidia, a pseudoparasite of *Didelphis aurita* (Marsupialia: Didelphoidea). *Braz J Vet Parasitol* **12**: 33–35

Upton SJ, Lindsay DS, Current WL, Ernst JV (1985) *Isospora masoni* sp. n. (Apicomplexa: Eimeriidae) from the Cotton Rat, *Sigmodon hispidus*. *Proc Helminthol Soc* **52**: 60–63

Zajac AM, Conboy GA (2006) Veterinary Clinical Parasitology. Blackwell Publishing, Iowa, USA, pp 3–8

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New record of *Microtus mystacinus* in eastern Kazakhstan: phylogeographical considerations

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Abstract

The Eastern European vole (*Microtus mystacinus*) is an arvicoline rodent distributed across northern and eastern Europe, the Balkans, Turkey, Armenia, NW and N Iran, Russia as far east as the Tobol River in W Siberia, and W and N Kazakhstan. We present a novel records from eastern Kazakhstan (the village of Dzhambul – 49°14'21.3"N, 86°18'29.9"E and the village of Sekisovka – 50°21'9.18"N, 82°35'46.5"E) based on mtDNA and we discuss implications of this findings on biogeography of eastern Kazakhstan populations. Marine Isotope Stage 11 is considered an important period for the diversification of the *arvalis* species group. In the context of our study, it is important to analyse genetically discontinuous Siberian populations, and the current distribution of *M. mystacinus* in new localities in eastern Kazakhstan.

Keywords

Microtus mystacinus, Kazakhstan

Introduction

The Eastern European vole, *Microtus mystacinus* De Filippi, 1865, is an arvicoline rodent with an unsettled nomenclature. It has been named most commonly as *M. subarvalis* Meyer, Orlov & Skholl, 1972, *M. epiroticus* Ondrias, 1966, *M. rossiaemeridionalis*

Ognev, 1924, and *M. levis* Miller, 1908 (e.g., Musser and Carleton 2005; Kryštufek and Vohralík 2005). We adhere to the name *M. mystacinus*, following the detailed study by Mahmoudi et al. (2017) and the review of Kryštufek (2017). Despite its nomenclature instability, there is a consensus about its phylogenetic affinities: this species has been traditionally attributed to the *arvalis* species group in the subgenus *Microtus* s. str. (Musser and Carleton 2005). This view has been strongly supported by chromosomal and genetic evidence (e.g., Mazurok et al. 2001, Jaarola et al. 2004, Mahmoudi et al. 2017). According to new studies, it is related to the following species: *M. ilaeus* Thomas, 1912 (syn. *M. kirgisorum* Ognev, 1950), *M. transcasicus* Satunin, 1905, *M. kermanensis* Roguin, 1988, *M. arvalis* (Pallas, 1778), and *M. obscurus* (Eversmann, 1841) (e.g., Golenishchev et al. 2000; Jaarola et al. 2004; Kryštufek and Vohralík 2005; Mahmoudi et al. 2017), but it is the closest relative of *M. arvalis* and *M. obscurus* based on available DNA data (cyt *b*; Mahmoudi et al. 2017).

In general, *M. mystacinus* represents one of the best cases of a cryptic species in arvicoline, because it was primarily recognized by chromosomal number (*M. mystacinus*: $2n = 54$; *M. arvalis*: $2n = 46$) (Meyer et al. 1969; Mazurok et al. 2001; Pavlova and Tchabovsky 2011). It is now generally considered a valid species of the genus *Microtus* based on hybridisation data, and chromosomal and genetic differences (for reviews see Kryštufek and Vohralík 2005 and Musser and Carleton 2005). Several authors have attempted to distinguish *M. mystacinus* from the common vole (*M. arvalis*), the Altai vole (*M. obscurus*), and the Middle Eastern vole (*M. transcasicus*) based on morphological data (Král et al. 1981; Zagorodnyuk 1991a, b; Masing 1999; Hotzi et al. 2008; Markova et al. 2009, 2012; Markov et al. 2012; Ghorbani et al. 2015). Although some diagnostic characters have been proposed (e.g., qualitative and quantitative cranial and dental morphology) and multivariate morphometric approaches have been applied (e.g., Markov et al. 2012; Markova et al. 2012), these approaches have been lacking in diagnostic power (Kryštufek and Vohralík 2005; Markov et al. 2012), except for characters proposed by Kryštufek and Vohralík (2005).

The distribution and habitat preferences of the Eastern European vole are relatively well known due to the intensive attention devoted to it (see Kryštufek and Vohralík 2005; Musser and Carleton 2005; Shenbrot and Krasnov 2005; Kryštufek 2017, and references therein). It prefers to live in places with high and dense herbaceous or grassy vegetation, hedgerows, and stands of reeds and it avoids short-grass meadows and dry areas (Kryštufek and Vohralík 2005; Aulagnier et al. 2009; Kryštufek 2017). The distribution range of the Eastern European vole, to date, extends from southern Finland, the Baltic eastwards to western Siberia with patches in the southern Urals, the Novosibirsk suburbs to the southwest margin of Lake Baikal and Buryatia, the southern Caucasus, northern Iran to Turkey, connecting to Greece and the majority of the Balkan Peninsula to Ukraine (Baskevich 1996; Gileva et al. 1996; Yakimenko and Kryukov 1997; Musser and Carleton 2005; Shenbrot and Krasnov 2005; Pavlova and Tchabovsky 2011; Ghorbani et al. 2015; Baskevich et al. 2016; Kryštufek 2017; Moroldoev et al. 2017).

Populations occupying the Arctic Svalbard Archipelago (Fredga et al. 1990; recently extinct according to Aulagnier et al. 2009), Jan Mayen Island in the N Atlantic (Kryštufek

2017), Olkhon Island in Lake Baikal (Pavlova and Tchabovsky 2011; Kryštufek 2017) and Far Eastern Russia (Khabarovsk Krai, near Sovetskaya Gavan City, see Kartavtseva et al. 2012; Tiunov et al. 2013) are probably introduced. *M. mystacinus*, *M. arvalis*, and *M. obscurus* broadly overlap in distribution and occur sympatrically in a few regions (e.g., Meyer et al. 1996; Musser and Carleton 2005; Shenbrot and Krasnov 2005 see also Tougaard et al. 2013).

When considering the distribution of *M. mystacinus* within Kazakhstan, there are records from the western or north-western parts. The easternmost record is from the Karabalyk district (Kovalskaya 1994; Meyer et al. 1996). Here, we report an additional record of *M. mystacinus* from eastern Kazakhstan and comment on it from a phylogeographic point of view.

Materials and methods

A survey of small mammals conducted in eastern Kazakhstan provided the surprising discovery of three specimens of *M. mystacinus*, that are characterized here based on molecular methods. The first sample (Kazakhstan 1) was collected in July 2006 on pasture land near the village of Dzhambul (GPS coordinates: 49°14'21.3"N, 86°18'29.9"E) by FS and two more specimens (Kazakhstan 2, 3) were collected in September 2017 near a pond not far from the village Sekisovka (GPS coordinates: 50°21'9.18"N, 82°35'46.5"E) by AM and JV.

DNA extraction was carried out using the Genomic DNA Mini Kit – tissue (Geneaid, New Taipei, Taiwan). We amplified the mitochondrial gene cytochrome *b* (cyt *b* hereinafter) using universal primers L14724, L15162, H15149 and H15915 (Irwin et al. 1991). Amplification conditions for cyt *b* consisted of 37 thermal cycles, an initial denaturation step at 94 °C for 3 min, denaturation at 94 °C for 30 seconds, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min. Sequences were obtained using the Sanger sequencing (Sanger et al. 1977) services at laboratory SEQme s.r.o. (Dobříš, Czech Republic).

We obtained 1137 base pairs long sequences that satisfied the quality of base pairs (GenBank access number LT970847-LT970849). These were compared using available sequences from GenBank, specifically with 250 specimens that comprise all available sequences of *M. mystacinus* (under names *M. levis*, *M. rossiameridionalis* and *M. mystacinus*), and representative sequences of particular clades in *M. arvalis* and *M. obscurus* associated with previous studies (Baker et al. 1996a, b; Haynes et al. 2003; Fink et al. 2004; Jaarola et al. 2004; Triant and DeWoody 2007; Bužan et al. 2010; Thanou et al. 2012; Tougaard et al. 2013; Stojak et al. 2016; Mahmoudi et al. 2017). Several more sequences (*M. kirgisorum*, accession number AY513809, AY513810; *M. socialis*, accession number AY513830, AY513831; and *M. transcaspicus*, accession number KX581067-KX581075) were downloaded from GenBank as potentially outgroups. The obtained sequences were aligned using the ClustalW algorithm implemented in GENEIOUS v.10.0.5 (Kearse et al. 2012). We employed a likelihood (ML) and Bayes-

ian inference method (BI) for phylogenetic analyses. Likelihood phylogenetic analyses were conducted using the PhyML plugin for GENEIOUS. Final Bayesian phylogenetic analyses were conducted in BEAST 2.4.5.0 (Drummond et al. 2012), where phylogenetic relationships were reconstructed under the Yule speciation process (Steel and McKenzie 2001) with the GTR model of evolution detected in JModelTest 2.1.7 (Nylander 2004) under the Akaike Information Criterion (AIC). The nucleotide data were run for 30 000 000 generations with a sampling frequency of every 1000th generation; with final burn-in set at 20%. Time estimations were also computed in BEAST2 (Drummond et al. 2012) for the topology detected by the Bayesian phylogenetic analysis. We adopted one fossil calibration point (0.475±0.025 Mya for the origin of *M. arvalis*; Miesenheimer I; Tougaard et al. 2013) to estimate divergence time in studied taxa and to compare estimations with Mahmoudi et al. (2017) (which are based on the following proposed molecular clock rate, 3.27×10^{-7} mutations/site/year for *M. arvalis*; Martínková et al. 2013). The split time with 95% highest posterior density was applied to a relaxed-clock model assuming a constant population size. The convergence and stability of estimated parameters was checked using TRACER 1.6 (Rambaut et al. 2017) and the maximum clade credibility trees were obtained with TREEANNOTATOR 2.4.5.0, and visualized in FIGTREE 1.4.3 (Rambaut 2009).

Some analyses were applied for *M. mystacinus* only. Specifically, haplotype characteristics were identified using DnaSP version 5.0 (Rozas et al. 2003) and the degree of diversification was estimated based on average pairwise distances using the Kimura two-parameters model of substitutions in MEGA5 (Tamura et al. 2011). The detailed haplotype network was conducted in POP ART 1.7 using the median-joining method (Bandelt et al. 1999).

Results and discussion

The obtained sequences of 1137 base pairs from three specimens exhibited close relationships with available cyt *b* sequences of *Microtus mystacinus*, in all comparisons. Specifically, they were nested inside this species, so our study identified this species in eastern Kazakhstan (see also below). All sequences of *M. mystacinus* form a sister group to the *M. obscurus* + *M. arvalis*, in accordance with previous comprehensive studies (e.g., Haynes et al. 2003; Fink et al. 2004; Jaarola et al. 2004; Triant and DeWoody 2007; Tougaard et al. 2013; Stojak et al. 2015, 2016; Mahmoudi et al. 2017).

Considering the intraspecific structure in *Microtus mystacinus*, we can distinguish two deep lineages (Iran, abbreviated as IR) and the rest of populations mostly from Europe, additionally divided into several sub-lineages (TU, EU, GK), concordantly in ML and BI phylogenetic trees and the haplotype network (see Figure 1). This structure, specifically groups IR, TU, and EU, were identified firstly by Mahmoudi et al. (2017). TU lineage consists of Turkish and Armenian samples (without specimen Armenia 1), EU lineage of samples from the majority of Europe, mainly from Ukraine and Romania except for specimens from Greece, which comprise GK lineage, as well as samples

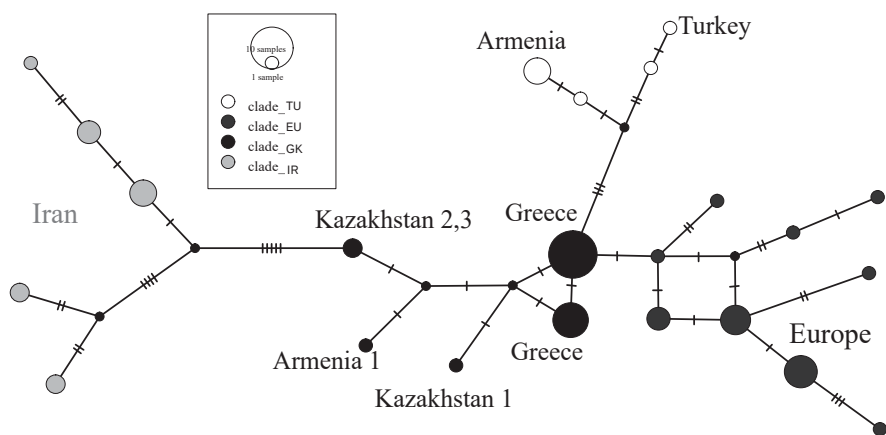


Figure 1. Median Joining Network based on the cyt *b* sequences of *M. mystacinus*.

Table 1. The K2P Inter – and intra-species average estimates of K2 genetic distance for cyt *b* in recognized lineages of *M. mystacinus* (TU – Turkey, Armenia; EU – Europe; GK – Greece, Kazakhstan; IR – Iran).

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1. TU	0.007									
2. EU	0.025	0.007								
3. GR	0.021	0.016	0.006							
4. Armenia_1	0.024	0.019	×	×						
5. Greece	0.016	0.011	×	0.009	0.001					
6. Kazakhstan	0.023	0.018	×	0.007	0.008	0.006				
7. IR	0.035	0.044	0.031	0.031	0.034	0.028	0.013			
8. <i>M. obscurus</i>	0.067	0.066	0.065	0.062	0.066	0.059	0.068	0.028		
9. <i>M. arvalis</i>	0.067	0.057	0.065	0.062	0.066	0.063	0.067	0.059	0.003	
10. <i>M. transcaspicus</i>	0.075	0.079	0.071	0.069	0.072	0.065	0.068	0.067	0.084	0.004

from eastern Kazakhstan and the specimen 1 from Armenia. This pattern indicates a complex diversification of *M. mystacinus* across its former and current distribution.

In general, *Microtus mystacinus* exhibited rather low intraspecific cyt *b* distances (except for the Iranian subset) and the obtained interspecific cyt *b* distances (see Table 1) are very similar to the values published in other studies (*M. arvalis* × *mystacinus*: Jaarola et al. (2004): 6–8%; Mahmoudi et al. (2017): 6–7%). As the intraspecific divergence for *Microtus mystacinus* and its cryptic diversity was intensively discussed by Mahmoudi et al. (2017), we would like to note only that the genetic distances cannot be presented as an absolute criterion for deciding whether two operational taxonomic units are distinct species (for detail see Groves et al. 2017), and in the case of species within the *arvalis*-group, some currently recognized species with rather low genetic distances exhibit infertile hybrids or hybrids with a reduced fertility (Meyer et al. 1985; Golenishchev et al. 2000; Jaarola et al. 2004).

The estimated clade divergence times varied substantially according to the calibration used (see Table 2). In summary, our estimations are more similar with other esti-

Table 2. Time to the most recent common ancestor (TMRCA and 95% HPD lower/upper limit – in million years) with BEAST2 for particular *Microtus* species (T – *M. transcasicus*, M – *M. mystacinus*, O – *M. obscurus*, A – *M. arvalis*) and recognized lineages of *M. levis* (TU – Turkey, Armenia; EU – Europe; GK – Greece, Kazakhstan; IR – Iran).

Nodes	Analysis 1 – fossil calibrations		Mahmoudi et al. 2017	Tougard et al. 2013
	TMRCA	95% HPD	TMRCA (95%HPD)	TMRCA (95%HPD)
a. T+M+O+A	1.102	0.77–1.28	0.238 (0.16–0.35)	–
b. M+O+A	0.797	0.60–1.05	0.217 (0.15–0.31)	0.531 (0.42–0.67)
c. O+A	0.616	0.51–0.78	0.184 (0.12–0.26)	0.478 (0.40–0.56)
d. T	0.537	0.32–0.57	0.040 (0.01–0.08)	–
e. O	0.410	0.27–0.58	0.119 (0.07–0.18)	0.173 (0.10–0.29)
f. A	0.490	0.48–0.54	0.146 (0.10–0.21)	0.446 (0.39–0.49)
g. IR+ EU+GK+TU	0.575	0.04–0.77	0.147 (0.09–0.22)	0.033 (0.00–0.08)
h. EU+GK+TU	0.408	0.28–0.57	0.092 (0.05–0.14)	–
i. EU+GK	0.332	0.23–0.47	–	–
j. TU	0.235	0.10–0.40	0.022 (0.01–0.04)	–
k. EU	0.219	0.14–0.32	0.075 (0.05–0.11)	–
l. GK	0.280	0.19–0.40	–	–
m. IR	0.390	0.24–0.47	0.117 (0.06–0.18)	–

mates based on fossil calibration points (albeit slightly higher) than with estimations based on mutation rates (see Table 2). Focusing on the most studied species, *M. arvalis*, we estimate its time to the most recent common ancestor (TMRCA) as 0.490 Mya, Tougard et al. (2008) 0.472 Mya and Tougard et al. (2013) 0.446 Mya, Stojak et al. (2015, 2016) 0.064–0.067 Myr and Mahmoudi et al. (2017) 0.146. Our estimation is similar to Tougard et al. (2008, 2013) as a logical result of the utilization of the same fossil calibration point, but all other specified estimations are much lower and associated with the same mutation rate (3.27×10^{-7} substitutions/site/year) proposed by Martínková et al. (2013) specifically for *Microtus arvalis* based on a recent geological event. It is not easy to judge which values are realistic, but our estimates seem to be compatible with other phylogenetic studies (e.g., Mazurok et al. 2001; Bannikova et al. 2010) and the fossil record (e.g., Cuenca-Bescós et al. 2001; Markova et al. 2012). Based on this compatibility, we adhere to the values of our estimations. In any case, it would be worth to compare different calibrations methods under different calibrations points and proposed mutations rates in future (e.g., methods of Baker et al. 1996a; Jaarola and Searle 2002), and also to consider the potential biases of the fossil record (e.g., incomplete nature, process of geological dating, reliability of species identification; cf. Ho 2007).

Evolution and diversification of arvicoline rodents, including the *arvalis*-group, has been closely related to Quaternary climatic oscillations and the associated abiotic and biotic environmental factors (e.g., Horáček and Ložek 1988; Horáček 1990; Chaline et al. 1999; Stojak et al. 2016; Tougard 2017 and references therein). For the *arvalis*-group, interglacial periods are considered to be periods of species expansions and glacials as periods of retractions with potential survival of particular species in refu-

gia (e.g., Golenishchev et al. 2000; Tougard et al. 2008; Stojak et al. 2015; Stojak et al. 2016). Golenishchev et al. (2000) considered one of the ancient alpine glaciations as responsible for disrupting the geographic range of *M. arvalis* and *M. obscurus*, whereas Tougard et al. (2008) considered interglacials as the agents of speciation. Based on our time estimations, the diversification of *M. mystacinus* + (*M. arvalis* + *M. obscurus*) group has happened within the last 0.79 Mya, thus comprising several interglacial and glacial periods (Gates 1993; Sirocko et al. 2007; Mahmoudi et al. 2017).

In our data, we observed synchronous, deep intraspecific divergences in all three species around 0.49–0.41 Mya (see Figure 2; in *M. mystacinus* we operated with separate timelines for the Iranian lineage (IR) and the remainder (sub-lineages TU, EU, GK) because the Iranian populations are divergent from the others; pairwise distance shows significant variation, see Table 1). This interval corresponds to the Holstein interglacial period (considering the stratigraphy of Western Europe) that is considered to be equivalent to Marine Isotope Stage (MIS) 11 (Sirocko et al. 2007; see Figure 2). The influence of the Holstein on the *arvalis*-group diversification can be explained by two historical scenarios. First, the preceding period, MIS 12, was characterized by a pronounced cold period (around 0.460 Mya), during which the earliest pan-Eurasian mammoth fauna associated with tundra-steppe habitats (called mammoth steppe, see Guthrie 2001) was formed. Second, the warmest phase of MIS 11 is the phase with the highest temperatures in the last 500 thousand years, persisting, persisting two times longer than the Eemian interglacial and three times longer than the Holocene (Sirocko et al. 2007). Interglacial conditions may have disrupted the mammoth steppe biome due to an increase in precipitation, temperature, and associated forest expansions (for Late Quaternary see Říčanová et al. 2018). Tougard et al. (2008) recognized that the evolutionary history of temperate small mammals is much more complex than previously suggested. Individual species responded to various factors in multiple ways, and at different times during the Pleistocene (Lorenzen et al. 2011). Therefore, we tend to be reserved about whether observed pulses in diversification could be interpreted as expansion alongside some geographical/biotope barriers or fragmentation of some particular populations.

To conclude, our study proved an additional occurrence of *Microtus mystacinus* in Kazakhstan. The studies of Kovalskaya (1994), Meyer et al. (1996) and Okulova et al. (2014) specified the distribution of this species from western or northwestern parts of Kazakhstan, with the easternmost observation from the Karabalyk district (Kovalskaya 1994). Other localities of this species are known around Novosibirsk, several hundred kilometres away from the Kazakhstani border (Pavlova and Tchabovsky 2011). Although our material is not suitable to establish the full distribution range in Kazakhstan, it enables us to extend the range of this species further south.

The distribution of *M. mystacinus* could be partly human-induced, as documented by Tiunov et al. (2013) when regarding the railway across Siberia and the Far East of Russia (e.g., Olkhon Island, Pavlova and Tchabovsky 2011; Buryatia, Moroldoev et al. 2017). If we consider this possibility, the locality near Sekisovka is approx. 30 km distant from the nearest railway from Ust-Kamenogorsk to Ridder, but our second locality (near Dzhambul) is more than 150 km distant from the nearest

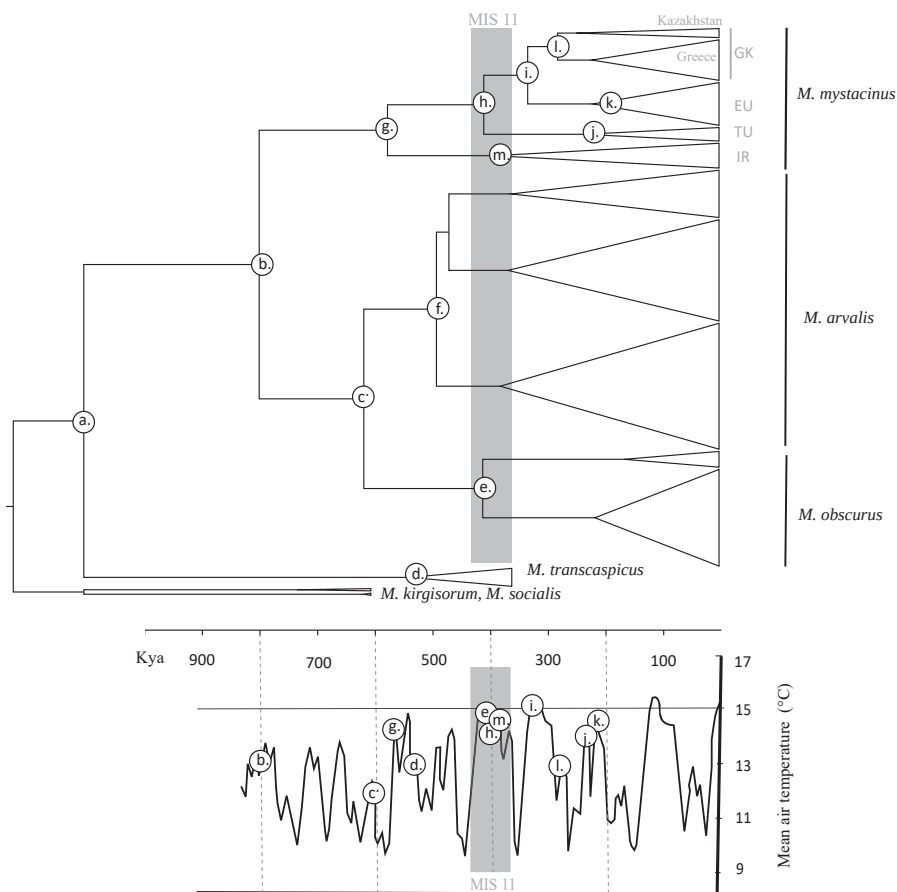


Figure 2. Time of the most recent common ancestor (TMRCA) for *Microtus* species and lineages of *M. mystacinus* using fossil calibrations. Nodes are plotted on a mean air temperature curve in last 800 thousand years (based on Gates 1993). See Table 2 for time estimates.

railway at Zyryanovsk (built after 1930; according to official web page of KTZ – КАЗАКСТАҢ ТЕМІР ЖОЛЫ). In Russian territory, this species shows pathways of invasion around the Transbaikalia railway and the surrounding agricultural landscape (e.g., Tiunov et al. 2013, Moroldoev et al. 2017). As the Kazakhstani specimens are significantly divergent from other available sequences (approx. 100 kya), we could consider the distribution of *M. mystacinus* in Kazakhstan as natural, but additional evidence is welcomed. Based on the presented network-phylogenetic relationship of samples it seems that a potential route of colonization for Kazakhstan populations could have originated somewhere between the Balkans and sites north of the Black

and Caspian seas, whereas populations in Turkey and parts of Armenia were colonized from a southern route.

Our study is the first genotyping of *M. mystacinus* from the eastern part of its distribution, where its occurrence is more discontinuous. In the context of our study, it is important to analyse genetically these Baikal and Far Eastern populations, and further map out the extent of *M. mystacinus* occurrence in East Kazakhstan.

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References

- Aulagnier S, Haffner P, Mitchell-Jones AJ, Moutou F, Zima J (2009) Mammals of Europe, North Africa and the Middle East. A and C Black Publishers, London, 272 pp.
- Baker RJ, Hamilton MJ, Van Den Bussche RA, Wright AJ, Wiggins LE, Hamilton MJ, Reat EP, Smith MH, Lomakin MD, Chesser RK (1996a) Small mammals from the most radioactive sites near the Chernobyl nuclear power plant. *Journal of Mammalogy* 77(1): 155–170. <https://doi.org/10.2307/1382717>
- Baker RJ, Van Den Bussche RA, Wright AJ, Wiggins LE (1996b) High levels of genetic change in rodents of Chernobyl. *Nature* 380(6576): 707–708. <https://doi.org/10.1038/380707a0>
- Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16(1): 37–48. <https://doi.org/10.1093/oxford-journals.molbev.a026036>
- Bannikova AA, Lebedev VS, Lisovsky AA, Matrosova V, Abramson NI, Obolenskaya EV, Tesakov AS (2010) Molecular phylogeny and evolution of the Asian lineage of vole genus *Microtus* (Rodentia: Arvicolinae) inferred from mitochondrial cytochrome *b* sequence. *Biological Journal of the Linnean Society* 99(3): 595–613. <https://doi.org/10.1111/j.1095-8312.2009.01378.x>
- Baskevich MI, Mironova TA, Cherepanova EV, Krivonogov DM (2016) New data on chromosomal variability, distribution of sibling species, and hybridization of 46-chromosomal forms of *Microtus arvalis* sensu lato (Rodentia, Arvicolinae) in the Upper Volga basin. *Biology Bulletin* 43(9): 1281–1291. <https://doi.org/10.1134/S1062359016110042>
- Baskevich MI (1996) On the karyological differentiation in Caucasian population of common vole (Rodentia, Cricetidae, *Microtus*). *Zoologičeskij Žurnal* 75(2): 297–308. [In Russian]
- Bužan EV, Förster DW, Searle JB, Kryštufek B (2010) A new cytochrome *b* phylogroup of the common vole (*Microtus arvalis*) endemic to the Balkans and its implications for the evolu-

- tionary history of the species. *Biological Journal of the Linnean Society* 100(4): 788–796. <https://doi.org/10.1111/j.1095-8312.2010.01451.x>
- Chaline J, Brunet-Lecomte P, Montuire S, Viriot L, Courant F (1999) Anatomy of the arvicoline radiation (Rodentia): palaeogeographical, palaeoecological history and evolutionary data. *Annales Zoologici Fennici* 36(4): 239–267.
- Cuenca-Bescós GC, Canudo JI, Laplana C (2001) La séquence des rongeurs (Mammalia) des sites du Pléistocène inférieur et moyen d'Atapuerca (Burgos, Espagne). *Anthropologie* 105(1): 115–130. [https://doi.org/10.1016/S0003-5521\(01\)80009-1](https://doi.org/10.1016/S0003-5521(01)80009-1)
- Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* 29(8): 1969–73. <https://doi.org/10.1093/molbev/mss075>
- Fink S, Excoffier L, Heckel G (2004) Mitochondrial gene diversity in the common vole *Microtus arvalis* shaped by historical divergence and local adaptations. *Molecular Ecology* 13(11): 3501–14. <https://doi.org/10.1111/j.1365-294X.2004.02351.x>
- Fredga K, Jaarola M, Ims RA, Steen H, Yoccoz NG (1990) The common vole in Svalbard identified as *Microtus epiroticus* by chromosome analysis. *Polar Research* 8: 283–290. <https://doi.org/10.3402/polar.v8i2.6818>
- Gates DM (1993) Climate change and its biological consequences. Sinauer Associates. Inc. Sunderland, MA, 280 pp.
- Ghorbani F, Mohammadi Z, Darvish J, Kami HG, Siah sarvie R (2015) Morphological and morphometric characterization of the new records of the East European vole (*Microtus mystacinus* Miller, 1908) from northeast Iran. *Journal of Asia-Pacific Biodiversity* 8(3): 233–237. <https://doi.org/10.1016/j.japb.2015.07.002>
- Gileva EA, Cheprakov MI, Nochrin DY (1996) Voles of the group *Microtus arvalis* (Rodentia, Cricetidae) in Urals. *Zoologičeskij Žurnal* 75(9): 1436–1439 [in Russian].
- Golenishchev FN, Malikov VG, Bulatova NSh, Vaziri ASH, Nazari F (2000) Some new data on morphology and karyology of *Microtus kermanensis* (Rodentia, Arvicolinae) and supposed phylogeographic history of the “arvaloid” voles. In: Agadjanian AK, Orlov VN (Eds) *Systematic and Phylogeny of the Rodents and Lagomorphs*. Teriologicheskoe Obshchestvo, Moscow, 34–36.
- Groves CP, Cotterill FPD, Gippoliti S, Robovský J, Roos C, Taylor PJ, Zinners D (2017) Species definitions and conservation: a review and case studies from African mammals. *Conservation Genetics* 18(6): 1247–1256. <https://doi.org/10.1007/s10592-017-0976-0>
- Guthrie RD (2001) Origin and causes of the mammoth steppe: a story of cloud cover, woolly mammal tooth pits, buckles, and inside-out Beringia. *Quaternary Science Reviews* 20(1): 549–574. [https://doi.org/10.1016/S0277-3791\(00\)00099-8](https://doi.org/10.1016/S0277-3791(00)00099-8)
- Haynes S, Jaarola M, Searle JB (2003) Phylogeography of the common vole (*Microtus arvalis*) with particular emphasis on the colonization of the Orkney archipelago. *Molecular Ecology* 12(4): 951–956. <https://doi.org/10.1046/j.1365-294X.2003.01795.x>
- Ho SYW (2007) Calibrating molecular estimates of substitution rates and divergence times in birds. *Journal of Avian Biology* 38: 409–414. [10.1111/j.0908-8857.2007.04168.x](https://doi.org/10.1111/j.0908-8857.2007.04168.x)
- Horáček I (1990) On the context of Quaternary arvicolid evolution: changes in community development. In: Fejfar O, Heinrich WD (Eds) *International Symposium ‘Evolution,*

- Phylogeny and Biostratigraphy of Arvicolids (Rodentia, Mammalia). Geological Survey, Prague, 201–222.
- Horáček I, Ložek V (1988) Palaeozoology and the Mid-European Quaternary past: scope of the approach and selected results. Rozpravy ČSAV, řada matematických a přírodních věd. 98: 1–102.
- Hotzi V, Markov G, Csorba G (2008) Taking steps to discover the East-European vole (*Microtus levis*) in Hungary. Állattani Közlemények 93: 47–57. [In Bulgarian]
- Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome *b* gene of mammals. Journal of Molecular Evolution 32(2): 128–144. <https://doi.org/10.1007/BF02515385>
- Jaarola M, Martínková N, Gündüz I, Brunhoff C, Zima J, Nadachowski A, Amorif G, Bultogovag NS, Chondropoulos B, Fragedakis-Tsolish S, Gonzalez-Estebani J, Lopez-Fuster MJ, Kandaurov AS, Kefelioğlu H, da Luz Mathias M, Villatei I, Searle JB (2004) Molecular phylogeny of the speciose vole genus *Microtus* (Arvicolinae, Rodentia) inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 33(3): 647–63. <https://doi.org/10.1016/j.ympev.2004.07.015>
- Jaarola M, Searle JB (2002) Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. Molecular Ecology 11(12): 2613–2621. <https://doi.org/10.1046/j.1365-294X.2002.01639.x>
- Kartavtseva IV, Tiunov MP, Lapin AS, Visotchina NP, Ryabkova AV (2012) Invasion of *Microtus rossiaemeridionalis* into the territory of the Russian Far East. Russian Journal of Biological Invasions 3(1): 11–15. <https://doi.org/10.1134/S2075111712010031>
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>
- Kovalskaya YM (1994) On the distribution of voles of the group *arvalis* (Rodentia) in Kazakhstan. Zoologičeskij Žurnal 73(3): 120–125. [In Russian]
- Král B, Zima J, Hrabě V, Libosvářský J, Šebela M, Červený J (1981) On the morphology of *Microtus epiroticus*. Folia Zoologica Brno 30(4): 317–330.
- Kryštufek B, Vohralík V (2005) Mammals of Turkey and Cyprus. Rodentia I: Sciuridae, Dipodidae, Gliridae, Arvicolinae. University of Primorska, Science and Research Centre Koper, Koper, 292 pp.
- Kryštufek B (2017) The East European Vole. In: Wilson DE, Lacher Jr TE, Mittermeier RA (Eds) Handbook of the mammals of the World, Volume 7. Lynx Edicions, Barcelona, 352.
- Lorenzen ED, Nogués-Bravo D, Orlando L, Weinstock J, Binladen J, Marske KA, Ugan A, Borregaard MK, Gilbert TP, Nielsen R, Ho SYW, Goebel T, Graf KE, Byers D, Stenderup JT, Rasmussen M, Campos PF, Leonard JA, Koepfli KP, Froese D, Zazula G, Stafford TV, Aaris-Sørensen K, Batra P, Haywood AM, Singarayer JS, Valdes PJ, Boeskorov G, Burns JA, Davydov SP, Haile J, Jenkins DL, Kosintsev P, Kuznetsova T, Lai X, Martin LD, McDonald HG, Mol D, Meldgaard M, Munch K, Stephan E, Sablin M, Sommer RS, Sipko T, Scott E, Suchard MA, Tikhonov A, Willerslev R, Wayne RK, Cooper A, Hofreiter M., Sher A., Shapiro B, Rahbek C, Willerslev E (2011) Species-specific responses of Late

- Quaternary megafauna to climate and humans. *Nature* 479(7373): 359–364. <https://doi.org/10.1038/nature10574>
- Mahmoudi A, Darvish J, Aliabadian M, Moghaddam FY, Kryštufek B (2017) New insight into the cradle of the grey voles (subgenus *Microtus*) inferred from mitochondrial cytochrome *b* sequences. *Mammalia* 81(6): 583–593. <https://doi.org/10.1515/mammalia-2016-0001>
- Markov G, Csorba G, Kocheva M, Gospodinova M (2012) Skull features of the common vole (*Microtus arvalis* sensu lato) from Hungary: craniometrical evidence for its taxonomic detachment. *Turkish Journal of Zoology* 36(3): 283–290. <https://doi.org/10.3906/zoo-1002-49>
- Markova E, Malygin V, Montuire S, Nadachowski A, Quéré JP, Ochman K (2009) Dental variation in sibling species *Microtus arvalis* and *M. rossiaemeridionalis* (Arvicolinae, Rodentia): between species comparisons and geography of morphotype dental patterns. *Journal of Mammalian Evolution* 17: 121–139. <https://doi.org/10.1007/s10914-009-9128-8>
- Markova E, Beeren Z, van Kolfschoten T, Strukova T, Vrieling K (2012) Differentiating sibling species in the Quaternary fossil record: a comparison of morphological and molecular methods to identify *Microtus arvalis* and *M. rossiaemeridionalis* (Arvicolinae, Rodentia). *Journal of Systematic Palaeontology* 10(3): 585–597. <https://doi.org/10.1080/14772019.2011.618146>
- Martínková N, Barnett R, Cucchi T, Struchen R, Pascal M, Fischer MC, Higham T, Brace S, Ho SYW, Quéré JP, O’ Higgins P, Excoffier L, Heckel G, Hoelzel RA, Dobney KM, Searle JB (2013) Divergent evolutionary processes associated with colonization of offshore islands. *Ecology* 22(20): 5205–5220. <https://doi.org/10.1111/mec.12462>
- Masing M (1999) The skull of *Microtus levis* (Arvicolidae, Rodentia). *Folia Theriologica Estonica* 4: 76–90.
- Mazurok NA, Rubtsova NV, Isaenko AA, Pavlova ME, Slobodyanyuk SY, Nesterova TB, Zakian SM (2001) Comparative chromosome and mitochondrial DNA Analyses and phylogenetic relationships within common voles (*Microtus*, Arvicolidae). *Chromosome Research* 9(2): 107–120. <https://doi.org/10.1023/A:1009226918924>
- Meyer MN, Golenishchev FN, Radjabli SI, Sablina OV (1996) Voles (subgenus *Microtus* Schrank) of Russia and adjacent territories. *Proceedings of the Zoological Institute of the Russian Academy of Sciences* (232): 1–320. [in Russian]
- Meyer MN, Orlov VN, Scholl ED (1969) Utilization of karyological, physiological and cytological analysis for the separation of new species of rodents (Rodentia, Mammalia). *Doklady Akademii Nauk USSR* 188: 1411–1414 [in Russian].
- Meyer MN, Radjabli SI, Bulatova NS, Golenishchev FN (1985) Karyological peculiarities and probable relations of common voles of the group “arvalis” (Rodentia, Cricetidae, *Microtus*). *Zoologičeskij Žurnal* 64(3): 417–428.
- Moroldov IV, Sheremeteyeva IN, Kartavtseva IV (2017) The first finding of East European vole (*Microtus rossiaemeridionalis*) in Buryatia. *Russian Journal of Biological Invasions* 8(3): 266–271. <https://doi.org/10.1134/S2075111717030109>
- Musser GG, Carleton MD (2005) Subfamily Arvicolinae. In: Wilson DE, Reeder DM (Eds) *Mammal species of the world: a taxonomic and geographic reference*. The Johns Hopkins University Press, Baltimore, 956–1039.
- Nylander JAA (2004) MrModeltest v2. Program distributed by the Author. Evolutionary Biology Centre, Uppsala University.

- Okulova NM, Khlyap LA, Bidashko FG, Warshavskiy AA, Grazhdanov AK, Neronov VV (2014) Rodent communities of the Western Kazakhstan oblast of the Republic of Kazakhstan 1: Maps of rodent communities and zoogeographic regionalization. *Arid Ecosystems* 4(2): 75–84. <https://doi.org/10.1134/S2079096114020073>
- Pavlova SV, Tchabovsky AV (2011) Presence of the 54-chromosome common vole (Mammalia) on Olkhon Island (Lake Baikal, East Siberia, Russia), and the occurrence of an unusual X-chromosome variant. *Comparative Cytogenetics* 5(5): 433–40. <https://doi.org/10.3897/CompCytogen.v5i5.1720>
- Rambaut A (2009) Computer program and documentation distributed by the author. <http://beast.bio.ed.ac.uk>
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2017) Tracer v1.7, Available from <https://github.com/beast-dev/tracer>
- Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* 19(18): 2496. <https://doi.org/10.1093/bioinformatics/btg359>
- Řičánková VP, Horsák M, Hais M, Robovský J, Chytrý M (2018) Environmental correlates of the Late Quaternary regional extinctions of large and small Palaearctic mammals. *Ecography* 41(3): 516–527. <https://doi.org/10.1111/ecog.02851>
- Sanger FS, Nicklen S, Coulson AE (1977) DNA sequencing with chain termination inhibitors. *Proceedings of the National Academy of Sciences of the USA* 74(12): 5463–5467. <https://doi.org/10.1073/pnas.74.12.5463>
- Shenbrot GI, Krasnov BR (2005) Atlas of the geographic distribution of the arvicoline rodents of the world (Rodentia, Muridae: Arvicolinae). Pensoft, Sofia-Moscow, 336 pp.
- Sirocko F, Claussen M, Litt T, Sanchez-Goni MF (2007) The climate of past interglacials (Vol. 7). Elsevier, Amsterdam, 622 pp.
- Steel M, McKenzie A (2001) Properties of phylogenetic trees generated by Yule-type speciation models. *Mathematical Biosciences* 170(1): 91–112. [https://doi.org/10.1016/S0025-5564\(00\)00061-4](https://doi.org/10.1016/S0025-5564(00)00061-4)
- Stojak J, McDevitt AD, Herman JS, Searle JB, Wójcik JM (2015) Post-glacial colonization of eastern Europe from the Carpathian refugium: evidence from mitochondrial DNA of the common vole *Microtus arvalis*. *Biological Journal of the Linnean Society* 115(4): 927–939. <https://doi.org/10.1111/bij.12535>
- Stojak J, McDevitt AD, Herman JS, Kryštufek B, Uhlíková J, Purger JJ, Lavrenchenko LA, Searle JB, Wójcik JM (2016) Between the Balkans and the Baltic: Phylogeography of a Common Vole mitochondrial DNA lineage limited to Central Europe. *PLoS ONE* 11(12): e0168621. <https://doi.org/10.1371/journal.pone.0168621>
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28(10): 2731–9. <https://doi.org/10.1093/molbev/msr121>
- Thanou E, Tryfonopoulos G, Chondropoulos B, Fraguédakis-Tsolis S (2012) Comparative phylogeography of the five Greek vole species infers the existence of multiple South Balkan subrefugia. *Hystrix Italian Journal of Mammalogy* 79(3): 363–376. <https://doi.org/10.1080/11250003.2011.651163>

- Tiunov MP, Kartavtseva IV, Lapin AS (2013) Morphotype analysis of the sibling vole (*Microtus rossiaemeridionalis*) casually introduced to the Russian Far East. *Acta Theriologica* 58(1): 79–82. <https://doi.org/10.1007/s13364-012-0092-y>
- Tougard C, Renvoise E, Petitjean A, Quéré JP (2008) New insight into the colonization processes of Common Voles: Inferences from molecular and fossil evidence. *PLoS ONE* 3(10): e3532. <https://doi.org/10.1371/journal.pone.0003532>
- Tougard C (2017) Did the Quaternary climatic fluctuations really influence the tempo and mode of diversification in European rodents? *Journal of Zoological Systematics and Evolutionary Research* 55(1): 46–56. <https://doi.org/10.1111/jzs.12152>
- Tougard C, Montuire S, Volobouev V, Markova E, Contet J, Aniskin V, Quere JP (2013) Exploring phylogeography and species limits in the Altai vole (Rodentia: Cricetidae). *Biological Journal of the Linnean Society* 108(2): 434–452. <https://doi.org/10.1111/j.1095-8312.2012.02034.x>
- Triant DA, DeWoody JA (2007) Extensive mitochondrial DNA transfer in a rapidly evolving rodent has been mediated by independent insertion events and by duplications. *Gene* 401(1): 61–70. <https://doi.org/10.1016/j.gene.2007.07.003>
- Yakimenko LV, Kryukov AP (1997) On karyotype variation in common vole *Microtus rossiaemeridionalis* (Rodentia, Cricetidae). *Zoologičeskij Žurnal* 76(3): 375–378. [In Russian]
- Zagorodnyuk IV (1991a) Polytypical Arvicolidae in Eastern Europe: taxonomy, distribution and diagnostics. Institute of Zoology, Kiev, 63 pp.
- Zagorodnyuk IV (1991b) Systematic position of *Microtus brevirostris* (Rodentiformes): materials toward the taxonomy and diagnostics of the "arvalis" group. *Vestnik Zoologii* 25(3): 26–35.

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Gastrointestinal parasites of arctic foxes (*Vulpes lagopus*) and sibling voles (*Microtus levis*) in Spitsbergen, Svalbard

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Abstract

The arctic fox (*Vulpes lagopus*), an apex predator with an omnipresent distribution in the Arctic, is a potential source of intestinal parasites that may endanger people and pet animals such as dogs, thus posing a health risk. Non-invasive methods, such as coprology, are often the only option when studying wildlife parasitic fauna. However, the detection and identification of parasites are significantly enhanced when used in combination with methods of molecular biology. Using both approaches, we identified unicellular and multicellular parasites in faeces of arctic foxes and carcasses of sibling voles (*Microtus levis*) in Svalbard, where molecular methods are used for the first time. Six new species were detected in the arctic fox in Svalbard, *Eucoleus aerophilus*, *Uncinaria stenocephala*, *Toxocara canis*, *Trichuris vulpis*, *Eimeria* spp., and *Enterocytozoon bieneusi*, the latter never found in the arctic fox species before. In addition, only one parasite was found in the sibling vole in Svalbard, the *Cryptosporidium aliticolis*, which has never been detected in Svalbard before.

Keywords Parasites · Svalbard · Arctic fox · Sibling vole · Coprology

Introduction

Arctic foxes have a circumpolar distribution and are apex predators in Arctic ecosystems (Audet et al. 2002). In the

High Arctic Svalbard archipelago, they are scavengers and top predators of both marine and terrestrial ecosystems (Ehrich et al. 2015; Eide et al. 2012). The arctic fox is an important vector of zoonotic pathogens, and knowledge of their health status is of great importance for the ecosystem (e.g. Andreassen et al. 2017; Skirnisson et al. 1993; Sørensen et al. 2005). Gastrointestinal parasites may cause effects on vital demographic parameters like decreased survival and fecundity rate (Anderson and May 1978). It can also contribute to documented increased mortality rates in arctic foxes, especially in juveniles (Meijer et al. 2011). In combination with other stressors such as harvesting, pollutant levels, and climate change, parasites may contribute to unknown cumulative effects on Arctic fox populations.

Arctic foxes are apex predators and scavengers with an opportunistic and generalistic feeding behaviour (Eide et al. 2012; Ehrich et al. 2015). They live in two main tundra types, inland and coastal, that differ in the type of available food sources (Braestrup 1941). Rodents represent the main food source for the inland fox populations, e.g. in North America, Eurasia, and in east Greenland, while the coastal ecotype lives in areas where rodents like lemmings are absent, with a diet mainly from the marine food web like seabirds, eggs, fish, crustaceans on the seashore, or caribou of seals and reindeer (Angerbjörn et al. 2004; Hersteinsson and Macdonald 1996;

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Prestrud et al. 1992). As top predators, living in both inland and coastal ecosystems, one might expect a broad species diversity of intestinal parasites in arctic foxes.

In Svalbard, arctic foxes belong to the coastal ecotype and they have to survive even in the absence of rodents, apart from a small introduced population of sibling voles (*Microtus levis*). The sibling voles were introduced with the cattle feed between 1920 and 1960, but with a very restricted area of distribution (Henttonen et al. 2001). Other year-round resident species in Svalbard that are important in the diet of the arctic fox are carcasses of Svalbard reindeer (*Rangifer tarandus platyrhynchus*) and the Svalbard rock ptarmigan (*Lagopus muta hyperborea*). In summer, the archipelago is visited by numerous numbers of migrating seabirds and terrestrial birds (e.g. geese), and there is a large variety of food items from the marine ecosystem (Eide et al. 2012). With the lack of small rodents like lemmings, Svalbard has relatively low biodiversity, making the number of parasite species in arctic foxes low, compared with other parts of the Arctic (e.g. Jónsdóttir 2005). However, arctic foxes may still carry a considerable number of parasites (e.g. Henttonen et al. 2001).

To date, several intestinal parasites have been recorded in Arctic foxes in Svalbard (Fuglei et al. 2008; Henttonen et al. 2001; Stien et al. 2010). The documented parasite fauna includes cestodes *Echinococcus multilocularis*, *Diphyllobothrium* sp., *Taenia ovis krabbei*, *T. crassiceps*, and *T. polyacantha*. The latter two cestodes together with *E. multilocularis* use rodents as intermediate hosts and depend on sibling voles that inhabit a very restricted area in Svalbard, while *T. ovis krabbei* uses reindeer and *Diphyllobothrium* sp. uses fish or invertebrates as intermediate hosts. Moreover, two additional parasites were found: an unidentified ascarid nematode and an unidentified acanthocephalan. Metacystodes of *E. multilocularis* were also detected in sibling voles in 1999 and 2001–2006, and in 2003, two voles were positive for *T. crassiceps* (Henttonen et al. 2001; Stien et al. 2010).

In the course of this study, faeces were collected around arctic fox dens during the annual den surveys conducted by the Norwegian Polar Institute as well as when found on the ground while doing field work. Sampling was followed by microscopy together with extended use of molecular methods for the detection of parasites. Part of the samples originated from the sibling vole distribution area in Longyearbyen, located 10 km east of Grumantbyen, the core area of the sibling voles (Henttonen et al. 2001). For that reason, we also captured sibling voles and included data based on their dissections.

Materials and methods

Faeces of Arctic foxes were collected during the summer seasons in 2012 ($n = 10$), 2013 ($n = 12$), and 2015 ($n = 40$) from

three different sites in Longyearbyen, Sassendalen, and Billefjorden in the central part of Spitsbergen, the largest island of the high Arctic Svalbard archipelago (Fig. 1). Faeces samples were collected from the ground; thus, it cannot be excluded that more samples were collected repeatedly from a single host individual. Faeces were stored in sealed plastic bags, and subsequently forwarded for laboratory analyses up to 3 weeks post collection to laboratories in Centre for Polar Ecology, University of South Bohemia in České Budějovice, Czech Republic). All samples were transported and stored at 4–8 °C prior to being used for laboratory analyses.

Sibling voles ($n = 63$) were trapped (spring-loaded bar mousetrap) during summer in 2017 (research registered in The Research in Svalbard Database, permission RiS-10852). Traps were baited with almond and placed at two locations in Longyearbyen the largest of the four human settlements in Svalbard. One site was located around a horse stable (78° 14' 32" N 15° 31' 42.5" E) and one in Nybyen by an abandoned dog yard (78° 11' 44" N 15° 33' 39.4" E). Traps were checked every 2–3 h with voles dissected immediately after capture. Vole carcasses from the 2014 and 2015 seasons were acquired from local people from whole Longyearbyen (caught during the winter and spring and kept frozen until summer) and were used for the purpose of this study. Vole organs were examined macroscopically for the presence of metacystodes, and with special attention to liver for the presence of *E. multilocularis*. The intestinal contents were stored in screw top plastic tubes in 5% potassium dichromate solution at 4–8 °C and subsequently forwarded for laboratory analyses (laboratories in Centre for Polar Ecology, University of South Bohemia in České Budějovice, Czech Republic).

Faecal samples of foxes and voles' intestinal content from necropsy were initially screened for helminth and protozoal stages using the light microscopy ($\times 200$ and $\times 400$ magnifications, Olympus BX51, camera Olympus Camedia C-5060, Quick PHOTO MICRO 2.3 software) following two methods of concentration (i) Sheather's sugar flotation method (Sheather 1923; Smith et al. 2007) and (ii) AMS III sedimentation (Hunter et al. 1960).

Faecal samples of foxes and vole intestinal contents were homogenised with 0.5-mm glass beads (Biospec Products, Inc., Bartlesville, OK, USA) in a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, CA, USA) at a speed of 5 m/s for 1 min followed by DNA extraction using the Exgene™ Stool DNA mini kit (GeneAid, South Korea) following the manufacturer's protocol. Nested PCR protocols were used to amplify the ITS (Internal Transcribed Spacer) region of the rRNA gene of *E. bieneusi* as previously described by Buckholt et al. (2002), the ITS region of the rRNA gene of *Encephalitozoon* spp. using the primer sets of INT580F and INT580R according to Didier et al. (1995) and MSP3 and MSP4A primer sets for the secondary PCR reaction according to Katzwinkel-Wladarsch et al. (1996), the partial sequence of

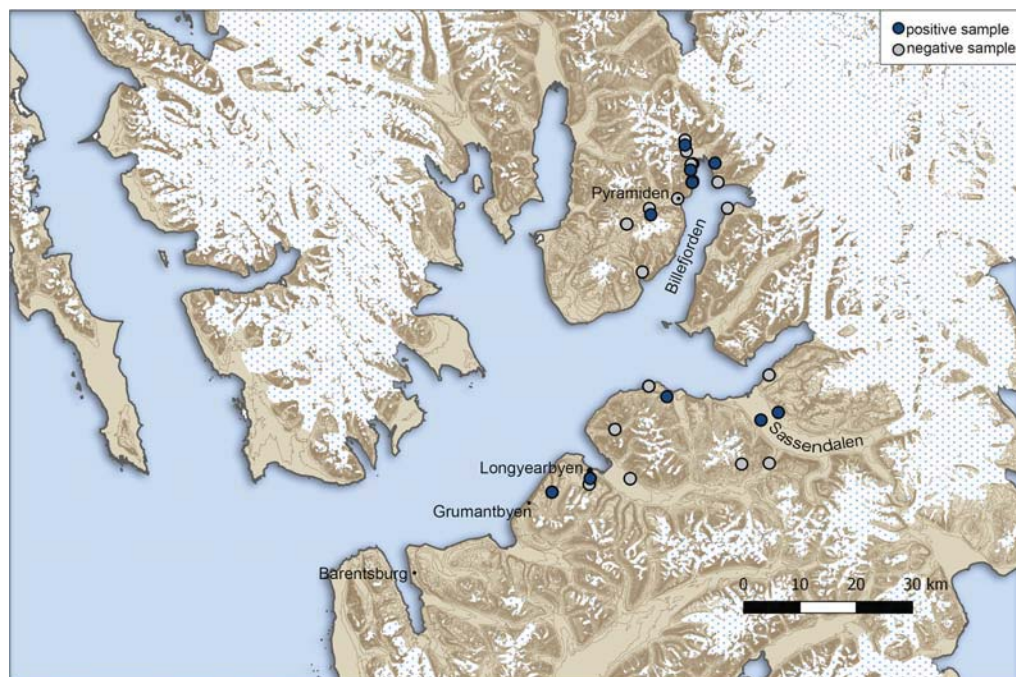


Fig. 1 Map showing the main sites (Longyearbyen, Sassendalen, and the Billefjorden area) of collected arctic fox faeces samples in Spitsbergen, Svalbard. Dark buttons, positive samples; light buttons, negative samples. Map created in QGIS Development Team (2018) QGIS Geographic

information system. Open source geospatial foundation project. Available at: <http://qgis.osgeo.org>; Basemap data from Norwegian Polar Institute. Available at: <https://geodata.npolar.no/>

TPI (Triosephosphate Isomerase) gene of *Giardia* spp. described by Sulaiman et al. (2003), the partial sequence of the *Cryptosporidium* small ribosomal subunit rRNA (18S rRNA) gene described by Jiang et al. (2005), the ITS region of nematodes of the family Ascaridae using the primer set AsITS1 described by Carlsgart et al. (2009), the partial sequence of COI (Cytochrome c oxidase I) and 18S rRNA genes of the family Eimeriidae (Kvičerová et al. 2008; Schwarz et al. 2009), and following individual genes were amplified by PCR using the two primer pairs for tapeworms: near the D1–D3 region of *IsrDNA* with LSU5 and 1500R (Littlewood et al. 2000; Olson et al. 2003) and mitochondrial gene NAD1 (Trachsel et al. 2007). The primary PCR reaction containing 12.5 µl 1× Plain PP master mix (Top-Bio, Praha, Czech Republic; contain Taq polymerase), 400 nM each primer, 1.0 µl of template DNA and molecular grade water up to a volume 25 µl. The reaction conditions for secondary PCR were similar to those described above for the primary PCR, with the exception that 2 µl of the primary PCR product was used as the template. A negative (molecular grade water) and positive controls (DNA of *C. varanii*, *E. cuniculi* genotype III, *E. bieneusi* genotype D, *G. intestinalis* assemblage E,

E. intestinalis, *E. multilocularis*, and *A. suum*) were included in each appropriate PCR reaction set. Each cycle consisted of denaturation of 94 °C for 45 s, annealing temperature that was specific for each of the targeted genus (according to mentioned reference publications), and extension of 72 °C for 60 s. Initial denaturation at 94 °C for 3 min and the final extension at 72 °C for 7 min/10 min were included. The number of cycles (35 for *Cryptosporidium*, *Giardia*, Eimeriidae COI, and microsporidia, 34 for Ascaridae, and 30 for Eimeriidae 18S rRNA) was set based on the above-mentioned reference publications. PCR products were visualised on 1% agarose gel containing 0.1 g/ml ethidium bromide, positive samples of expected sizes were isolated from the gel by the ExpinTM Combo GP (GeneAll, South Korea) and sequenced with PCR primers for Ascarids and secondary PCR primers for the remaining three parasite taxa on an ABI 3130 sequence analyzer (SEQme, Dobříš, Czech Republic). Each sample was sequenced in both directions. Sequences were aligned and assembled using the Geneious 9.1.5 software (<http://www.geneious.com>, Kearse et al. 2012), and compared with sequences in the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences obtained in this study were

deposited in the GenBank database under the accession numbers MK315211-MK315221.

Ethical approval

The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia, and also by the Ministry of the Environment of the Czech Republic (Permit Numbers MZP/2017/630/854). All procedures performed in studies involving animals were in accordance with the ethical standards of the Norwegian Animal Welfare Act. The research was also registered in The Research in Svalbard Database, RiS-10852. All handling/usage with biological samples were allowed by the University of South Bohemia in České Budějovice in accordance with the law of the Czech Republic (Act No. 166/1999), regulation of European Parliament (Act. No. 1069/2009), and Commission Regulation (EU) (No. 142/2011).

Results

Out of the 62 samples collected from arctic fox faeces, 17 were microscopically positive for endoparasites. Specifically, four samples were positive for eggs of *Toxascaris leonina* 13.3 % (Fig. 2a), one for *Trichuris vulpis* 1.7 % (Fig. 2c), two for *Eucoleus* sp. 3.3 % (Fig. 2b), and one sample for *Uncinaria stenocephala* 1.7 % (Fig. 2d; Table 1). In addition, five samples were positive for several different morphotypes of *Eimeria* spp. oocysts 8.3 % (Fig. 3; Table 1).

Only two samples had more than one species of parasite: *T. leonina* with *T. canis* (season 2015), and *T. leonina* together with *Eimeria* spp. (season 2013). Nevertheless, three positive samples (each containing only single parasitic taxon, *Eucoleus* sp., *U. stenocephala*, and *Eimeria* spp.) collected in 2013 originated from the same locality in Billefjorden. These samples could originate from a single individual. Summary of positive samples from the seasons 2012, 2013, and 2015 are provided in Fig. 1.

Three sequences of eimerians found in the faeces of arctic foxes were obtained (8.3 %; Table 1). The partial cytochrome c oxidase I (COI) sequence of eimerians present in the sample 63A clustered inside the eimerians from gallinaceous birds. The partial COI sequence of eimerians found in sample 153 clustered individually, being most closely related to the eimerians infecting rodents and pangolin. The partial sequence of 18S rDNA of eimerians occurring in sample 125 also clustered individually, on the basal position of the eimeriids infecting warm-blooded vertebrates (Online Resource1-2).

No sample was microscopically positive for spores of microsporidia. However, *Enterocytozoon bienersi*-specific

DNA was detected by molecular tools in one arctic fox (1.7 %; Table 1).

Additionally, specific DNA of *T. leonina* and *Toxocara canis* was detected in four (13.3 %) and one (1.7 %) microscopically negative samples, respectively (Table 1). All samples from foxes and voles were molecularly negative for any tapeworm (Table 1).

None of the 63 sibling voles was microscopically positive for any endoparasites (Table 1). A sample from one sibling vole contained specific DNA of *Cryptosporidium* (1.7 %) and following phylogenetic analysis of the 18S rRNA gene showed the presence of *Cryptosporidium alticolis* (100 % identity with the bootstrap value 100, Table 1). However, none of the samples from foxes was positive neither for *Cryptosporidium* spp. nor for *Giardia* spp. and also none of the sibling voles contained DNA of *Giardia* spp., eimerians or microsporidia.

Discussion

We were able to successfully detect exogenous life stages and specific DNA of endoparasites in collected samples via the microscopy and molecular tools, respectively. Parasitological examination of faecal material always poses a risk of underestimation of the parasite species richness. Limitation of coprological examination resides in the fact that it is able to detect only those parasites that shed eggs/cysts/oocysts in sufficient quantity over the detection limit of the method used during the sampling period (Martínez-Carrasco et al. 2007). Therefore, we also employed molecular methods to complement the traditional method, and to be able to detect parasites that were not detected during the microscopical examination due to low infection intensity or stage sizes that are difficult to be detected (e.g. microsporidia spores). Specifically, arctic fox samples that were microscopically negative for *T. leonina* and *E. bienersi* were molecularly positive, showing the usefulness and the improvement by using different parasitological approaches.

In contrast to Stien et al. (2010) that focused on materials from arctic fox GI tracts that reported high abundance of tapeworms, we did not detect any tapeworm stages in the collected arctic fox faecal material. On the other hand, we recorded six new parasites that have not been previously reported in Svalbard, specifically, a respiratory tract worm *Eucoleus* sp. (Fig. 2b), hookworm *U. stenocephala* (Fig. 2d), whipworms *Trichuris vulpis* (Fig. 2c), *T. canis*, *Eimeria* spp., and *E. bienersi*. All of them are common parasites of foxes, and although they have been detected in other Arctic areas (Aguirre et al. 2000; Meijer et al. 2011; Skirnisson et al. 1993), this is the first record in Svalbard.

Toxascaris leonina was the most prevalent parasite for arctic foxes in this study, detected at all sites and it was more

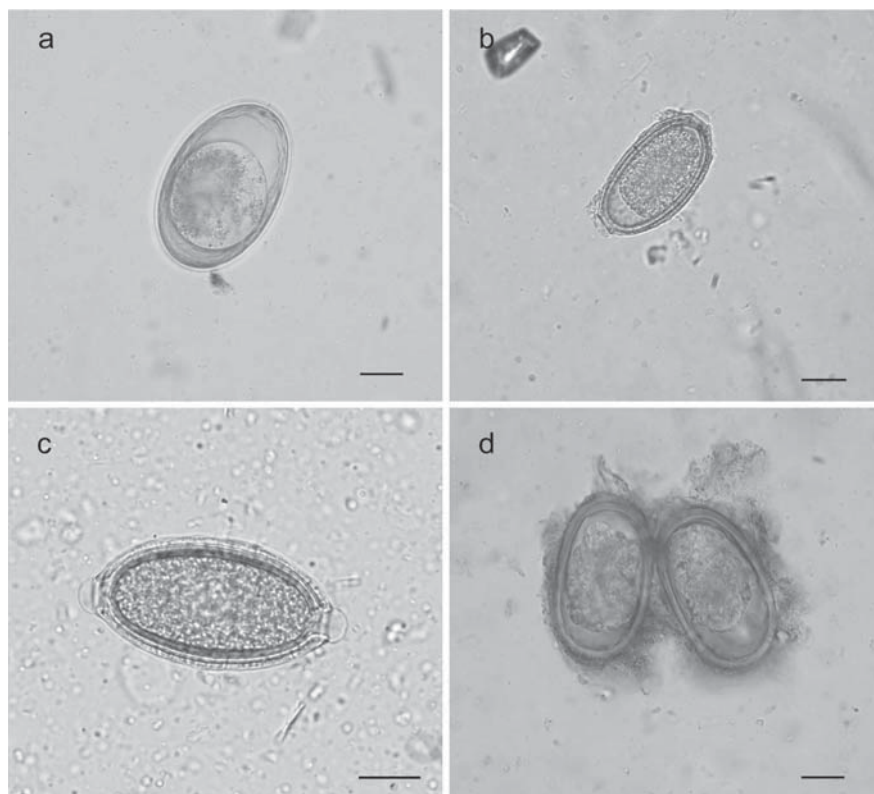


Fig. 2 Nematode eggs observed in the arctic fox faecal samples. **a** *Toxascaris leonina*. **b** *Eucoleus* sp.. **c** *Trichuris* sp.. **d** *Uncinaria stenocephala*. The scale bar 20 μ m

abundant than *T. canis* (Meijer et al. 2011; Elmore et al. 2013). In general, ascarid nematodes (mostly *T. leonina* and *T. canis*) are frequent intestinal parasites of arctic foxes. The prevalence found in this study (13.3%) is lower compared with the study (33 %) of Stien et al. (2010), both from Svalbard. The difference may be caused by a lower number of examined samples in our study or year to year differences. *Toxascaris leonina* may use rodents as paratenic hosts, and Stien et al. (2010) found a decreased prevalence of *T. leonina* with distance to the sibling vole core area of Grumantbyen (see the map in Fig. 1). Because of our low samples size, we were not able to analyse for such an effect. The presence of *T. canis* is for the first time documented in arctic foxes in Svalbard and is striking. Predominant species in high latitudes is *T. leonina*, an ascarid roundworm that is able to survive at low temperatures and can generally adapt to a greater variety of climate conditions than *Toxocara* spp. (Okoshi and Usui 1968). *Toxocara canis* has previously been detected in arctic foxes only in Iceland (one single case), and in Sweden (common parasite,

several cases) (Meijer et al. 2011; Skírnisson et al. 1993). Stien et al. (2010) detected *T. leonina* but there was also large numbers of unidentified nematode individuals present.

The differentiation of *Eucoleus* spp. from *Calodium hepaticum* eggs was performed according to Fugassa et al. (2008). *Eucoleus* sp., a respiratory nematode common in foxes and other carnivores, is documented for the first time in Svalbard. Its pathogenic role in foxes is not well recognised, but it usually causes only minor clinical signs (Lalošević et al. 2013). However, it has also been considered as a cause of massive mortality in farmed silver foxes (*Vulpes vulpes*; Skryabin et al. 1957). The life cycle is direct; animals become infected by ingesting the embryonated eggs from the environment (Taylor et al. 2007). It is difficult to distinguish via microscopy whether these are eggs of the genus *E. aerophilus* or *E. boehmi* because of the high morphological similarity of their eggs (Traversa et al. 2010). The first mentioned has been reported in arctic foxes from Iceland and Sweden (Aguirre et al. 2000; Meijer et al. 2011; Skírnisson et al. 1993) and

Table 1 Parasite species in arctic foxes (*Vulpes lagopus*) and sibling voles (*Microtus levis*) in central Spitzbergen, Svalbard. Samples are characterised by microscopy and PCR analysis with prevalence (%) of parasites found in 62 faecal samples and in 63 trapped sibling voles. Positive samples are completed with locality and year of sampling (2 = 2012; 3 = 2013; 5 = 2015). Parasites written in bold are new species to Svalbard

Arctic foxes	Microscopy (n = 62)	Molecular analysis (n = 62)	Prevalence (%)	Positive sample	
				Year	Locality
<i>Toxascaris leonina</i>	4	5 *one	12.9	2; 3; 5	All locations [†]
<i>Eucoleus</i> sp.	2	0	3.2	2; 3	Billefjorden
<i>Trichuris</i> sp.	1	-	1.6	5	Billefjorden
<i>Toxocara canis</i>	0	1	1.6	5	Sassendalen
<i>Uncinaria stenocephala</i>	1	-	1.6	3	Billefjorden
<i>Eimeria</i> spp.	5	3 *all	8.1	2; 3; 5	Billefjorden
<i>Enterocytozoon bieneusi</i>	0	1	1.6	2	Billefjorden
<i>Encephalitozoon</i> sp.	0	0	0	-	-
<i>Giardia</i> sp.	0	0	0	-	-
<i>Cryptosporidium</i> sp.	0	0	0	-	-
Cestodes	0	0	0	-	-
Sibling voles	Microscopy (n = 63)	Molecular analysis (n = 63)	Prevalence (%)	Positive sample	
				Year	Locality
<i>Cryptosporidium alticolis</i>	0	1	1.6	5	Longyearbyen

0 = negative sample; - = not tested; * = microscopically positive; [†] = Billefjorden, Sassendalen, Longyearbyen

the latter from Greenland (Andreassen et al. 2017). The hookworm *U. stenocephala* has been previously reported in arctic foxes in Iceland, Greenland, and Sweden (Meijer et al. 2011; Rausch et al. 1983; Skirnisson et al. 1993). Unlike another hookworm *Ancylostoma caninum*, this parasite has a higher tolerance to lower temperatures (Balasingam 1964). The life cycle of *U. stenocephala* usually follows a faecal-oral route of transmission, ingestion of paratenic host (rodents), or less frequent the larvae migrate through the skin (Chu et al. 2013; Zajac and Conboy 2012). Eggs of *Trichuris vulpis*, a common parasite of red foxes (*Vulpes vulpes*; Borecka et al. 2013; Míterpáková et al. 2009), have only been reported in arctic foxes in Sweden (Meijer et al. 2011). The life cycle of this parasite is also direct, and animals become infected through a faecal-oral transmission (Venco et al. 2011).

Stien et al. (2010) recorded several species of tapeworms (*Taenia ovis krabbei*, *T. polyacantha*, *T. crassiceps*, *Diphyllobothrium* sp., and *Echinococcus multilocularis*), and acanthocephala, whereas all of our samples were negative for these parasites despite the same sites of origin of some samples for both studies.

Fuglei et al. (2008) found a strong spatial pattern in the occurrence of the tapeworm *E. multilocularis* in arctic fox faeces on Svalbard. They found a high *E. multilocularis* prevalence within the core area of the sibling vole, namely the Grumantbyen. However, similar to our study, in the area from Bjørmdalen (2–6 km from the vole core area Grumantbyen) and in more distant areas on Nordenskiöld Land, no faeces

were positive for *E. multilocularis*, except one faeces found at a very far distance (110–130 km). Also, the prevalence of the *E. multilocularis* has been analysed in sibling voles in Svalbard. Henttonen et al. (2001) and Stien et al. (2010) trapped 224 (26 % positive) and 506 (19 % positive) voles in the vole core area in Grumantbyen, respectively, between the years 1999 and 2006. Their findings were different compared with this study, as all 63 captured voles in our study were negative. The reason for this is most probably because our sibling voles came from Longyearbyen about 20 km from Grumantbyen, and Grumantbyen is the only locality where *E. multilocularis* is found in voles in Svalbard. The reason for this may be that in the vicinity of Longyearbyen, compared with Grumantbyen, both hosts are fewer, the intermediate host (the sibling vole) and arctic foxes, the definitive hosts of this tapeworm, and therefore less infected voles (Fuglei et al. 2008).

Based on the molecular analyses (Online Resource1-2), *Eimeria* sp. occurred in one faecal sample (63A) from the arctic fox. Our sample clustered inside with *Eimeria* spp. infecting gallinaceous birds. Because eimerians tend to be usually highly host specific (Vrba and Pakandl 2015), it points to a probable origin from birds, and passages through the gastrointestinal tract of the fox. In the case of sample 63A, several different morphotypes of *Eimeria* spp. oocysts were present (Fig. 3c, d) and it is not possible to distinguish which one the sequence belongs. The second eimerian sample (sample 153) clustered individually, close to the eimerians infecting rodents

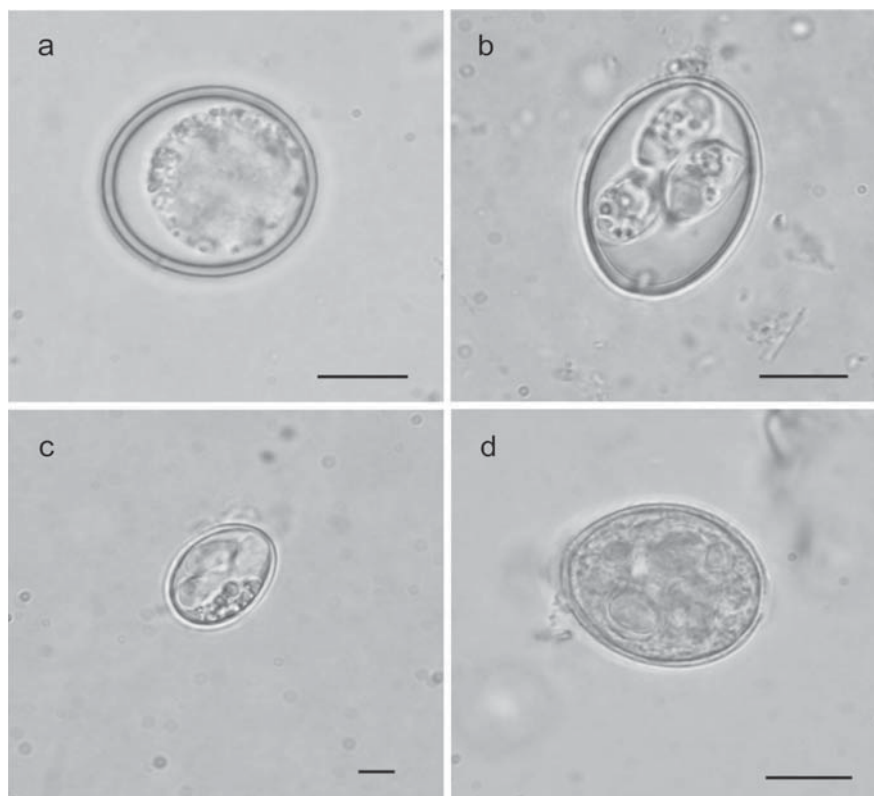


Fig. 3 Different morphotypes of *Eimeria* spp. oocysts observed in the arctic fox faecal samples. **a, b** From sample number 153. **c, d** From sample number 63A from Billefjorden. In the case of **a** and **d**, these are nonsporulated oocysts. The scale bar 10 µm

(field mice, squirrels, dormouse) and pangolin; thus, we suppose that these eimerians may infect the foxes, or may represent rodent eimerians just passed through the gastrointestinal tract of the fox. Eimerians present in the third sample (sample 125) clustered individually, on the basal position of the eimeriids infecting warm-blooded vertebrates; thus, we presume that these are real parasites of arctic foxes.

Encephalitozoon spp. and *Enterocytozoon bieneusi* are the most widely spread microsporidia in mammals (Hinney et al. 2016). Only *E. bieneusi* was molecularly positive in one arctic fox sample. However, this is the first report for arctic foxes. The sequence share 99 % sequence identity (bootstrap support 88) with genotype WL15 (GenBank AY237223) that has been found both in humans and animals. Arctic foxes may be infected by contaminated water with spores or from their prey (birds, rodents; Thellier and Breton 2008). Alternatively, the detection of a single case of *E. bieneusi* in fox could also be explained by simple passage of spores through the digestive tract without any ongoing infection.

Even though series of previous reports characterised *E. cuniculi* as a predominant parasite in farmed blue foxes in the Scandinavian countries (Arnesen and Nordstoga 1977; Mohn et al. 1974; Nordstoga and Westbye 1976), wild populations of arctic foxes in Iceland (Hersteinsson et al. 1993) and red foxes in England, Ireland, and the Czech Republic (Wilson 1979; Murphy et al. 2007; Hůrková and Modrý 2006), we have not detected this parasite in the samples in this study.

None of our samples tested positive for *Cryptosporidium* spp. The prevalence of *Cryptosporidium* seems to be low in the Arctic in general, and in some areas, the parasite has not been detected at all. For example, in Greenland, there is no record of this protist neither from animals nor from humans (Jenkins et al. 2013).

However, we molecularly detected the presence of *Cryptosporidium* in one sibling vole from Longyearbyen, which is the first detection in Svalbard. The sequence was 100 % identical to *C. alticolis* isolated from a common vole (*M. arvalis*) from the Czech Republic (Horčíčková et al. 2018;

Genbank KY644657). Low prevalence correlates with the environmental effects of the *Cryptosporidium* life cycle: oocysts of *Cryptosporidium* are highly sensitive to extreme temperatures, and in temperatures under zero they remain viable only for a short period of time (Fayer et al. 1998).

Even though Svalbard is situated at high latitudes with a high degree of isolation, the parasite species richness in arctic foxes is comparable with populations from other areas of their distribution such as Iceland, Greenland, and Canada, at least with regard to herein examined groups of parasites (Aguirre et al. 2000; Andreassen et al. 2017; Eaton and Secord 1979; Elmore et al. 2013; Kapel and Nansen 1996; Meijer et al. 2011; Rausch et al. 1983; Skirnisson et al. 1993). One of the reasons for this resemblance is probably that the Svalbard fox population is connected to other Arctic continents over the sea ice (Geffen et al. 2007; Norwegian Ice Service – MET Norway: <http://polarview.met.no/>; Carmichael et al. 2007; Norén et al. 2011) and the foxes can migrate over long distances and shed their parasites. However, only from locations that are connected with sea ice.

Eucoleus sp., *U. stenocephala*, *T. canis*, *Trichuris vulpis*, and *Eimeria* have never been detected in Svalbard; however, they are parasites of arctic foxes in other arctic locations (Meijer et al. 2011; Rausch et al. 1983; Skirnisson et al. 1993). We declined the possibility of transmission via dogs present in the island as they are under strict veterinary control (SYSSELMANNEN: <http://www.sysselmannen.no/en/Shortcuts/Pets/>). The occurrence of these parasites in Svalbard may be explained by transmission over the sea ice inside the GI tract of an arctic fox. Foxes can migrate over long distances, and Svalbard serves as a “meeting” point from both the east and west (Norén et al. 2010).

As this study shows, the coprological studies are keys for assessing the parasitic fauna and potential health risk of wild animals in the Arctic. The present documentation of as many as six new parasites for Svalbard shows the importance of the host-parasite studies in the Arctic ecosystem. It is also crucial for mapping the parasite richness of arctic foxes throughout their distribution range, enabling us to evaluate the impact of climate change that will affect the Arctic most profoundly.

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Compliance with ethical standards The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia, and also by the Ministry of the Environment of the Czech Republic (Permit Numbers MZP/2017/630/854). All procedures performed in studies involving animals were in accordance with the ethical standards of the Norwegian Animal Welfare Act. The research was also registered in The Research in Svalbard

Database, RiS-10852. All handling/usage with biological samples were allowed by the University of South Bohemia in České Budějovice in accordance with the law of the Czech Republic (Act No. 166/1999), regulation of European Parliament (Act. No. 1069/2009), and Commission Regulation (EU) (No. 142/2011).

Conflict of interest The authors declare that they have no conflict of interest.

References

- Aguirre AA, Angerbjörn A, Tannerfeldt M, Möner T (2000) Health evaluation of Arctic fox (*Alopex lagopus*) cubs in Sweden. *J Zoo Wildl Med* 31:36–40. [https://doi.org/10.1638/1042-7260\(2000\)031\[0036:HEOAFJ\]2.0.CO;2](https://doi.org/10.1638/1042-7260(2000)031[0036:HEOAFJ]2.0.CO;2)
- Anderson RM, May RM (1978) Regulation and stability of host–parasite population interactions 1. Regulatory processes. *J Anim Ecol* 47: 219–247. <https://doi.org/10.2307/3933>
- Andreassen PNS, Schmidt NM, Kapel CMO, Christensen MU, Sittler B, Gilg O, Enemark H, Al-Sabi MNS (2017) Gastrointestinal parasites of two populations of Arctic foxes (*Vulpes lagopus*) from Northeast Greenland. *Polar Res*. <https://doi.org/10.1080/17518369.2017.1308667>
- Angerbjörn A, Hersteinsson P, Tannerfeldt M (2004) Arctic fox. In: Sillero-Zubiri C, Hoffmann M, Macdonald DW (eds) Canids: foxes, wolves, jackals and dogs. Status Survey and Conservation Action Plan. IUCN/SSC Canid Specialist Group, Gland and Cambridge, pp 117–123
- Arnesen K, Nordstoga K (1977) Ocular encephalitozoonosis (nosematosis) in blue foxes. *Acta Ophthalmol* 55:641–651. <https://doi.org/10.1111/j.1755-3768.1977.tb05662.x>
- Audet MA, Robbins CB, Larivière S (2002) *Alopex lagopus*. *Mamm Species* 31:1–10. [https://doi.org/10.1644/1545-1410\(2002\)713<0001:AL>2.0.CO;2](https://doi.org/10.1644/1545-1410(2002)713<0001:AL>2.0.CO;2)
- Balasingam E (1964) Comparative studies of the effects of temperature on free-living stages of *Placoconus lotoris*, *Dochmoides stenocephala* and *Ancylostoma caninum*. *Can J Zool* 42:907–918. <https://doi.org/10.1139/z64-088>
- Borecka A, Gawor J, Zięba F (2013) A survey of intestinal helminths in wild carnivores from the Tatra National Park, southern Poland. *Ann Parasitol* 59:169–172
- Braestrup FW (1941) A study on the arctic fox in Greenland: immigrations, fluctuations in numbers based mainly on trading statistics. *Medd Grønland* 131:1–101
- Buckholt MA, Lee JH, Tzipori S (2002) Prevalence of *Enterocytozoon bieneusi* in swine: an 18-month survey at a slaughterhouse in Massachusetts. *Appl Environ Microbiol* 68:2595–2599. <https://doi.org/10.1128/AEM.68.5.2595-2599.2002>
- Carlsgrat J, Roepstorff A, Nejsup P (2009) Multiplex PCR on single unembryonated *Ascaris* (roundworm) eggs. *Parasitol Res* 104: 939–943. <https://doi.org/10.1007/s00436-008-1307-7>
- Carmichael LE, Krizan J, Nagy JA, Fuglei E, Dumond M, Johnson D, Veitch A, Bertreux D, Strobeck C (2007) Historical and ecological determinants of genetic structure in arctic canids. *Mol Ecol* 16: 3466–3483. <https://doi.org/10.1111/j.1365-294X.2007.03381.x>
- Chu S, Myers SL, Wagner B, Snead EC (2013) Hookworm dermatitis due to *Uncinaria stenocephala* in a dog from Saskatchewan. *Can Vet J* 194:743–747
- Didier ES, Vossbrinck CR, Baker MD, Rogers LB, Bertucci DC, Shadduck JA (1995) Identification and characterization of three *Encephalitozoon cuniculi* strains. *Parasitology* 111:411–421. <https://doi.org/10.1017/S00318200065914>

- Eaton RDP, Secord DC (1979) Some intestinal parasites of arctic fox, Banks Island, N. W. T. Can J Comp Med 43:229–230
- Ehrich D, Ims RA, Yoccoz NG, Lecomte N, Killengreen ST, Fuglei E, Rodnikova AY, Ebinger BS, Menyushina IE, Nolet BA, Pokrovsky IG, Popov IY, Schmidt NM, Sokolov AA, Sokolova NA, Sokolov VA (2015) What can stable isotope analysis of top predator tissues contribute to monitoring of tundra ecosystems? Ecosystems 18:404–416. <https://doi.org/10.1007/s10021-014-9834-9>
- Eide NE, Stien A, Prestrud P, Yoccoz NG, Fuglei E (2012) Reproductive responses to spatial and temporal prey availability in a coastal Arctic fox population. J Anim Ecol 81:640–648. <https://doi.org/10.1111/j.1365-2656.2011.01936.x>
- Elmore SA, Lalonde LF, Samelius G, Alisauskas RT, Gajadhar AA, Jenkins EJ (2013) Endoparasites in the feces of arctic foxes in a terrestrial ecosystem in Canada. Int J Parasitol Parasites Wildl 2: 90–96. <https://doi.org/10.1016/j.ijppaw.2013.02.005>
- Fayer R, Trout JM, Jenkins MC (1998) Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. J Parasitol 84:1165–1169. <https://doi.org/10.2307/3284666>
- Fugassa MH, Taglioretti V, Gonçalves MLC, Araújo A, Sardella NH, Denegri GM (2008) *Capillaria* spp. eggs in Patagonian archaeological sites: statistical analysis of morphometric data. Mem Inst Oswaldo Cruz 103:104–105. <https://doi.org/10.1590/S0074-02762008000100016>
- Fuglei E, Stien A, Yoccoz NG, Ims RA, Eide NE, Prestrud P, Oksanen A (2008) Spatial distribution of *Echinococcus multilocularis*, Svalbard, Norway. Emerg Infect Dis 14:73–75. <https://doi.org/10.3201/eid1401.070565>
- Geffen E, Waidyaratne S, Dalén L, Angerbjörn A, Vila C, Hersteinsson P, Fuglei E, White PA, Goltzman M, Kapel CM, Wayne RK (2007) Sea ice occurrence predicts genetic isolation in the Arctic fox. Mol Ecol 16:4241–4255. <https://doi.org/10.1111/j.1365-294X.2007.03507.x>
- Henttonen H, Fuglei E, Gower CN, Haukioja V, Ims RA, Niemimäa J, Yoccoz NG (2001) *Echinococcus multilocularis* on Svalbard: introduction of an intermediate host has enabled the local life-cycle. Parasitology 123:547–552. <https://doi.org/10.1017/S0031182001008800>
- Hersteinsson P, Macdonald DW (1996) Diet of arctic foxes (*Alopex lagopus*) in Iceland. J Zool 240:457–474. <https://doi.org/10.1111/j.1469-7998.1996.tb05298.x>
- Hersteinsson P, Gunnarsson E, Hjartardóttir S, Skirnisson K (1993) Prevalence of *Encephalitozoon cuniculi* antibodies in terrestrial mammals in Iceland, 1986 to 1989. J Wildl Dis 29:341–344. <https://doi.org/10.7589/0090-3558-29.2.341>
- Hinney B, Sak B, Joachim A, Kváč M (2016) More than a rabbit's tale - *Encephalitozoon* spp. in wild mammals and birds. Int J Parasitol Parasites Wildl 5:76–87. <https://doi.org/10.1016/j.ijppaw.2016.01.001>
- Horčíková M, Čondlová Š, Holubová N, Sak B, Květoňová D, Hlášková L, Konečný R, Sedláček F, Clark M, Giddings C, McEvoy J, Kváč M (2018) Diversity of *Cryptosporidium* in common voles and description of *Cryptosporidium alticolis* sp. n. and *Cryptosporidium microti* sp. n. (Apicomplexa: Cryptosporidiidae). Parasitology 17:1–14. <https://doi.org/10.1017/S0031182018001142>
- Hunter GW, Frye WW, Swartzwelder J (1960) A manual of tropical medicine, 3rd edn. W. B. Saunders Co., Ltd., London
- Hůrková L, Modrý D (2006) PCR detection of *Neospora caninum*, *Toxoplasma gondii* and *Encephalitozoon cuniculi* in brains of wild carnivores. Vet Parasitol 137:150–154. <https://doi.org/10.1016/j.vetpar.2006.01.005>
- Jenkins E, Castrodale L, de Rosemond S, Dixon B, Elmore S, Gesy K, Hoberg E, Polley L, Schurer J, Simard M, Thompson RCA (2013) Tradition and transition: parasitic zoonoses of people and animals in Alaska, northern Canada and Greenland. Adv Parasitol 82:33–204. <https://doi.org/10.1016/B978-0-12-407706-5.00002-2>
- Jiang J, Alderisio KA, Xiao L (2005) Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl Environ Microbiol 71:4446–4454. <https://doi.org/10.1128/AEM.71.8.4446-4454.2005>
- Jónsdóttir IS (2005) Terrestrial ecosystems on Svalbard: heterogeneity, complexity and fragility from an Arctic island perspective. Proc R Irish Acad 105:155–165. <https://doi.org/10.3318/BIOE.2005.105.3.155>
- Kapel CMO, Nansen P (1996) Gastrointestinal helminths of arctic foxes (*Alopex lagopus*) from different bioclimatological regions in Greenland. J Parasitol 82:17–24. <https://doi.org/10.2307/3284109>
- Katzwinkel-Wladarsch S, Lieb M, Helse W, Löscher T, Rinder H (1996) Direct amplification and species determination of microsporidian DNA from stool specimens. Tropical Med Int Health 1:373–378. <https://doi.org/10.1046/j.1365-3156.1996.d01-51.x>
- Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>
- Kvičerová J, Pakandl M, Hypša V (2008) Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. Parasitology 135:443–452. <https://doi.org/10.1017/S0031182007004106>
- Lalošević V, Lalošević D, Capo I, Simin V, Galfi A, Traversa D (2013) High infection rate of zoonotic *Eucolus aerophilus* infection in foxes from Serbia. Parasite. <https://doi.org/10.1051/parasite/2012003>
- Littlewood DTJ, Curini-Galletti M, Herniou EA (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. Mol Phylogenet Evol 16:449–466. <https://doi.org/10.1006/mpev.2000.0802>
- Martínez-Carrasco C, Berriatua M, Garijo E, Martínez J, Alonso FD, Ruiz de Ybáñez R (2007) Epidemiological study of non-systematic parasitism in dogs in southeast Mediterranean Spain assessed by coprological and post-mortem examination. Zoonoses Public Hlth 54:195–203. <https://doi.org/10.1111/j.1863-2378.2007.01047.x>
- Meijer T, Mattson R, Angerbjörn A, Osterman-Lind E, Fernández-Aguilar X, Gavriel-Widén D (2011) Endoparasites in the endangered Fennoscandian population of Arctic foxes (*Vulpes lagopus*). Eur J Wildl Res 57:923–927. <https://doi.org/10.1007/s10344-011-0505-2>
- Miterpáková M, Hurníková Z, Antolová D, Dubinský P (2009) Endoparasites of red fox (*Vulpes vulpes*) in the Slovak Republic with the emphasis on zoonotic species *Echinococcus multilocularis* and *Trichinella* spp. Helminthologia 46:73–79. <https://doi.org/10.2478/s11687-009-0015-x>
- Mohn SF, Nordstoga K, Krogsrud J, Helgebostad A (1974) Transplacental transmission of *Nosema cuniculi* in the blue fox (*Alopex lagopus*). Acta Pathol Microbiol Scand B: Microbiol Immunol 82:299–300. <https://doi.org/10.1111/j.1699-0463.1974.tb02328.x>
- Murphy TM, Walochnik J, Hassl A, Moriarty J, Mooney J, Toolan D, Sanchez-Miguel C, O'Loughlin A, McAuliffe A (2007) Study on the prevalence of *Toxoplasma gondii* and *Neospora caninum* and molecular evidence of *Encephalitozoon cuniculi* and *Encephalitozoon (Septata) intestinalis* infections in red foxes (*Vulpes vulpes*) in rural Ireland. Vet Parasitol 146:227–234. <https://doi.org/10.1016/j.vetpar.2007.02.017>
- Nordstoga K, Westbye K (1976) Polyarteritis nodosa associated with nomenclature in blue foxes. Acta Pathol Microbiol Scand A 84: 291–296
- Norén K, Carmichael L, Dalén L, Hersteinsson P, Samelius G, Fuglei E, Kapel CMO, Menyushina I, Strobeck C, Angerbjörn A (2010)

- Arctic fox *Vulpes lagopus* population structure: Circumpolar patterns and processes. *Oikos* 120:873–885. <https://doi.org/10.1111/j.1600-0706.2010.18766.x>
- Norén K, Carmichael L, Fuglei E, Eide NE, Hersteinsson P, Angerbjörn A (2011) Pulses of movement across the sea ice: population connectivity and temporal genetic structure in the Arctic fox. *Oecologia* 166:973–984. <https://doi.org/10.1007/s00442-011-1939-7>
- Okoshi S, Usui M (1968) Experimental studies on *Toxascaris leonina*. IV. Development of eggs of three ascarids, *T. leonina*, *Toxocara canis* and *Toxocara cati*, in dogs and cats. *Jpn J Vet Sci* 30:29–38. <https://doi.org/10.1292/jvms1939.30.29>
- Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). *Int J Parasitol* 33:733–755. [https://doi.org/10.1016/S0020-7519\(03\)00049-3](https://doi.org/10.1016/S0020-7519(03)00049-3)
- Prestrud P, Krogsrud J, Gjert I (1992) The occurrence of rabies in the Svalbard Islands of Norway. *J Wildl Dis* 28:57–63. <https://doi.org/10.7589/0090-3558-28.1.57>
- Rausch RL, Fay FH, Williamson FSL (1983) Helminths of the Arctic fox, *Alopex lagopus* (L.), in Greenland. *Can J Zool* 61:1847–1851. <https://doi.org/10.1139/z83-237>
- Schwarz RS, Jenkins MC, Klopp S, Miska KB (2009) Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *J Parasitol* 95:871–880. <https://doi.org/10.1645/GE-1898.1>
- Sheather AL (1923) The detection of intestinal protozoa and mange parasites by a flotation technique. *J Comp Pathol Ther* 36:266–275. [https://doi.org/10.1016/S0368-1742\(23\)80052-2](https://doi.org/10.1016/S0368-1742(23)80052-2)
- Skirnisson K, Eydal M, Gunnarsson E, Hersteinsson P (1993) Parasites of the arctic fox (*Alopex lagopus*) in Iceland. *J Wildl Dis* 29:440–446. <https://doi.org/10.7589/0090-3558-29.3.440>
- Skryabin KI, Shikhobalova NP, Orlov IV (1957) [Trichocephalids and Capillariids of animals and man and the diseases caused by them.] In: *Essentials of Nematodology* 6th edn. House of the USSR Acad. Sci., Moscow (In Russian)
- Smith PH, Seklau BS, Wiles E, Malone JB, Monahan MC (2007) Collection, preservation, and diagnostic methods. In: Baker DG (ed) *Flynn's Parasites of Laboratory Animals*, 2nd edn. Wiley-Blackwell, Iowa
- Sørensen KK, Mørk T, Sigurdardóttir OG, Asbakk K, Akerstedt J, Bergsjø B, Fuglei E (2005) Acute toxoplasmosis in three wild arctic foxes (*Alopex lagopus*) from Svalbard; one with co-infections of *Salmonella* Enteritidis PT1 and *Yersinia pseudotuberculosis* serotype 2b. *Res Vet Sci* 78:161–167. <https://doi.org/10.1016/j.rvsc.2004.07.010>
- Stien A, Voutilainen L, Haukisalme V, Fuglei E, Mørk T, Yoccoz NG, Ims RA, Henttonen H (2010) Intestinal parasites of the Arctic fox in relation to the abundance and distribution of intermediate hosts. *Parasitology* 137:149–157. <https://doi.org/10.1017/S0031182009990953>
- Sulaiman IM, Fayer R, Bern C, Gilman RH, Trout JM, Schantz PM, Das P, Lal AA, Xiao L (2003) Triphosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. *Emerg Infect Dis* 9:1444–1452. <https://doi.org/10.3201/eid0911.030084>
- Taylor MA, Coop RL, Wall RL (2007) *Veterinary Parasitology*, 3rd edn. Blackwell Publishing Ltd., Oxford
- Thellier M, Breton J (2008) *Enterocytozoon bienersi* in human and animals, focus on laboratory identification and molecular epidemiology. *Parasite* 15:349–358. <https://doi.org/10.1051/parasite/2008153349>
- Trachsel D, Deplazes P, Mathis A (2007) Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. *Parasitology* 134:911–920. <https://doi.org/10.1017/S0031182007002235>
- Traversa D, Di Cesare A, Conboy G (2010) Canine and feline cardiopulmonary parasitic nematodes in Europe: emerging and underestimated. *Parasit Vectors* 3:62. <https://doi.org/10.1186/1756-3305-3-62>
- Venco L, Valenti V, Genchi M, Grandi G (2011, 2011) A dog with Pseudo-Addison disease associated with *Trichuris vulpis* infection. *J Parasitol Res*. <https://doi.org/10.1155/2011/682039>
- Vrba V, Pakandl M (2015) Host specificity of turkey and chicken *Eimeria*: controlled cross-transmission studies and a phylogenetic view. *Vet Parasitol* 208:118–124. <https://doi.org/10.1016/j.vetpar.2015.01.017>
- Wilson JM (1979) *Encephalitozoon cuniculi* in wild European rabbits and a fox. *Res Vet Sci* 26:114
- Zajac AM, Conboy GA (2012) *Veterinary clinical parasitology*, 8th edn. Wiley-Blackwell, Oxford

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Generalist *Eimeria* species in rodents: Multilocus analyses indicate inadequate resolution of established markers

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Abstract

- Intracellular parasites of the genus *Eimeria* are described as tissue/host-specific. Phylogenetic classification of rodent *Eimeria* suggested that some species have a broader host range than previously assumed. We explore whether *Eimeria* spp. infecting house mice are misclassified by the most widely used molecular markers due to a lack of resolution, or whether, instead, these parasite species are indeed infecting multiple host species.
- With the commonly used markers (18S/COI), we recovered monophyletic clades of *E. falciformis* and *E. vermiformis* from *Mus* that included *E. apionodes* identified in other rodent host species (*Apodemus* spp., *Myodes glareolus*, and *Microtus arvalis*). A lack of internal resolution in these clades could suggest the existence of a species complex with a wide host range infecting murid and cricetid rodents. We question, however, the power of COI and 18S markers to provide adequate resolution for assessing host specificity. In addition to the rarely used marker ORF470 from the apicoplast genome, we present multilocus genotyping as an alternative approach. Phylogenetic analysis of 35 nuclear markers differentiated *E. falciformis* from house mice from isolates from *Apodemus* hosts. Isolates of *E. vermiformis* from *Mus* are still found in clusters interspersed with non-*Mus* isolates, even with this high-resolution data.
- In conclusion, we show that species-level resolution should not be assumed for COI and 18S markers in coccidia. Host-parasite cospeciation at shallow phylogenetic nodes, as well as contemporary coccidian host ranges more generally, is still open questions that need to be addressed using novel genetic markers with higher resolution.

KEYWORDS

18S, COI, *Eimeria*, multilocus sequence typing, phylogenetics, rodents

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1 | INTRODUCTION

Coccidians of the genus *Eimeria* have been described as monoxenous, intracellular parasites (Becker, 1934; Long & Joyner, 1984; Marquardt, 1981). Two different characteristics extensively used to delineate *Eimeria* species are their assumed high degree of host and tissue specificity. It is not clear, however, whether host specificity is the same for *Eimeria* species infecting hosts in different clades. *Eimeria* species of rodents show a degree of host specificity (Ball & Lewis, 1984; De Vos, 1970; Duszynski, 2011; Wilber, Duszynski, Upton, Seville, & Corliss, 1998), but individual isolates can experimentally infect different species and even genera of rodents (Levine & Ivens, 1988; Upton, McAllister, Brillhart, Duszynski, & Wash, 1992).

Descriptions of *Eimeria* species are based on the size and shape of sporulated oocysts and their internal structures. The life cycles of a few species have additionally been studied and data on their dynamics (e.g., the patent period, the time before oocysts are shed in feces) are available (Duszynski, Eastham, & Yates, 1982; Hnida, Wilson, & Duszynski, 1998; Lainson & Shaw, 1990; Levine & Ivens, 1965; Mesfin & Bellamy, 1978; Todd & Hammond, 1968; Todd & Lepp, 1971; Turner, Penzhorn, & Getz, 2016; Wash, Duszynski, & Yates, 1985). For field studies, the morphology of sporulated oocysts alone is considered insufficient to infer species identity because of inadequate reference descriptions (MacPherson & Gajadhar, 1993; Tenter et al., 2002).

Genetic markers from nuclear (nu) and mitochondrial (mt) genomes, and less frequently of the apicoplast (ap) genome, have been used to complement morphological taxonomy with phylogenetic analyses (Hnida & Duszynski, 1999a, 1999b; Kvičarová, Mikeš, & Hypša, 2011; Ogedengbe, Ogedengbe, Hafeez, & Barta, 2015; Zhao & Duszynski, 2001a). Based on the assumption of host specificity of individual *Eimeria* species, phylogenetic analysis of nuclear small subunit ribosomal (18S) rDNA and cytochrome c oxidase I (COI) fragments supports predominant host-parasite cospeciation (Ogedengbe, El-Sherry, Ogedengbe, Chapman, & Barta, 2018). Species infecting rodents, however, are found in two separate clades, generating marked discrepancy between parasite and host phylogeny at deeper nodes (Kvičarová & Hypša, 2013). At shallow nodes of the phylogeny for rodent coccidians, cases of host generalism have been suggested (Máková et al., 2018). Host specificity of *Eimeria* species infecting rodents is not as undisputed as in other hosts such as poultry (Barta et al., 1997) or rabbits (Kvičarová, Pakandl, & Hypša, 2008). Kvičarová and Hypša (2013) suggested that adaptation rather than cospeciation is shaping rodent *Eimeria* cophylogenies. Máková et al. (2018) added that host ecology and distribution may favor host-switches among closely related rodent species. A high specificity of *E. apionodes* naturally infecting *Apodemus flavicollis* was originally suggested based on failed attempts to experimentally infect other rodents: *Myodes (Clethrionomys) glareolus*, *Microtus arvalis*, or *Mus musculus* (Pellérdy, 1954). It is, however, unclear if this result holds for the multiple isolates that have been assigned as *E. apionodes*.

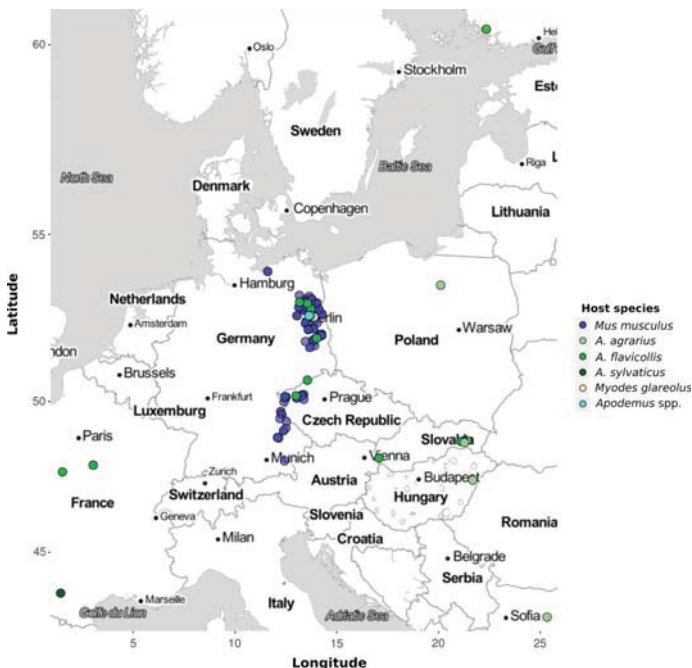


FIGURE 1 Location of rodent samples. *Mus musculus* samples were collected from the German federal states of Mecklenburg-Vorpommern, Bavaria, and Brandenburg and in Bohemia (Czech Republic). Non-*Mus* samples were collected from different countries within Europe. Color in the points indicates the host species

We studied wild populations of *Mus musculus* and other rodents to assess the diversity of *Eimeria* isolates at shallow depth of phylogenetic relationships. We test host specificity based on phylogenetic analysis using established markers (nu 18S, mt COI, and ap ORF470). We question in how far these markers are polymorphic enough to resolve between genetic clusters with different host usage (and whether a negative result for genetic differentiation therefore suggests cases of generalism). We develop and apply multilocus sequence typing to disentangle relationships unresolved by 18S and COI markers.

2 | MATERIAL AND METHODS

2.1 | Origin of samples

DNA was extracted from the colon content or gastrointestinal tissue of house mice (*Mus musculus*) infected with *Eimeria*. These samples came from rodents captured in farms and private properties in the German federal states of Mecklenburg-Vorpommern, Bavaria, and Brandenburg (capture permit No. 2347/35/2014) and in Bohemia (Czech Republic) between 2014 and 2017 (Jarquín-Díaz et al., 2019). Additionally, DNA from gastrointestinal tract and tissue or feces of *Apodemus* spp. from different regions in Europe (including areas overlapping with those sampled for house mice) were also included (Máková et al., 2018) (Figure 1) (Data S1).

2.2 | Host identification

Rodents were first identified visually based on their morphology. Identification of *Mus musculus* at the subspecies-level was confirmed based on a set of previously described markers (Đureje, Macholán, Baird, & Piálek, 2012). In order to confirm the species of non-*Mus* rodents, a fragment of cytochrome *b* (~900 bp) was amplified from host DNA. PCRs were performed according to the protocols described by Reutter, Petit, Brünner, and Vogel (2003) for *Apodemus* spp., Abramson, Rodchenkova, and Kostygov (2009) (primers UCBO_F/LM_R), and Jaarola and Searle (2002) (primers L14641M/H15408M) for rodents belonging to the subfamily Arvicolinae (*Myodes* spp. and *Microtus* spp.).

2.3 | PCR amplification (nu 18S rDNA, mt COI, and ap ORF470)

For phylogenetic analysis, nuclear small subunit ribosomal DNA (18S; ~1,500 bp), a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI; ~800 bp) gene and apicoplast ORF470 (~800 bp) were amplified using primers previously reported by Kvičerová et al. (2008), Ogedengbe, Hanner, and Barta (2011) and Zhao and Duszynski (2001b), respectively.

When COI failed to amplify with this protocol, an alternative pair of primers was used: Eim_COI_M_F (ATGTCACNTCTCCAACCTCAGT)

and Eim_COI_M_R (GAGCAACATCAANAGCAGTGT). These primers amplify a ~700 bp fragment of COI and were designed based on the mitochondrial genome of *E. falciformis* (CM008276.1) (Heitlinger, Spork, Lucius, & Dieterich, 2014; Jarquín-Díaz et al., 2019).

PCRs were carried out in a Labcycler (SensoQuest GmbH, Göttingen, Germany) using 0.025 U/μL of DreamTaqTMDNA Polymerase (Thermo Scientific, Waltham, USA), 1X DreamTaq Buffer, 0.5 mM dNTP Mix, 0.25 μM from each primer, and 1–20 ng/μL of DNA template in 25 μL reaction. A concentration of 0.25 mM dNTP mix and a supplementation with 0.5 mM MgCl₂ was used for the ap ORF470 amplification. The thermocycling protocol consisted of 95°C initial denaturation (4 min) followed by 35 cycles of 92°C denaturation (45 s), annealing at 52°C (30 s/Eim_COI); 53°C (45 s/18S); 55°C (30 s/COI); 50°C (45 s/ORF470); 72°C extension 90 s (18S/ORF470), 20 s (COI/Eim_COI), and a final extension at 72°C (10 min). DNA from oocysts of *E. falciformis* BayerHaberKorn1970 and DNA from colon content of a noninfected laboratory (NMRI) mouse were used as positive and negative controls, respectively.

All PCR products from nu 18S, mt COI, and ap ORF470 of the expected size were purified using the SAP-Exo Kit (Jena Bioscience GmbH, Jena, Germany) and sequenced in both directions by LGC Genomics (Berlin, Germany). Quality assessment and sequence assembly was performed in Geneious v6.1.8. All sequences were submitted to the NCBI GenBank database (Accession numbers: nu 18S rDNA [MH751925-MH752036, MK246860-MK246868, and MK625202-MK625210]; mt COI [MH777467-MH777593, MH75302-MH755324, MK257106-MK257114, and MK631866-MK631868] and ap ORF470 [MH755325-MH755450, MK257115-MK257125, and MK631869-MK631884]).

2.4 | Phylogenetic analysis and inference of intraspecific genetic diversity

Datasets for each gene and a concatenated alignment (nu 18S, mt COI, and ap ORF470) were created adding closely related reference sequences available in the GenBank (Data S2).

Protein-coding sequences (mt COI and ap ORF470) were aligned by translation using the Multiple Align algorithm and translation frame 1 with the genetic code for "mold protozoan mitochondrial," 18S sequences were aligned using MUSCLE (Edgar, 2004), both through Geneious v6.1.8.

Phylogenetic trees for all datasets were constructed using maximum likelihood (ML) and Bayesian inference (BI) methods, implemented in PhyML v3.0 (Guindon et al., 2010) and MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012), respectively. Sequence evolution models most appropriate for each dataset were determined in JModelTest v2.1.10 (Posada, 2008). For ML trees, a bootstrap analysis with 1,000 replicates was performed, whereas MCMC for BI was run with two cold and two hot chains for 1,000,000 generations or until the split freq value was below 0.05. The concatenated dataset was analyzed using partitions and locus-specific models. Trees were visualized with FigTree v1.4.2

(Rambaut, 2012). A haplotype network of mt COI sequences was inferred using a codon-based alignment trimmed to 500 bp available for all isolates. Haplotype frequencies were calculated and a network was constructed with the R package "pegas" v0.11 (Paradis et al., 2018).

2.5 | Multimer genotyping PCR and high-throughput sequencing

Samples positive for *E. falciformis* and *E. vermiformis* from *Mus musculus* and *Eimeria* spp. from *Apodemus* with indistinguishable 18S and COI sequences were used for a multimer amplification using the microfluidics PCR system Fluidigm Access Array 48 x 48 (Fluidigm, San Francisco, California, USA). We used target-specific primers (Data S3) that were designed based on the genome of *E. falciformis* (Heitlinger et al., 2014) to amplify exons of nuclear genes (Data S4) and coding and noncoding regions from the apicoplast genome (Data S5). Library preparation was performed according to the protocol Access Array Barcode Library for Illumina Sequencers (single direction indexing) as described by the manufacturer (Fluidigm, San Francisco, California, USA). The library was purified using Agencourt AMPure XP Reagent beads (Beckman Coulter Life Sciences, Krefeld, Germany). Quality and integrity of the library was confirmed using the Agilent 2200 Tape Station with D1000 ScreenTapes (Agilent Technologies). Sequences were generated at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv) on the Illumina MiSeq platform (Illumina) in two runs, one using "v3 chemistry" with 600 cycles, the other "v2 chemistry" with 500 cycles. All sequencing raw data can be accessed through the BioProject PRJNA548431 in the NCBI Short Read Archive (SRA).

2.6 | Bioinformatic analysis of multilocus sequence typing

Screening and trimming of sequencing reads was performed using the package dada2 v1.2.1 (Callahan et al., 2016). All reads were trimmed to 245 bases, while allowing a maximum of 4 expected errors (maxEE). Sorting and assignment to amplicons was performed with the package MultiAmplicon v0.1 (Heitlinger, 2019) and the most abundant sequence was recorded for each marker in each sample (recording but disregarding minority sequence in nonclonal infection for further analysis; see Data S6). Sequences were aligned using the function "AlignSeqs" from the package DECIPHER v2.10.0 (Wright, 2016) and nontarget sequences were excluded from alignments if >20% divergence was observed with other sequences (such as in cases off-target amplification of mostly bacterial sequences). Alignments were controlled for the absence of insertions/deletions (indels) that distort the open reading frame. Prevalent multiple-of-3-mere indels corresponding to homopolymeric amino acid repeats (HAARs; Heitlinger et al., 2014) of diverse length were coded as missing data due to their unclear model of evolution. The function

"dudi.pcr" from the packages ade4 v1.7-13 (Dray & Dufour, 2007) and adegenet v2.1.1 (Jombart, 2008) was used to visualize genetic distances between samples based on all markers. The code for this pipeline is available at https://github.com/VictorHJD/AA_Eimeria_Genotyping.

The alignments of the concatenated sequences were then exported. The number of informative sites was summarized using the tool DIVEIN (Deng et al., 2010) and phylogenetic trees were computed by Bayesian inference in MrBayes v3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012). A partitioned model was implemented to estimate the tree considering each gene separately. The analysis was performed with two runs, with 1,000,000 generations leading to a split frequency value below 0.05, and 200,000 generations were discarded as burn-in when estimating posterior probability. Additionally, maximum likelihood trees were inferred with 1,000 bootstrap replicates in PhyML v3.0 (Guindon et al., 2010).

The topology of ML and BI trees was compared and summarized into a consensus tree with minimum clade frequency threshold of 0.95 using the program SumTrees v4.3.0 (Sukumaran & Holder, 2010).

3 | RESULTS

3.1 | Established markers do not recover clades corresponding to species with different host usage

We performed phylogenetic analyses using nuclear, mitochondrial, and apicoplast markers to assess the clustering of our sequences into groups of previously described species.

We inferred a phylogenetic tree of nu 18S based on 215 sequences (509–1,795 bp). Of these, 111 from parasites in house mice (*M. musculus*) (3 from ileum tissue, 16 from cecum tissue, and 92 from colon content) and 18 from parasites in non-*Mus* rodents were generated in the present study (3 from ileum tissue, 3 from cecum tissue, 3 from colon content, and 9 from feces). To test for host specificity of house mouse *Eimeria*, we included reference sequences from related *Eimeria* species described in murid and cricetid rodents. *Isosporasp.* sequences identified in *Talpa europaea* moles were used as an outgroup. Both ML and BI rooted trees shared the general topology (Figure 2).

The sequences derived from *Mus musculus* samples clustered in three well-supported monophyletic groups: one comprising reference sequences of *E. falciformis* (*E. falciformis* group), another of *E. ferrisi* (*E. ferrisi* group), and the third of *E. vermiformis* (*E. vermiformis* group). All three groups, however, included sequences of *Eimeria* from other cricetid and murid hosts without showing internal substructure linked to the observed host species infected (host usage) (Figure 2).

The phylogenetic tree of mt COI was based on 233 sequences (381–804 bp), 149 of which were obtained from *Eimeria* infecting house mice (3 from ileum, 16 from cecum tissue, and 130 from colon content) and 12 from non-*Mus* rodents in our study (2 from ileum,

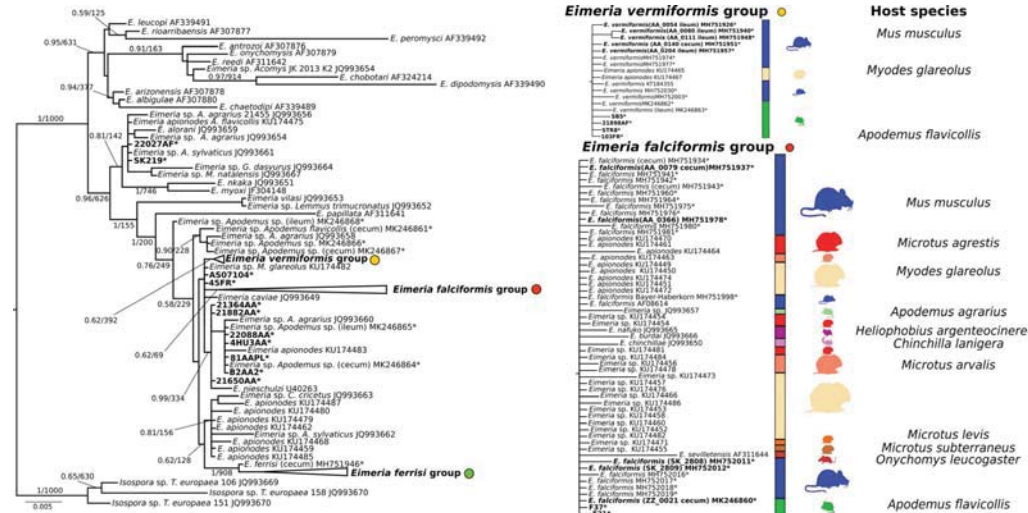


FIGURE 2 Phylogenetic trees inferred from nuclear small ribosomal subunit (18S rDNA). Phylogenetic tree based on 18S rDNA sequences. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* of this study. Reference sequences from other rodents were included. The scale bar represents sequence divergence. Hosts for closely related sequences of *E. falciformis* and *E. vermiformis* are indicated in the expanded form of the group. * represents sequences generated in the present study. Tissue of origin is indicated in brackets. Sequences in bold were included in the multimer phylogenetic inference

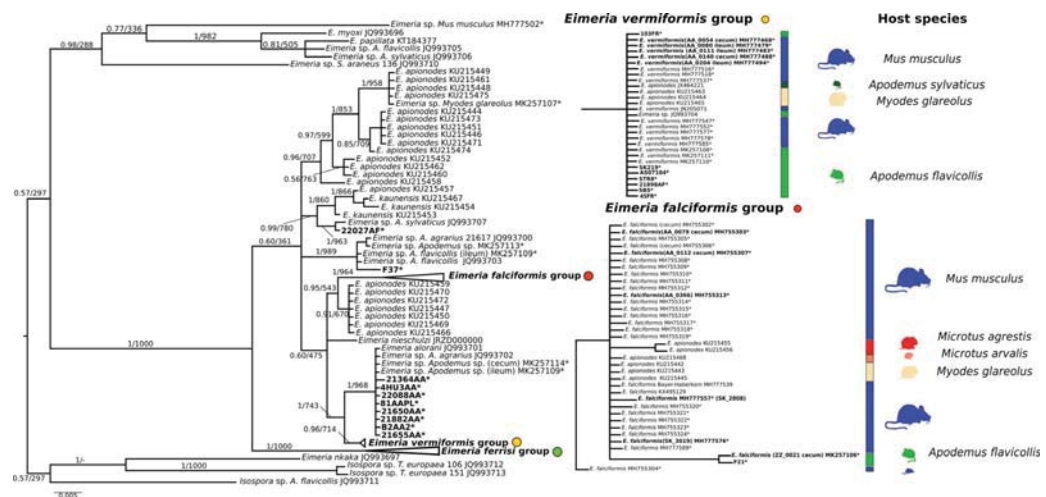


FIGURE 3 Phylogenetic trees inferred from mitochondrial cytochrome c oxidase (COI) sequences. Phylogenetic tree based on COI. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* of this study. Reference sequences from other rodents were included. The scale bar represents sequence divergence. Hosts for closely related sequences of *E. falciformis* and *E. vermiformis* are indicated in the expanded form of the group. * represents sequences generated in the present study. Tissue of origin is indicated in brackets. Sequences in bold were included in the multimer phylogenetic inference

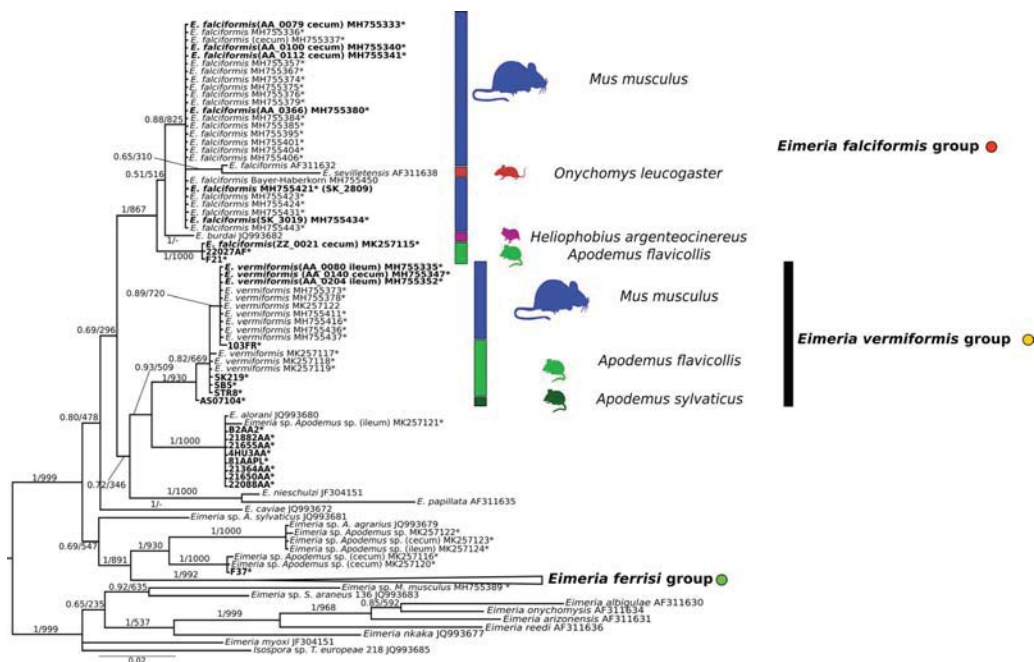


FIGURE 4 Phylogenetic trees inferred from apicoplast open reading frame 470 (ORF470) sequences. Phylogenetic tree based on ORF470 sequences. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* of this study. Reference sequences from other rodents were included. The scale bar represents sequence divergence. Hosts for closely related sequences of *E. falciformis* and *E. vermiformis* are indicated in the expanded form of the group. * represents sequences generated in the present study. Tissue of origin is indicated in brackets. Sequences in bold were included in the multimer phylogenetic inference

1 from cecum, 6 from colon content, and 3 from feces) (Figure 1). Similar to 18S, COI sequences derived from house mice clustered in three monophyletic groups including reference sequences of *E. falciformis* ($n = 26$), *E. ferrisi* ($n = 109$), and *E. vermiformis* ($n = 13$). Groups of *E. falciformis* and *E. vermiformis* also include sequences derived from *Eimeria* isolates of common voles (*Mi. arvalis*), bank voles (*My. glareolus*), short-tailed voles (*Mi. agrestis*), yellow-necked mice (*A. flavicollis*), or wood mice (*A. sylvaticus*). In addition to our isolates from *M. musculus*, the *E. ferrisi* groups contain sequences of *E. burdai* and *E. nafuko*, species described from sub-Saharan mole rats (*Heliophobius argenteocinereus*). Again, the clades do not show further substructure indicative of host usage (Figure 3).

A phylogenetic tree of ORF470 was based on 172 sequences (Figure 4) and showed a similar topology to the COI and 18S trees. Sequences derived from *Eimeria* isolates from *Mus musculus* (n = 125) also clustered into the same three groups. For this marker, the number of sequences available in databases from other cricetid and murid rodents is very limited, and none of the available sequences clustered within the highly supported "species clusters" of our isolates. In contrast to nu 18S and mt COI, our newly generated sequences from isolates detected in *A. flavicollis* and *A. sylvaticus*

formed separate clusters that were basal to the *E. falciformis* group ($n = 3$), and outside of the *E. vermiformis* group ($n = 4$) (Figure 4).

To combine all available information into a single phylogenetic analysis, we used a concatenated alignment. In the tree constructed from this alignment (Data S7), the clusters from *E. vermiformis* and *E. ferrisi* observed in the individual phylogenies with 18S, COI, and ORF470 were confirmed in the concatenated tree. Sequences from the *E. falciformis* group were found in an unresolved basal position with *E. apionodes* isolates derived from *Myodes* sp. and *Microtus* sp. This result probably indicates conflicting signals for different markers and missing data.

3.2 | Low genetic diversity of mt COI in rodent *Eimeria* isolates

With the aim to estimate the genetic diversity of isolates of *Eimeria* from different rodent hosts, we constructed a haplotype network (Figure 5) from 161 COI sequences obtained in this study combined with 59 previously published sequences (alignment of 459 bp without gaps). The network comprised 20 different haplotypes with up

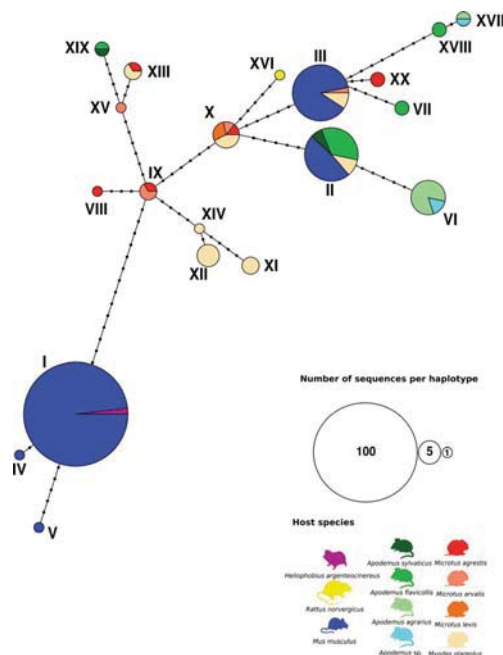


FIGURE 5 Statistical parsimony network of *Eimeria* spp. haplotypes for COI sequences. Network based on a 459 bp region of the gene coding for the mitochondrial cytochrome c oxidase from *Eimeria* isolates detected in rodents (*Mus musculus*, *Apodemus flavicollis*, *A. sylvaticus*, *A. agrarius*) caught in Europe. Previously published sequences from different species of *Eimeria* infecting cricetid and murid rodents were also included. Coloring of each haplotype is based on the host species from the *Eimeria* isolate. Every haplotype is marked with a consecutive number and its size indicates the number of sequences included on it. Each node represents a mutational step between two haplotypes

to 14 polymorphic nucleotide sites among them. The network confirms the lack of genetic differentiation of *E. falciformis* and *E. vermiformis* from some isolates described as *E. apionodes* in non-*Mus* hosts using sequences of COI.

3.3 | Multilocus genotyping

To determine whether markers with a higher resolution could distinguish host usage patterns for the "rodent parasite models" *E. falciformis* and *E. vermiformis*, we designed a multilocus sequence typing approach. Thirty-five markers targeting exons in the nuclear genome (Data S4) and 5 regions of the apicoplast genome were amplified for 19 samples from *Apodemus* spp. hosts, 12 samples from house mice and corresponding regions from the reference genome of *E. falciformis* and *E. vermiformis* were included. All the isolates used

correspond to *Eimeria* species with different morphology (Data S10 and S11).

A multivariate analysis identified three clusters of isolates for the nuclear markers: one group included the laboratory isolate of *E. vermiformis*, another the isolate of *E. falciformis*, and a third group only contained *Eimeria* isolates from *Apodemus agrarius* (Figure 6b). This result was corroborated by phylogenetic analysis of SNPs (2019 informative alignment columns). We excluded prevalent indels from this analysis. Indels in protein-coding genes (all "in-frame" with a length divisible by three) correspond to homopolymeric amino acid repeats (HAARs) and are expected in protein-coding genes of *Eimeria* spp. (Heitlinger et al., 2014; Reid et al., 2014) (Data S8). Three clades were recovered in this tree (Figure 6a): Despite the apparently morphological differences (Data S10 and S11), the laboratory isolate of *E. vermiformis* from *Mus musculus* was indistinguishable from a field isolate from *Apodemus flavicollis*. Other isolates from house mouse and *A. flavicollis* and *A. sylvaticus* clustered in an unresolved internal relationship with house mouse *E. vermiformis* isolates (Group II). We note that four of five sequences for *E. vermiformis* from house mice were amplified from ileum tissue, the primary location of infection with this species (in contrast, *E. falciformis* infects primarily the cecum; Jarquín-Díaz et al., 2019). A second clade recovered by nuclear multilocus analysis contained *E. falciformis* from house mice. This clade showed a well-supported substructure in which 7 house mouse field isolates grouped with the laboratory isolate BayerHaberKorn1970 but were separated from 4 *Eimeria* isolates from *A. flavicollis* (Group III). This substructure agrees with the morphological difference previously observed based on the presence of polar granule in *E. falciformis* and its absence in *E. apionodes* (Data S10 and S11).

Analyses based on apicoplast markers (both multivariate clustering and phylogenetic analyses; Figure 7) identified similar groups: a well-separated cluster with isolates from *A. agrarius*, a cluster containing *E. vermiformis*, and another containing *E. falciformis* isolates. Some differences between the apicoplast and nuclear markers were obvious, though. *Eimeria* isolates from *M. musculus* (AA_0054_IL, AA_0080_IL, AA_0111_IL, and AA_0112_CE) were less similar to the *E. vermiformis* group, leading to a multivariate clustering between the *E. falciformis* and *E. vermiformis* groups (Figure 5b). This was recovered in a phylogenetic tree as isolates appeared at the end of a long branch in the *E. vermiformis* group (Figure 7a). In an analysis of apicoplast markers, the *E. falciformis* isolates from *Mus* were not differentiated from those from *A. flavicollis*. Inspection of phylogenetic trees for individual markers (Data S12) highlighted problems with the apicoplast dataset: samples that had been previously reported as coinfecting with *E. ferrisi* (AA_0080_IL, AA_0111_IL, and AA_0112_CE), showed an aberrant clustering for different markers. Samples AA_0080_IL and AA_0111_IL clustered in the group of *E. falciformis* with Ap12, while AA_0112_CE clustered with Ap5, in disagreement with the consensus species trees for other markers. We conclude that for these samples *E. ferrisi* or even *E. falciformis* apicoplast sequences were likely amplified and recovered as the majority sequence.

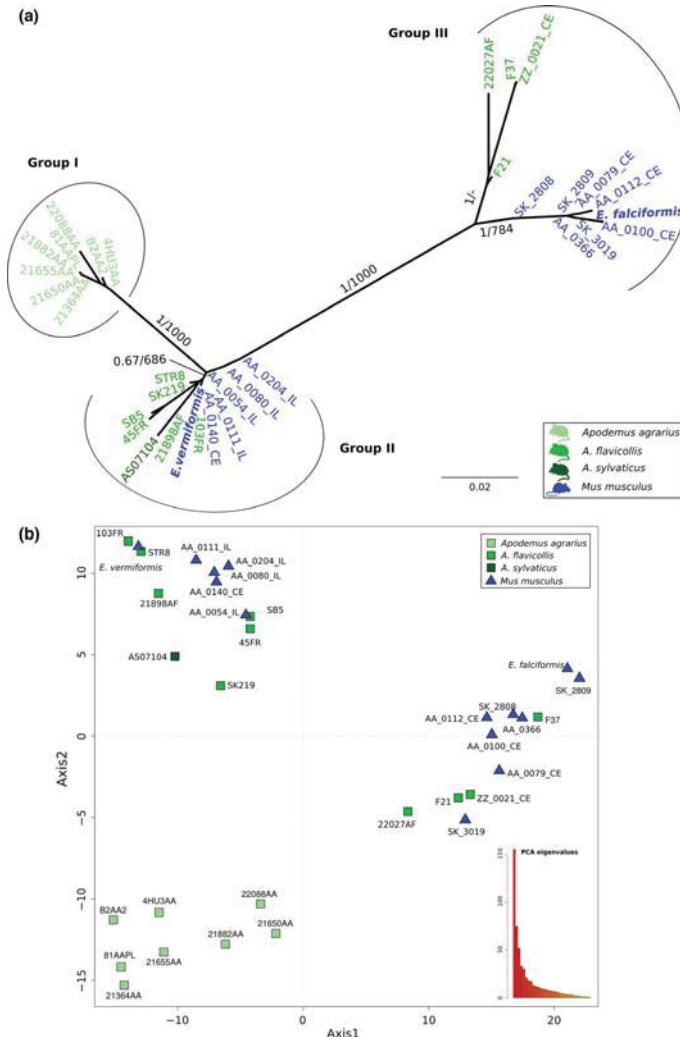


FIGURE 6 Nuclear multilocus genotyping of *Eimeria* isolates from *Mus musculus* and *Apodemus*. (a) The phylogenetic tree was estimated with a multilocus dataset formed with 35 nuclear markers from 31 *Eimeria* isolates derived from wild *Mus musculus* and three species of *Apodemus* (*A. agrarius*, *A. sylvaticus*, *A. flavicollis*). *Eimeria falciformis* and *E. vermiformis* sequences were included as reference. The scale bar represents sequence divergence. Color represents the host of origin for the isolates. Bootstrap support values and Bayesian posterior probabilities are shown on branches. (b) Principal component analysis based on single nucleotide polymorphisms (SNPs) from the same *Eimeria* isolates. Samples form three clusters. Shape indicates the genus of host and colors the species. Eigenvalues of the dimensions are shown in an insert to visualize the proportion of variance explained by the axes

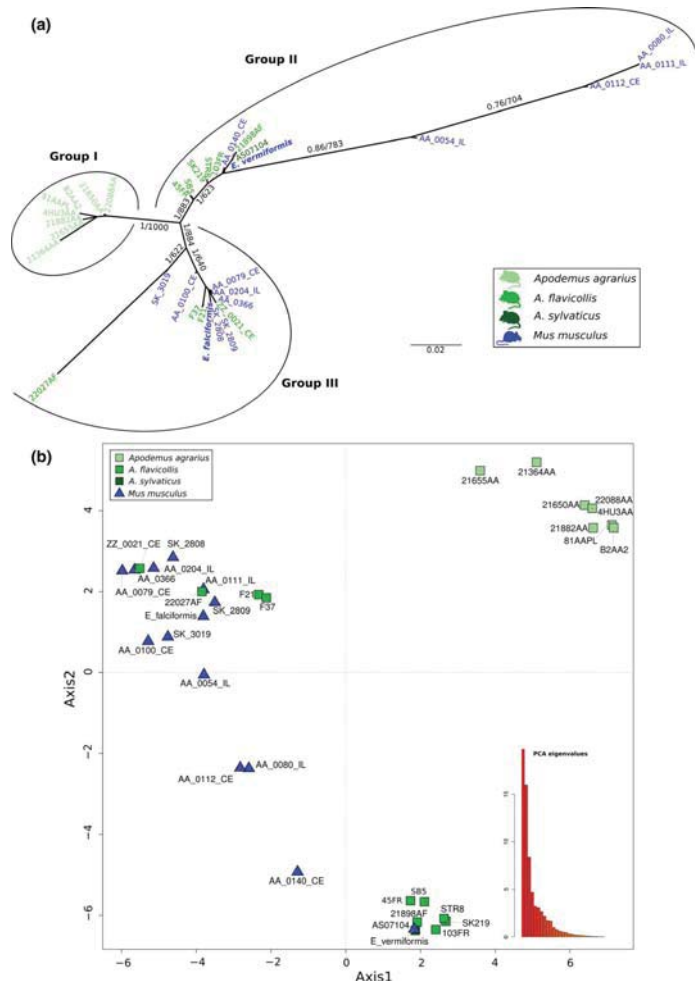
4 | DISCUSSION

We studied whether coccidia with different host usage can be distinguished with currently used molecular markers, using the example of *Eimeria* species in house mice and related rodents. We found that commonly used phylogenetic markers, nu 18S rDNA and mt COI, are not sufficiently variable to differentiate parasite isolates that would be regarded as separate species based on host usage. The relatively rarely used marker ap ORF470 from the apicoplast genome seems to provide slightly better resolution. We developed a multilocus genotyping approach to show that *E. falciformis* from the house mouse can likely be distinguished from related isolates from other hosts

based on nuclear markers. In contrast, even with this high-resolution approach *E. vermiformis* from house mice and isolates from other host species were found in a nested and unresolved cluster.

Phylogenies derived from each of the analyzed markers (esp. 18S) confirmed the topology of rodent *Eimeria* species observed before at deeper nodes of the phylogeny (Kvičarová et al., 2008; Ogedengbe et al., 2018; Zhao & Duszynski, 2001a). At the tips of the phylogeny, 18S sequences of *E. falciformis* and *E. vermiformis* isolates clustered with isolates from hosts of different genera or even families (Figure 2). This result was expected to some extent, as phylogenetic analyses with 18S sequences usually fail to separate closely related parasites isolated from closely related hosts (Ogedengbe et al., 2018).

FIGURE 7 Apicoplast multilocus genotyping of *Eimeria* isolates from *Mus musculus* and *Apodemus*. (a) The phylogenetic tree was estimated with a multimarker dataset formed with 5 apicoplast markers from 31 *Eimeria* wild isolates derived from *Mus musculus* and three species of *Apodemus* (*A. agrarius*, *A. sylvaticus*, *A. flavicollis*). *Eimeria falciformis* and *E. vermiformis* sequences were included as reference. The scale bar represents the sequence divergence. Color represents the host of origin for the isolates. Bootstrap support values and Bayesian posterior probabilities are shown on branches. (b) Principal component analysis based on single nucleotide polymorphisms (SNPs) from the same *Eimeria* isolates. Samples form three clusters based on the similarities for all the SNPs. Shape indicates the genus of host and colors the species. Eigenvalues of the dimensions are shown in an insert to visualize the proportion of variance explained by the axes



Previous studies described COI as a universal barcode variable enough to resolve relationships between coccidians, including *Eimeria* (Ogedengbe et al., 2011, 2018). We therefore expected to differentiate our house mouse isolates from species found in other hosts using COI. Neither phylogenetic (Figure 3) nor haplotype inference (Figure 5), however, supported differentiation of *E. falciformis* and *E. vermiformis* from some of the isolates described as *E. apionodes*. Many of the COI sequences were even identical for isolates from different hosts. Limited resolution of COI outside of metazoans has been reported before (Meyer & Paulay, 2005). Rodent hosts of *Eimeria*, in the families Muridae (*Mus*, *Rattus*, *Apodemus*) and Cricetidae (*Myodes*, *Microtus*), diverged around 25 million years ago (Churakov et al., 2010; Steppan, Adkins, & Anderson, 2004) and it seems possible that COI of coccidia evolves

at such slow rates that it fails to differentiate *Eimeria* species with similar divergence. We stress that for rodent coccidia, COI should not be assumed to resolve *bona fide* species with different host usage.

The potential of the apicoplast marker ORF470 to distinguish rodent *Eimeria* species has been highlighted before (Ogedengbe et al., 2015; Zhao & Duszynski, 2001b), but few studies have followed the recommendation to use this marker. Consequently, few database sequences are available. Phylogenetic analysis of these sequences (Figure 4) separates our three species clusters well and shows hints of internal structure separating *E. apionodes* derived from *A. flavicollis* from house mouse isolates. Our work increases the number of sequences available for ORF470 and supports its use as a marker for discrimination of *Eimeria* species.

To distinguish *E. falciformis*, *E. vermiformis* (from house mice), and *E. apionodes* (from *Apodemus* spp.), we established and used a multilocus sequence typing protocol. Our multilocus approach supports a differentiation of *E. falciformis* (infecting the house mouse; Eimer, 1870; Haberkorn, 1970) from *E. apionodes* (infecting *A. flavicollis*; Pellérdy, 1954). The same approach was unable to distinguish *M. musculus*-derived *E. vermiformis* isolates from one "*E. apionodes*" isolate from *A. flavicollis* (Figures 2, 3, 4, 6 and 7). This suggests a broad host range of genetically indistinguishable *Eimeria* isolates which have been assigned to paraphyletic species *E. apionodes* and *E. vermiformis*.

Multilocus genotyping using apicoplast markers showed some discrepancies with the nuclear analysis. These discrepancies can be attributed to double infections previously discovered in those particular isolates (Jarquín-Díaz et al., 2019). Compared to the nuclear genome, the apicoplast genome is present in much higher copy numbers (Heitlinger et al., 2014). This, combined with more conserved primer binding sites, can lead to amplification of nontarget sequences such as those of the prevalent *E. ferrisi* (Jarquín-Díaz et al., 2019) creating artificial "chimeric" isolates in case of double infections.

We use our system also as a test case whether the commonly used markers (18S, COI) provide enough resolution to assess parasite specificity. We conclude that unresolved genetic clusters and monomorphic haplotypes currently identified via 18S and COI genotyping should not be assumed to indicate parasite species with generalist host usage. Novel nuclear markers are needed in addition to ORF470 to analyze host species specificity of rodent *Eimeria*. Care must be taken to avoid potential artifacts introduced by double infection and mixed amplification.

Whether other *Eimeria* species from different rodent hosts are indeed phylogenetically distinguishable species (or whether genetically differentiable clusters show different host usage) is still an open question. This question needs to be addressed more broadly with markers providing higher resolution than 18S or COI. This question is highly relevant as hypotheses, assumptions, and predictions concerning host-parasite interactions from evolutionary (Adamson & Caira, 1994; Combes, 2001; Poulin, Krasnov, & Mouillot, 2011; Schmid-Hempel, 2011), ecological (Fenton & Brockhurst, 2008; Forbes, Muma, & Smith, 2002; Kassen, 2002), and mechanistic (Rathore et al., 2003) perspectives depend on the placement of parasite species in the specialist-generalist continuum (Schmid-Hempel, 2011).

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

VHJD and EH designed the project and obtained funding. VHJD, AM, TRS, JJ, KB, ST, and JK obtained data, VHJD, AB, and EH designed the analysis, VHJD, AB, and EH performed the analysis. VHJD, EH, and JK interpreted the results. VHJD and EH wrote the manuscript with contributions from all other authors. EH supervised the project.

DATA AVAILABILITY STATEMENT

- DNA sequences: nu 18S rDNA [MH751925-MH752036, MK246860-MK246868 and MK625202-MK625210]; mt COI [MH777467-MH777593, MH755302-MH755324, MK257106-MK257114, and MK631866-MK631868], and ap ORF470 [MH755325-MH755450, MK257115-MK257125, and MK631869-MK631884].
- All sequencing raw data can be accessed through the BioProject PRJNA548431 in the NCBI Short Read Archive (SRA).
- The code for the pipeline used in the multilocus type analysis is available on github at https://github.com/VictorHJD/AA_Eimeria_Genotyping.

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REFERENCES

- Abramson, N. I., Rodchenkova, E. N., & Kostygov, A. Y. (2009). Genetic variation and phylogeography of the bank vole (*Clethrionomys glareolus*, Arvicolinae, Rodentia) in Russia with special reference to the introgression of the mtDNA of a closely related species, red-backed vole (*Cl. rutilus*). *Russian Journal of Genetics*, 45(5), 533. <https://doi.org/10.1134/S1022795409050044>
- Adamson, M. L., & Caira, J. N. (1994). Evolutionary factors influencing the nature of parasite specificity. *Parasitology*, 109(S1), S85–S95. <https://doi.org/10.1017/S0031182000085103>
- Ball, S. J., & Lewis, D. C. (1984). Eimeviu (Protozoa: Coccidia) in wild populations of some British rodents. *Journal of Zoology*, 202(3), 373–381.
- Barta, J. R., Martin, D. S., Liberator, P. A., Dashkevich, M., Anderson, J. W., Feighner, S. D., ... Profous-Juchelka, H. (1997). Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. *The Journal of Parasitology*, 83(2), 262–271. <https://doi.org/10.2307/3284453>
- Becker, E. R. (1934). *Coccidia and coccidiosis of domesticated, game and laboratory animals and of man*. Berlin; Verlag Paul Parey: Budapest, Hungary: Akademiai Kiado.
- Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference

- from Illumina amplicon data. *Nature Methods*, 13(7), 581. <https://doi.org/10.1038/nmeth.3869>
- Churakov, G., Sadasivuni, M. K., Rosenbloom, K. R., Huchon, D., Brosius, J., & Schmitz, J. (2010). Rodent evolution: Back to the root. *Molecular Biology and Evolution*, 27(6), 1315–1326. <https://doi.org/10.1093/molbev/msq019>
- Combes, C. (2001). *Parasitism: The ecology and evolution of intimate interactions*. Chicago, IL: University of Chicago Press.
- De Vos, A. J. (1970). Studies on the host range of *Eimeria chinchillae* de Vos & van der Westhuizen, 1968. *The Onderstepoort Journal of Veterinary Research*, 37(1), 29–36.
- Deng, W., Maust, B., Nickle, D., Learn, G., Liu, Y. I., Heath, L., ... Mullins, J. (2010). DIVEIN: A web server to analyze phylogenies, sequence divergence, diversity, and informative sites. *BioTechniques*, 48(5), 405–408. <https://doi.org/10.2144/000113370>
- Dray, S., & Dufour, A. B. (2007). The ade4 package: Implementing the duality diagram for ecologists. *Journal of Statistical Software*, 22(4), 1–20.
- Đureje, L., Macholán, M., Baird, S. J., & Piálek, J. (2012). The mouse hybrid zone in Central Europe: From morphology to molecules. *Folia Zoologica*, 61(3–4), 308–319. <https://doi.org/10.25225/fozo.v61.i3.a13.2012>
- Duszynski, D. W. (Ed.). (2011). *Eimeria*. eLS (pp. 1192–1196). Chichester, UK: John Wiley & Sons, Ltd. <https://doi.org/10.1002/9780470015902.a0001962.pub2>
- Duszynski, D. W., Eastham, G., & Yates, T. L. (1982). *Eimeria* from jumping mice (*Zapus* spp.): A new species and genetic and geographic features of *Z. hudsonius luteus*. *The Journal of Parasitology*, 68(6), 1146–1148.
- Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 32(5), 1792–1797. <https://doi.org/10.1093/nar/gkh340>
- Eimer, T. (1870). Ueber Die Ei-oder Kugelförmigen Sogenannten Psorospermien Der Wirbelthiere: Ein Beitrag Zur Entwicklungsgeschichte Der Gregarinen Und Zur Kenntniss Dieser Parasiten Als Krankheitsursache. A. Stuber.
- Fenton, A., & Brockhurst, M. A. (2008). The role of specialist parasites in structuring host communities. *Ecological Research*, 23(5), 795–804. <https://doi.org/10.1007/s11284-007-0440-6>
- Forbes, M. R., Muma, K. E., & Smith, B. P. (2002). Diffuse co-evolution: Constraints on a generalist parasite favor use of a dead end host. *Ecography*, 25(3), 345–351. <https://doi.org/10.1034/j.1600-0587.2002.250311.x>
- Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology*, 59(3), 307–321. <https://doi.org/10.1093/sysbio/syq010>
- Haberkorn, A. (1970). Die Entwicklung von *Eimeria falciformis* (Eimer 1870) in der weißen Maus (*Mus musculus*). *Zeitschrift Für Parasitenkunde*, 34(1), 49–67.
- Heitlinger, E. (2019). MultiAmpliconv0.1. Retrieved from: <https://derele.github.io/MultiAmplicon/index.html>
- Heitlinger, E., Spork, S., Lucius, R., & Dieterich, C. (2014). The genome of *Eimeria falciformis*-reduction and specialization in a single host apicomplexan parasite. *BMC Genomics*, 15(1), 696.
- Hnida, J. A., & Duszynski, D. W. (1999a). Taxonomy and systematics of some *Eimeria* species of murid rodents as determined by the ITS1 region of the ribosomal gene complex. *Parasitology*, 119(4), 349–357.
- Hnida, J. A., & Duszynski, D. W. (1999b). Taxonomy and phylogeny of some *Eimeria* (Apicomplexa: Eimeriidae) species of rodents as determined by polymerase chain reaction/restriction-fragment-length polymorphism analysis of 18s rDNA. *Parasitology Research*, 85(11), 887–894.
- Hnida, J. A., Wilson, W. D., & Duszynski, D. W. (1998). A New *Eimeria* Species (Apicomplexa: Eimeriidae) Infecting *Onychomys* Species (Rodentia: Muridae) in New Mexico and Arizona. *The Journal of Parasitology*, 84(6), 1207–https://doi.org/10.2307/3284675
- Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17(8), 754–755. <https://doi.org/10.1093/bioinformatics/17.8.754>
- Jaarola, M., & Searle, J. B. (2002). Phylogeography of field voles (*Microtus agrestis*) in Eurasia inferred from mitochondrial DNA sequences. *Molecular Ecology*, 11(12), 2613–2621. <https://doi.org/10.1046/j.1365-294X.2002.01639.x>
- Jarquín-Díaz, V. H., Balard, A., Jost, J., Kraft, J., Dikmen, M. N., Jana, K., & Heitlinger, E. (2019). Detection and quantification of house mouse *Eimeria* at the species level—challenges and solutions for the assessment of *Coccidia* in wildlife. *International Journal for Parasitology: Parasites and Wildlife*, 10, 29–40. <https://doi.org/10.1016/j.ijppaw.2019.07.004>
- Jombart, T. (2008). adegenet: A R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24(11), 1403–1405. <https://doi.org/10.1093/bioinformatics/btn129>
- Kassen, R. (2002). The experimental evolution of specialists, generalists, and the maintenance of diversity. *Journal of Evolutionary Biology*, 15(2), 173–190. <https://doi.org/10.1046/j.1420-9101.2002.00377.x>
- Kvičerová, J., & Hypša, V. (2013). Host-parasite incongruences in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. *PLoS ONE*, 8(7), e63601. <https://doi.org/10.1371/journal.pone.0063601>
- Kvičerová, J., Mikeš, V., & Hypša, V. (2011). Third lineage of rodent eimerians: Morphology, phylogeny and re-description of *Eimeria myoxi* (Apicomplexa: Eimeriidae) from *Eliomys quercinus* (Rodentia: Gliridae). *Parasitology*, 138(10), 1217–1223.
- Kvičerová, J., Pakandl, M., & Hypša, V. (2008). Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: Evolutionary significance of biological and morphological features. *Parasitology*, 135(4), 443–452.
- Lainson, R., & Shaw, J. J. (1990). *Coccidia* of Brazilian mammals: *Eimeria corticulata* n. sp. (Apicomplexa: Eimeriidae) from the anteater *Tamandua tetradactyla* (Xenarthra: Myrmecophagidae) and *Eimeria zygodontomyis* n. sp. from the cane mouse *Zygodontomys lasiurus* (Rodentia: Cricetidae). *The Journal of Protozoology*, 37(1), 51–54.
- Levine, N. D., & Ivans, V. (1965). The coccidian parasites (Protozoa, Sporozoa) of rodents 33. *Illinois Biological Monographs*, 33.
- Levine, N. D., & Ivans, V. (1988). Cross transmission of *Eimeria* spp. (Protozoa, Apicomplexa) of rodents—a review. *The Journal of Protozoology*, 35(3), 434–437.
- Long, P. L., & Joyner, L. P. (1984). Problems in the identification of species of *Eimeria*. *The Journal of Protozoology*, 31(4), 535–541. <https://doi.org/10.1111/j.1550-7408.1984.tb05498.x>
- Máková, A., Hoblíková, A., Hypša, V., Stanko, M., Martinů, J., & Kvičerová, J. (2018). Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations. *Molecular Phylogenetics and Evolution*, 127, 179–189. <https://doi.org/10.1016/j.ympev.2018.05.009>
- MacPherson, J. M., & Gajadhar, A. A. (1993). Differentiation of seven *Eimeria* species by random amplified polymorphic DNA. *Veterinary Parasitology*, 45(3–4), 257–266. [https://doi.org/10.1016/0304-4017\(93\)90080-7](https://doi.org/10.1016/0304-4017(93)90080-7)
- Marquardt, W. C. (1981). Host and site specificity in the coccidia: A perspective 1. *The Journal of Protozoology*, 28(2), 243–244. <https://doi.org/10.1111/j.1550-7408.1981.tb02841.x>
- Mesfin, G. M., & Bellamy, J. E. (1978). The life cycle of *Eimeria falciformis* var. *pragensis* (Sporozoa: Coccidia) in the mouse, *Mus musculus*. *The Journal of Parasitology*, 64(4), 696–705.
- Meyer, C. P., & Paulay, G. (2005). DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology*, 3(12), e422. <https://doi.org/10.1371/journal.pbio.0030422>
- Ogedengbe, J. D., Hanner, R. H., & Barta, J. R. (2011). DNA barcoding identifies *Eimeria* species and contributes to the phylogenetics

- of coccidian parasites (Eimeriorina, Apicomplexa, Alveolata). *International Journal for Parasitology*, 41(8), 843–850. <https://doi.org/10.1016/j.ijpara.2011.03.007>
- Ogedengbe, J. D., Ogedengbe, M. E., Hafeez, M. A., & Barta, J. R. (2015). Molecular phylogenetics of eimeriid coccidia (Eimeriidae, Eimeriorina, Apicomplexa, Alveolata): A preliminary multi-gene and multi-genome approach. *Parasitology Research*, 114(11), 4149–4160. <https://doi.org/10.1007/s00436-015-4646-1>
- Ogedengbe, M. E., El-Sherry, S., Ogedengbe, J. D., Chapman, H. D., & Barta, J. R. (2018). Phylogenies based on combined mitochondrial and nuclear sequences conflict with morphologically defined genera in the eimeriid coccidia (Apicomplexa). *International Journal for Parasitology*, 48(1), 59–69. <https://doi.org/10.1016/j.ijpara.2017.07.008>
- Paradis, E., Jombart, T., Brian, K., Schliep, K., Potts, A., Winter, D., & Kamvar, Z. N. (2018). Population and evolutionary genetics analysis system. *R Package Version 11, 1*.
- Pellérdy, L. (1954). Zur Kenntnis der Coccidien aus *Apodemus flavicollis*. *Acta Veterinaria Academiae Scientiarum Hungaricae*, 4, 187–191.
- Posada, D. (2008). jModelTest: Phylogenetic model averaging. *Molecular Biology and Evolution*, 25(7), 1253–1256. <https://doi.org/10.1093/molbev/msn083>
- Poulin, R., Krasnov, B. R., & Moullot, D. (2011). Host specificity in phylogenetic and geographic space. *Trends in Parasitology*, 27(8), 355–361. <https://doi.org/10.1016/j.pt.2011.05.003>
- Rambaut, A. (2012). FigTree v1.4. Available at: <http://tree.bio.ed.ac.uk/software/figtree/> (Accessed October 2017).
- Rathore, D., Hrstka, S. C., Sacci, J. B., De la Vega, P., Linhardt, R. J., Kumar, S., & McCutchan, T. F. (2003). Molecular mechanism of host specificity in *Plasmodium falciparum* infection role of circumsporozoite protein. *Journal of Biological Chemistry*, 278(42), 40905–40910. <https://doi.org/10.1101/gr.168955.113>
- Reid, A. J., Blake, D. P., Ansari, H. R., Billington, K., Browne, H. P., Bryant, J., ... Pain, A. (2014). Genomic analysis of the causative agents of coccidiosis in domestic chickens. *Genome Research*, 24(10), 1676–1685. <https://doi.org/10.1101/gr.168955.113>
- Reutter, B. A., Petit, E., Brünner, H., & Vogel, P. (2003). Cytochrome b haplotype divergences in West European *Apodemus*. *Mammalian Biology-Zeitschrift Für Säugetierkunde*, 68(3), 153–164. <https://doi.org/10.1078/1616-5047-00077>
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539–542.
- Schmid Hempel, P. (2011). *Evolutionary parasitology the integrated study of infections, immunology, ecology, and genetics* (No. 574.5249 S2). Reprinted. New York: Oxford University Press.
- Steppan, S. J., Adkins, R. M., & Anderson, J. (2004). Phylogeny and divergence-date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. *Systematic Biology*, 53(4), 533–553. <https://doi.org/10.1080/10635150490468701>
- Sukumaran, J., & Holder, M. T. (2010). DendroPy: A Python library for phylogenetic computing. *Bioinformatics*, 26(12), 1569–1571. <https://doi.org/10.1093/bioinformatics/btq228>
- Tenter, A. M., Barta, J. R., Beveridge, I., Duszynski, D. W., Mehlhorn, H., Morrison, D. A., ... Conrad, P. A. (2002). The conceptual basis for a new classification of the coccidia. *International Journal for Parasitology*, 32(5), 595–616. [https://doi.org/10.1016/S0020-7519\(02\)00021-8](https://doi.org/10.1016/S0020-7519(02)00021-8)
- Todd, K. S. Jr, & Hammond, D. M. (1968). Life cycle and host specificity of *Eimeria callospermophilii* Henry, 1932 from the Uinta ground squirrel *Spermophilus armatus*. *The Journal of Protozoology*, 15(1), 1–8.
- Todd, K. S. Jr, & Lepp, D. L. (1971). The life cycle of *Eimeria vermiformis* Ernst, Chobotar and Hammond, 1971 in the Mouse *Mus musculus* L. *The Journal of Protozoology*, 18(2), 332–337.
- Turner, W. C., Penzhorn, B. L., & Getz, W. M. (2016). Description of 3 new species of *Eimeria* (Apicomplexa: Eimeriidae) from Springbok (*Antidorcas marsupialis*) in Namibia. *Comparative Parasitology*, 83(2), 202–212.
- Upton, S. J., McAllister, C. T., Brillhart, D. B., Duszynski, D. W., & Wash, C. D. (1992). Cross-transmission studies with *Eimeria arizonensis*-like oocysts (Apicomplexa) in New World rodents of the genera *Baiomys*, *Neotoma*, *Onychomys*, *Peromyscus*, and *Reithrodontomys* (Muridae). *The Journal of Parasitology*, 78, 406–413. <https://doi.org/10.2307/3283636>
- Wash, C. D., Duszynski, D. W., & Yates, T. L. (1985). Eimerians from different karyotypes of the Japanese wood mouse (*Apodemus* spp.), with descriptions of two new species and a redescription of *Eimeria montgomeryae* Lewis and Ball, 1983. *The Journal of Parasitology*, 71, 808–814.
- Wilber, P. G., Duszynski, D. W., Upton, S. J., Seville, R. S., & Corliss, J. O. (1998). A revision of the taxonomy and nomenclature of the *Eimeria* spp. (Apicomplexa: Eimeriidae) from rodents in the Tribe Marmotini (Sciuridae). *Systematic Parasitology*, 39(2), 113–135.
- Wright, E. S. (2016). Using DECIPHER v2.0 to analyze big biological sequence data in R. *R Journal*, 8(1), 352–359.
- Zhao, X., & Duszynski, D. (2001a). Molecular phylogenies suggest the oocyst residuum can be used to distinguish two independent lineages of *Eimeria* spp in rodents. *Parasitology Research*, 87(8), 638–643.
- Zhao, X., & Duszynski, D. W. (2001b). Phylogenetic relationships among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. *International Journal for Parasitology*, 31(7), 715–719.

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Additional supporting information may be found online in the Supporting Information section.

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Abstract

Interplay between conserved host specificity and occasional host switches is an important process determining the evolution of host-parasite systems. Here, we address the dynamics of host switches at the population level in rodent-associated *Eimeria*. Focusing mainly on two ecologically similar host groups, Murinae and Arvicolinae, we show that their *Eimeria* form a complex system of many genetic lineages with different host specificities. The broad geographic distribution of lineages indicates that they are well-established genetic forms which retained their host specificities while spreading across large geographic areas. We also demonstrate that genetic structure is only partially reflected by morphological traits.

Keywords

coccidia; Arvicolinae; Murinae; host specificity; phylogeny; coevolution

1. Introduction

During the last two decades the research on host-parasite coevolution has experienced a significant shift in perspective. The traditional view recognized host specificity as an important phylogenetically conserved trait, often treated as part of the characterization of a particular group of parasites (Poulin, 2007). Due to this view, a cospeciation process resulting in congruence between host and parasite phylogenies has been considered a typical feature of host-parasite coevolution (Hafner and Nadler, 1988, 1990; Page, 2003; for review see Brooks et al., 2015). However, studies based on molecular data have revealed that in many cases host-parasite congruences are surprisingly weak, indicating that host switching is a frequent and important process shaping parasite evolution (Ricklefs et al., 2004; Johnson et al., 2011; Toit et al., 2013). This change of perspective has also affected the long-lasting debate on the “parasite paradox”, i.e. the ability of parasites, as presumably strict specialists, to undergo frequent changes of their hosts (Agosta et al., 2010). Several authors have recently expressed the need to find a new framework for coevolutionary research in parasitology. For example, Nylin et al. (2018) pointed out similarities between parasite-host and plant-insect associations and suggested to establish a common framework for both research areas, emphasizing host switches rather than cospeciation as the main evolutionary driver. A complex framework unifying various views on host-parasite coevolution dynamics (e.g., ecological fitting, Agosta and Klemens (2008), and oscillation hypothesis, Janz and Nylin (2008)) was recently formulated as a Stockholm paradigm (Agosta and Brooks, 2020).

The flexibility of parasites in changing their host ranges is even more pronounced when analyzed at the population level (Banks et al., 2006; Štefka and Hypša, 2008; Štefka et al., 2009; Demanche et al., 2015). In contrast to typical phylogenetic-level analyses which use host/parasite species as units, the population-wide approach, based on the screening of a large number of closely related individuals, provides a more complex insight into the evolutionary changes undergone during genetic diversification. For example, it may reveal that a presumed generalist (i.e. polyxenous parasite) is in fact an assemblage of different monoxenous lineages, each of them specialized on a different host (McCoy et al., 2001). This may be particularly true when a species is defined by morphology rather than by genetics or host specificity. On the other hand, the host-based delimitation and description of parasite species may lead to fragmentation of a biological species into artificial units (Dahlgren and Gjerde,

2010; Thiele et al., 2018). To understand the evolutionary conditions underlying the rapid changes of host specificities even among closely related lineages of parasites, we need to explore these processes at the population level in different parasites and host taxa.

In our recent studies, we analyzed the evolution of host specificity in *Eimeria* infecting several genera of rodents. We first showed that rodent-specific lineages arose independently at least three times within the genus *Eimeria* and are thus a polyphyletic ecological assemblage composed of distinct clusters with different specificities (Kvičerová and Hypša, 2013). By extending the survey to the population level we revealed high flexibility within some of the rodent-*Eimeria* lineages in respect to both the width of the host spectrum (i.e., how many host taxa the parasite can successfully infect) and species specificity (i.e., which particular host taxon or taxa the parasite can successfully infect) (Máková et al., 2018). The study also revealed a complicated relationship between oocyst morphology (hence the traditional classification of these *Eimeria*) and phylogeny. Two species from rodents, *E. uptoni* and *E. jerfinica*, were represented by well-formed, distantly-related monophyletic clusters. However, an additional four morphospecies were arranged in a complex manner, so that *E. alorani* and *E. kaunensis* clustered as monophyletic branches within *E. apionodes*, making the latter paraphyletic and split into four monophyletic branches with different specificities. In this study, we designate this large and complex group (*apionodes*, *alorani*, and *kaunensis*) as an “*apionodes* supercluster”. The overall pattern indicates that the distribution of different host specificities across the phylogenetic structure of this supercluster is shaped by the interplay of two opposing evolutionary processes. On one hand, the host specificity is conservative enough to be retained by distinct monophyletic clusters, on the other hand it is flexible enough to allow the clusters to differ in the host spectrum width and species specificity.

While the Máková et al. (2018) study was focused mainly on *Eimeria* from the rodent genus *Apodemus* (Muridae: Murinae), the sampling also covered several ecologically similar (i.e. living in the same habitats and found in the traps together with the *Apodemus* samples) species of the subfamily Arvicolinae (Cricetidae), which allowed the placement of *Apodemus* samples into a broader ecological and taxonomic context. Interestingly, these samples did not form separate Arvicolinae-specific clusters but were scattered across several of the

Apodemus-derived lineages. Altogether, this indicates that within the *apionodes* supercluster the parasites are capable of frequent, rapid switches, resulting in a whole spectrum of specificities, from a single host (*alorani* in *A. agrarius*) to several genera (e.g. *apionodes* I, *apionodes* III, or *apionodes* IV according to the designation in Mácová et al. (2018); Fig. 1).

To further explore the dynamics and specificity patterns in *Eimeria* from ecologically similar rodents, and to obtain a complex evolutionary picture of the host switches in these parasites, we extended in this study our data with a new set of samples focusing mainly on Arvicolinae hosts.

2. Materials and methods

2.1 Sampling

Rodent sampling was carried out from spring 2015 to spring 2018 using the standard wooden snap traps baited with lard or sardine oil. The traps were set in the late evening, left in the field overnight, and picked up in the early morning. Field studies were carried out under official permits provided by the Czech Republic/European Union or collaborating institutions; the protocol was approved by the Committee on the Ethics of Animal Experiments of the University of South Bohemia and by the Ministry of the Environment of the Czech Republic (Permit Numbers KUJCK 11134/2010 OZZL/2/Ou, 27873/ENV/11, 51304/ENV/14-2981/630/14, and MZP/2017/630/854). Gut content and faeces were collected from each trapped animal and preserved individually in 2.5% potassium dichromate ($K_2Cr_2O_7$) solution. Samples were allowed to sporulate on air at room temperature ($\sim 22^\circ C$) for 5 days, then stored at $4^\circ C$.

2.2 Sample examination, morphology

The samples were examined using the centrifugation-flotation concentration method with modified Sheather's sucrose solution with gravity 1.30 (Modrý et al., 2018) followed by light microscopy. Samples positive for coccidian oocysts were analyzed morphologically and morphometrically according to the guidelines and criteria published by Duszynski and Wilber (1997) and Berto et al. (2014) using the Olympus BX53 microscope with Nomarski interference contrast equipped with DP-73-1-51 high resolution image cooled digital camera Olympus, and Olympus cellSens Standard 1.13 imaging software.

2.3 Molecular analyses

Genomic DNA was extracted from the samples using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, LLC, Santa Ana, California, USA) following the manufacturer's instructions. A part of the small subunit of the 18S rRNA gene and the mitochondrial gene for cytochrome c oxidase subunit I (COI) were amplified using the PCR protocols and PCR primers published in Kvičerová et al. (2008) and Schwarz et al. (2009), respectively. Each PCR reaction was performed in a 25 µl volume and using HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany). The resulting PCR products were visualized by electrophoresis in 1% agarose gel stained with SYBR[®] Green (Bio-Rad, Inc., USA). PCR products of expected sizes were enzymatically purified using the alkaline phosphatase and exonuclease I (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and directly sequenced (Sanger sequencing carried out in Macrogen, Inc., Amsterdam, the Netherlands, on an automated ABI3730XL DNA analyzer). After verification of the taxonomic origin of the obtained sequences by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), the sequences were assembled using the Sequence Scanner v.1.0 (Applied Biosystems) and DNASTAR (DNASTAR Inc., Madison, Wisconsin, USA) programs, and aligned in BioEdit v.7.0.5 (Hall, 1999) together with additional sequences downloaded from the GenBank database. The 18S rDNA sequences were aligned in the nucleotide mode; the COI sequences were aligned in the amino acid mode, then switched to nucleotide mode and used for analyses. Since the alignments contained sequences of different lengths, they were trimmed to obtain the set of sufficient sequence lengths together with reasonable taxonomical representation. The obtained sequences were deposited in the GenBank database under the accession numbers MT919398-MT919400, MT921431-MT921454, MT 921798 (18S rDNA), and MT934366-MT934399 (COI) (Table S1).

2.4 Phylogeny

Phylogeny was reconstructed using two approaches (Maximum likelihood (ML) and Bayesian inference (BI)), computed in Phyml v.2.4.3 (Guindon and Gascuel, 2003) and MrBayes v.3.2.2 (Huelsenbeck and Ronquist, 2001), respectively. The best fitting model of evolution was calculated using SMS: Smart Model Selection (<http://www.atgc-montpellier.fr/phyml-sms/>). ML was computed with the GTR + Γ + I model and non-parametric bootstrap analysis of

1,000 replicates. BI was performed using the GTR + Γ + I model, mcmc run for 10 million generations, and with tree sampling every 100 generations. The trees were visualized in TreeView v.1.6.6 (Page, 1996), and then graphically exported into Adobe Illustrator CC 2019 v.23.0 (Adobe Systems, Inc.).

3. Results

The following 685 specimens of Arvicoline rodents were sampled from 38 localities across Europe: *Clethrionomys glareolus* (494 individuals), *Microtus arvalis* (87), *Microtus agrestis* (54), *Microtus mystacinus* (44), *Microtus oeconomus* (4), *Microtus subterraneus* (1), and *Arvicola amphibius* (1). Of the 685 animals, 218 (31.8 %) were microscopically positive for oocysts of *Eimeria*: *C. glareolus* (149/30.2 %), *M. arvalis* (41/47.1 %), *M. agrestis* (21/38.9 %), *M. oeconomus* (3/75.0 %), *M. mystacinus* (2/4.5 %), *M. subterraneus* (1/100 %), and *A. amphibius* (1/100 %) (for more details see Supplementary data - Fig. S1, and Tables S1, S2).

The final alignment of COI yielded a 600bp long matrix containing 69 sequences of coccidia from Arvicoline rodents together with 160 sequences of coccidia from *Apodemus* spp. The final alignment of 18S rDNA was 1,349bp long and contained 111 sequences of coccidia from Arvicoline rodents together with 75 sequences of coccidia from *Apodemus* spp.

Phylogenetic analysis of the COI matrix produced a well-resolved tree with a basic structure corresponding to the phylogeny presented in Mácová et al. (2018) (Figs. 2, 3, S2). The *Apodemus* samples branched within three phylogenetically distinct clusters which we hereafter refer to as *uptoni*, *jerfinica*, and “*apionodes* supercluster”. While *uptoni* and *jerfinica* are relatively small clusters (i.e., they encompass only a few of our samples) almost exclusively associated with *Apodemus*, the *apionodes* supercluster is a large and diverse group composed of several clusters associated with different host genera of Murinae and Arvicolinae. Most of the clusters in the *apionodes* supercluster correspond to those reported in Mácová et al. (2018), designated here as *apionodes* I-IV, *kaunensis* I-II, and *alorani*. An additional cluster, tentatively named as *coahuiliensis* (see below for details), was composed of several Arvicolinae samples. The phylogeny based on the 18S rRNA gene was less resolved but highly compatible with the COI tree (Figs. S3, S4).

At the morphological level, the addition of new samples made the overall evolutionary picture within the *apionodes* supercluster even more complex. For example, the cluster designated by Mácová et al. (2018) as *kaunensis* was split in the presented analysis into two different groups. The morphotype *E. kaunensis*, originally described from *Apodemus agrarius* from Russia by Arnastauskene, Kazlauskas and Maldzhyunaite (1978) with an oocyst residuum (OR), corresponds to the lineage collected exclusively from the *Apodemus* hosts, which we now designate as *kaunensis* I. Its sister group, collected from arvicolids and designated here as *kaunensis* II, has very similar morphology but lacks the OR (Fig. 4). So, technically speaking, only the *Apodemus*-specific *kaunensis* I corresponds taxonomically to *E. kaunensis*, while *kaunensis* II may be a sister species with different morphology and host specificity. Several of the Arvicolinae samples represented a new morphotype which did not correspond to any of the morphotypes described in Mácová et al. (2018) and clustered as a separate monophyletic cluster. Morphologically, these samples resembled *Eimeria coahuiliensis* described from *Microtus mexicanus subsimus* from Mexico, by big oocysts, a rough oocyst wall, and the presence of a micropyle, although the original description of *E. coahuiliensis* by Vance and Duszynski (1985) lacked the OR. Therefore, we tentatively designated it as *Eimeria* cf. *coahuiliensis* (Fig. 4).

4. Discussion

Regarding the complex structure of the *apionodes* supercluster, the two following questions are particularly important for its evolutionary interpretation. The first question concerns the origin and nature of different host specificities observed in the clusters. It is notable that despite the addition of a substantial number of samples, particularly from Arvicolinae (often sampled from the same localities as *Apodemus*), some lineages remained specific only to *Apodemus* hosts (*alorani*, *apionodes* II, *jerfinica*, and *uptoni*) while others were specific only to Arvicolinae (*apionodes* I, *kaunensis* II, *coahuiliensis*). Even in such a case as *apionodes* III, the samples from different subfamilies (i.e., Arvicolinae, Murinae) formed distinct, small monophyletic (sub)lineages. The only exception was the *Apodemus*-specific cluster *apionodes* IV where three Arvicolinae samples were scattered among the *Apodemus* samples. However, all three samples originated from a single sampling event in Slovakia, and no

other Arvicolinae sample fell into this cluster. Therefore, we conclude that these three samples likely represent artifacts either due to misidentification of the host or due to passive passage of the parasite, or simply mistaken samples, rather than genuine members of the cluster.

Since the clusters within the *apionodes* supercluster are often of very low genetic diversity (even a single haplotype in *apionodes* IV), one may ask whether they are stable genetic lineages with fixed specificities or just an artifact biased by sampling local populations of otherwise polyxenous species. The broad geographic distribution of the lineages, as shown in Fig. 3 (for details see Tables S1 and S2), indicates that the clusters are well-established genetic forms which retained their host specificities while spreading across large geographic areas. On the other hand, low genetic distances among the clusters (evidenced by extremely short branches within the *apionodes* supercluster) imply their recent origin and, therefore, rapid and repeated switching of host specificity.

The second question is concerned with the determination of the actual host spectrum. It is important to realize that the *specificity* defined by a phylogenetic analysis is always derived from a particular limited set of hosts. For example, our focal taxa (Arvicolinae and *Apodemus*) represent only parts of the families Cricetidae and Muridae. The reason behind this design is ecological, since the sampled taxa share the same habitats and were collected under the same conditions. Among other small mammals, only insectivores were rarely found in the traps, and the few *Eimeria* samples isolated from these hosts never fell within the *Apodemus*/Arvicolinae clusters. Few samples of other rodent taxa have been obtained from our past field studies (e.g., *E. burdai* and *E. nafuko* from *Heliophobius argenteocinereus*, *E. cahirinensis* from *Acomys cahirinus*, *E. callospermophili* from *Spermophilus citellus*, *E. myoxi* from *Eliomys quercinus*), or from the GenBank database (e.g. *E. falciformis*, *E. ferrisi*, and *E. vermiformis* from *Mus musculus*). Considering this design, we cannot exclude the possibility that the *Eimeria* from *Apodemus*/Arvicolinae clusters are also able to use other Cricetidae/Muridae or even more phylogenetically distant hosts. The clustering of the few samples obtained from other than our focal host taxa indicates that this is indeed very likely and that the phylogenetic arrangement of host specificities will be even more complex once additional host taxa are screened. Some of these non-focal samples branched outside our

Apodemus/Arvicolinae clusters (e.g., *E. cahirinensis* and *E. callospermophili* clustering as a long-branched sister taxa of the *uptoni* cluster, or *E. myoxi* at the base of the *jerfinica* cluster). Since these non-focal host taxa are only represented by a limited number of samples in our analyses, it is not possible to draw any conclusion as to whether they represent additional rodent-associated clusters that have yet to be thoroughly sampled, or just occasional infections (or even passive passages).

An opportunity to address phylogenetic affiliation of the non-focal taxa in a more concise manner has arisen from a recent study on three *Eimeria* spp. from *M. musculus*, namely *E. falciformis*, *E. ferrisi*, and *E. vermiformis*, each represented by multiple samples from a large geographic area in Germany (Jarquín-Díaz et al., 2019). When included into our dataset, all of these species clustered within the *apionodes* supercluster. Specifically, *E. vermiformis* within *apionodes* IV, *E. falciformis* within *apionodes* III (interestingly within the Arvicolinae sublineage), and *E. ferrisi* together with our samples from *Mus* and *Heliophobius*. It is pertinent to note that while formally described as distinct species, these eimerians from *Mus* are morphologically similar (almost indistinguishable) to each other as well as to *E. apionodes* (except for *E. vermiformis* which is more ellipsoidal). Based on the current limited data, it is impossible to infer the taxonomic status of these morphotypes. However, we want to stress that the lack of knowledge of the non-focal taxa is irrelevant with respect to the main evolutionary interpretation we present here. We show that the sampled focal host taxa form at least two mutually exclusive groups, each of them infected by different genetic clusters. This exclusive host specificity (i.e., *Apodemus* vs. Arvicolinae) manifests itself not only at the typical phylogenetic level in the form of deep branching clusters (*jerfinica*, *uptoni*) but also at the shallow level of individual haplotypes within the *apionodes* supercluster. In some cases, it is not possible to decide whether samples from different hosts are intermixed or form separated monophyletic groups, since they are all represented by an identical haplotype based on our markers (i.e., short sequences). This is illustrated by the large but uniform *apionodes* IV cluster encompassing 54 identical sequences from three *Apodemus* species and *M. musculus*.

A particularly interesting case which illustrates the flexibility of the *apionodes* supercluster is the position of *Eimeria caviae* from the domesticated guinea pig

(*Cavia aperea* f. *porcellus*) among the *Apodemus*/Arvicolinae clusters (Figs. 1, 3, and S2). This position is obviously not a methodological artifact, since the *E. caviae* monophyletic group is represented by 4 isolates from different breeders from various parts of the Czech Republic and Slovakia, which were collected over several years. In addition, oocysts of *E. caviae* are morphologically similar to oocysts of *E. apionodes*, to a certain degree (Pellérdy, 1974; Flausino et al., 2014).

When summarized, the overall pattern obtained from this extended sample set shows that at least some groups of *Eimeria* possess remarkable versatility in their biological characteristics, including morphological traits and host specificity. However, unlike the cases where morphological variability is due to phenotypic plasticity or where the variability of hosts reflects a broad spectrum of generalist parasites, this versatility is coupled with a clear phylogenetic structure. This pattern stresses that even a fundamental trait like host specificity can change and become genetically fixed very rapidly, and should therefore be studied at the population level.

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References

Agosta, S.J., Brooks, D.R., 2020. The Stockholm Paradigm. In: The Major Metaphors of Evolution. Springer, Cham, pp. 219-242.

Agosta, S.J., Janz, N., Brooks, D.R., 2010. How specialists can be generalists: resolving the "parasite paradox" and implications for emerging infectious disease. *Zoologia* 27, 151-162.

Agosta, S.J., Klemens, J.A., 2008. Ecological fitting by phenotypically flexible genotypes: implications for species associations, community assembly and evolution. *Ecol. Lett.* 11, 1123-1134.

Arnastauskene, T., Kazlauskas, Y., Maldzhyunaite, S., 1978. The natural groupings of the intestinal parasites of mouse rodents of the Kamsa Preserve and their dependence on host biotope, species and its population structure. *Acta Parasitol. Litu.* 16, 15-32. [In Russian].

Banks, J.C., Palma, R.L., Paterson, A.M., 2006. Cophylogenetic relationships between penguins and their chewing lice. *J. Evol. Biol.* 19, 156-166.

Berto, B.P., McIntosh, D., Lopez, C.W.G., 2014. Studies on coccidian oocysts (Apicomplexa: Eucoccidiorida). *Braz. J. Vet. Parasitol.* 23, 1-15.

Brooks, D.R., Hoberg, E.P., Boeger, W.A., 2015. In the eye of the cyclops: the classic case of cospeciation and why paradigms are important. *Comp. Parasitol.* 82, 1-8.

Dahlgren, S.S., Gjerde, B., 2010. Molecular characterization of five *Sarcocystis* species in red deer (*Cervus elaphus*), including *Sarcocystis hjorti* n. sp., reveals that these species are not intermediate host specific. *Parasitology* 137, 815-840.

Demanche, C., Deville, M., Michaux, J., Barriel, V., Pinçon, C., Aliouat-Denis, C.M., Pottier, M., Noël, C., Viscogliosi, E., Aliouat, M., Dei-Cas, E., Morand, S., Guillot, J., 2015. What do *Pneumocystis* organisms tell us about the phylogeography of their hosts? The case of the woodmouse *Apodemus sylvaticus* in Continental Europe and western Mediterranean islands. *PLoS One* 10, e0120839.

Duszynski, D.W., Wilber, P.G., 1997. A guideline for the preparation of species descriptions in the Eimeriidae. *J. Parasitol.* 83, 333-336.

Flausino, G., Berto, B.P., McIntosh, D., Furtado, T.T., Teixeira-Filho, W.L., Lopes, C.W.G., 2014. Phenotypic and genotypic characterization of *Eimeria caviae* from guinea pigs (*Cavia porcellus*). Acta Protozool. 53, 269-276.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenesis by maximum likelihood. Syst. Biol. 52, 696-704.

Hafner, M.S., Nadler, S.A., 1988. Phylogenetic trees support the coevolution of parasites and their hosts. Nature 332, 258-259.

Hafner, M.S., Nadler, S.A., 1990. Cospeciation in host-parasite assemblages: comparative analysis of rates of evolution and timing of cospeciation events. Syst. Zool. 39, 192-204.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95-98.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755.

Janz, N., Nylin, S., 2008. The oscillation hypothesis of host-plant range and speciation. In: Tilmon, K.J. (ed) Specialization, speciation, and radiation: the evolutionary biology of herbivorous insects. University of California Press, Berkeley, pp. 203-215.

Jarquín-Díaz, V.H., Balard, A., Jost, J., Kraft, J., Dikmen, M.N., Kvičerová, J., Heitlinger, E., 2019. Detection and quantification of house mouse *Eimeria* at the species level - challenges and solutions for the assessment of Coccidia in wildlife. Int. J. Parasitol. - Parasit. Wildl. 10, 29-40.

Johnson, K.P., Weckstein, J.D., Meyer, M.J., Clayton, D.H., 2011. There and back again: switching between host orders by avian body lice (Ischnocera: Goniodidae). Biol. J. Linn. Soc. 102, 614-625.

Kvičerová, J., Hypša, V., 2013. Host-parasite incongruences in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. PLoS ONE 8, e63601.

Kvičerová, J., Pakandl, M., Hypša, V., 2008. Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. Parasitology 135, 443-452.

Máková, A., Hoblíková, A., Hypša, V., Stanko, M., Martinů, J., Kvičerová, J., 2018. Mysteries of host switching: diversification and host specificity in rodent-coccidia associations. Mol. Phyl. Evol. 127, 179-189.

McCoy, K.D., Boulinier, T., Tirard, C., Michalakakis, Y., 2001. Host specificity of a generalist parasite: Genetic evidence of sympatric host races in the seabird tick *Ixodes uriae*. J. Evol. Biol. 14, 395-405.

Modrý, D., Pafčo, B., Petrželková, K.J., Hasegawa, H., 2018. Parasites of Apes. An Atlas of Coproscopic Diagnostics. 1st ed. Edition Chimaira, Frankfurt.

Nylin, S., Agosta, S., Bensch, S., Boeger, W., Braga, M.P., Brooks, R.D., Forister, M.L., Hambäck, P.A., Hoberg, E.P., Nyman, T., Schäpers, A., Stigall, A.L., Wheat, C.W., Österling, M., Janz, N., 2018. Embracing colonizations: A new paradigm for species association dynamics. Trends Ecol. Evol. 33, 4-14.

Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Applic. Biosci. 12, 357-358.

Page, R.D.M., 2003. Tangled Trees: Phylogeny, Cospeciation, and Coevolution. University of Chicago Press, Chicago, Illinois.

Pellérdy, L.P., 1974. Coccidia and Coccidiosis. Akadémiai Kiadó, Publishing House of the Hungarian Academy of Sciences, Budapest.

Poulin, R., 2007. Evolutionary Ecology of Parasites, 2nd Edition. Princeton University Press, New Jersey.

Ricklefs, R.E., Fallon, S.M., Bermingham, E., 2004. Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Syst. Biol.* 53, 111-119.

Schwarz, R.S., Jenkins, M.C., Klopp, S., Miska, K.B., 2009. Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. *J. Parasitol.* 95, 871-880.

Štefka, J., Hypša, V., 2008. Host specificity and genealogy of the louse *Polyplox serrata* on field mice, *Apodemus* species: a case of parasite duplication or colonisation? *Int. J. Parasitol.* 38, 731-741.

Štefka, J., Hypša, V., Scholz, T., 2009. Interplay of host specificity and biogeography in the population structure of a cosmopolitan endoparasite: microsatellite study of *Ligula intestinalis* (Cestoda). *Mol. Ecol.* 18, 1187-1206.

Thiele, E.A., Eberhard, M.L., Cotton, J.A., Durrant, C., Berg, J., Hamm, K., Ruiz-Tiben, E., 2018. Population genetic analysis of Chadian Guinea worms reveals that human and non-human hosts share common parasite populations. *PLoS Negl. Trop. Dis.* 12, e0006747.

Toit, N.D., van Vuuren, B.J., Matthee, S., Matthee, C.A., 2013. Biogeography and host-related factors trump parasite life history: limited congruence among the genetic structures of specific ectoparasitic lice and their rodent hosts. *Mol. Ecol.* 22, 5185-5204.

Vance, T.L., Duszynski, D.W., 1985. Coccidian parasites (Apicomplexa: Eimeriidae) of *Microtus* spp. (Rodentia: Arvicolidae) from the United States, Mexico, and Japan, with descriptions of five new species. *J. Parasitol.* 71, 302-311.

Declarations of interest: none.

Legends to figures

Figure 1

Phylogenetic relationships of rodent-associated *Eimeria*. The tree is based on the Bayesian analysis of the COI matrix (Fig. 3), polytomies are resolved according to the ML analysis (Fig. S1). Bold labels designate the lineages present in Mácová et al. (2018).

Colours of terminal clusters indicate host specificities: light blue = Murinae, red = Arvicolinae, shades of violet = mixed specificity. In addition to this basic indication of host specificity, particular hosts are indicated at the terminal taxa. If formally described, the parasite name is stated in parentheses. Aa = *A. agrarius*, Af = *A. flavicollis*, As = *A. sylvaticus*, Au = *A. uralensis*; Cg = *C. glareolus*, Ma = *M. arvalis*, Mag = *M. agrestis*, Ml = *M. levis*.

Figure 2

Phylogenetic relationships inferred by BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities; posterior probabilities lower than 0.50 are not provided. *Eimeria ranae* is used as an outgroup.

Figure 3

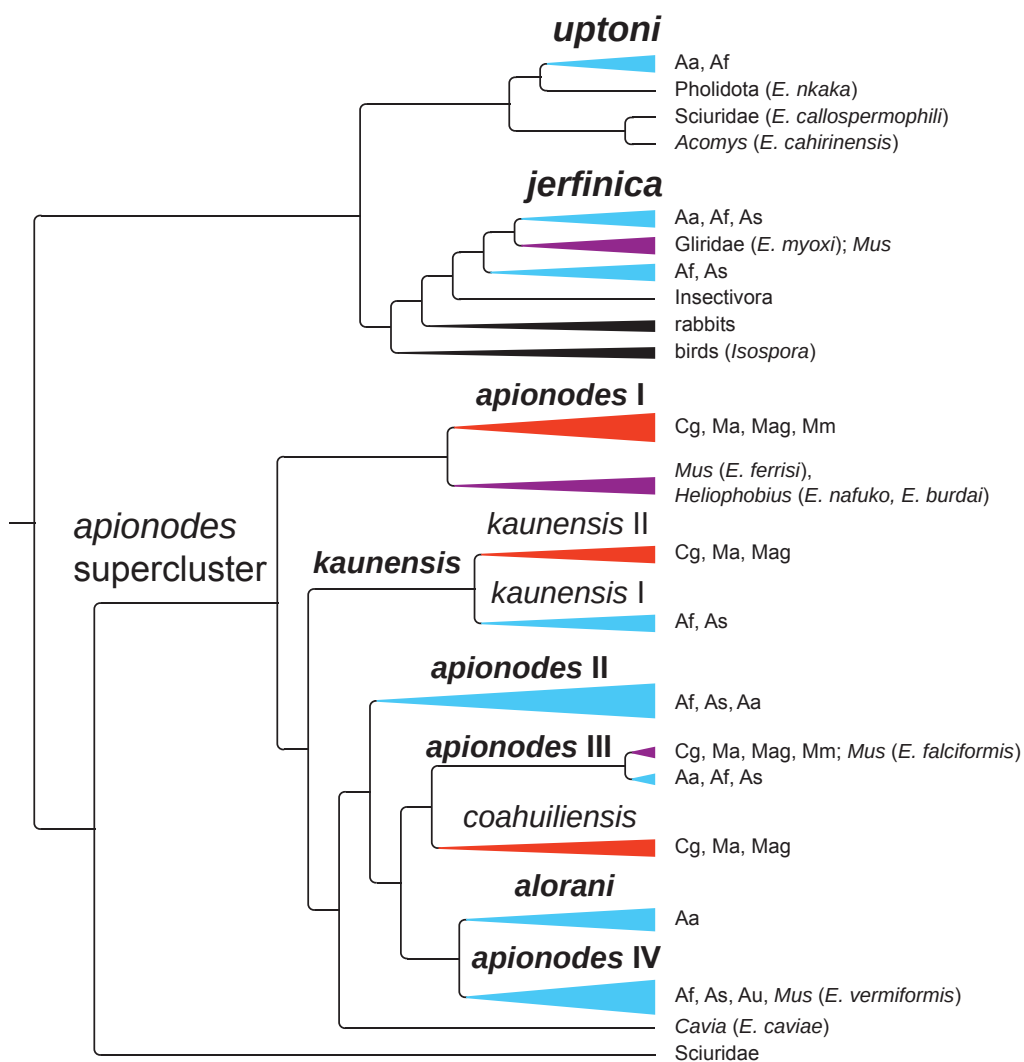
Phylogenetic relationships inferred by BI analysis of the COI sequences – detailed structure of the “*apionodes* supercluster”. Numbers at the nodes show posterior probabilities; posterior probabilities lower than 0.50 are not provided.

Figure 4

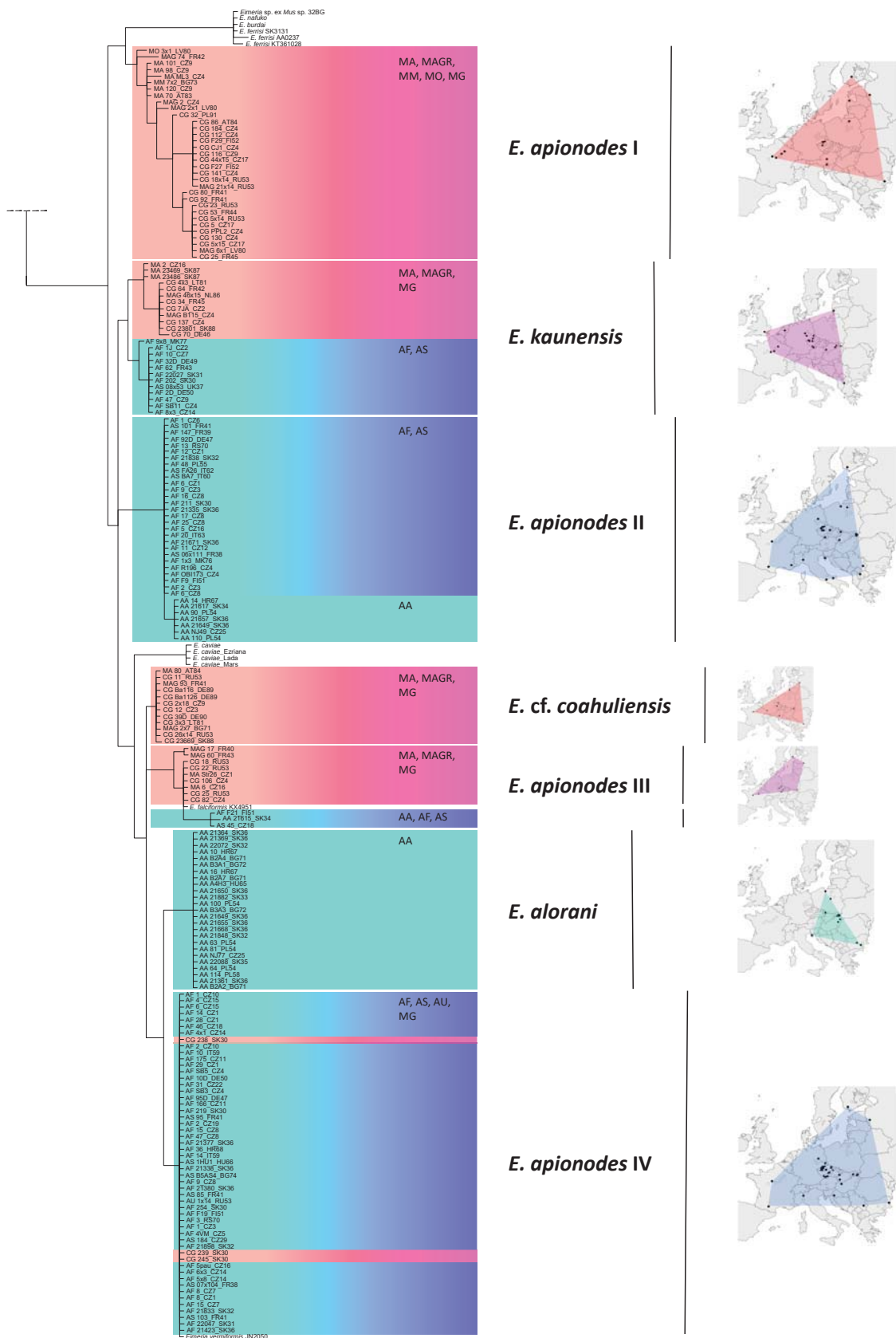
Photomicrographs of the recorded *Eimeria* lineages/morphotypes, and the main morphological features of their oocysts.

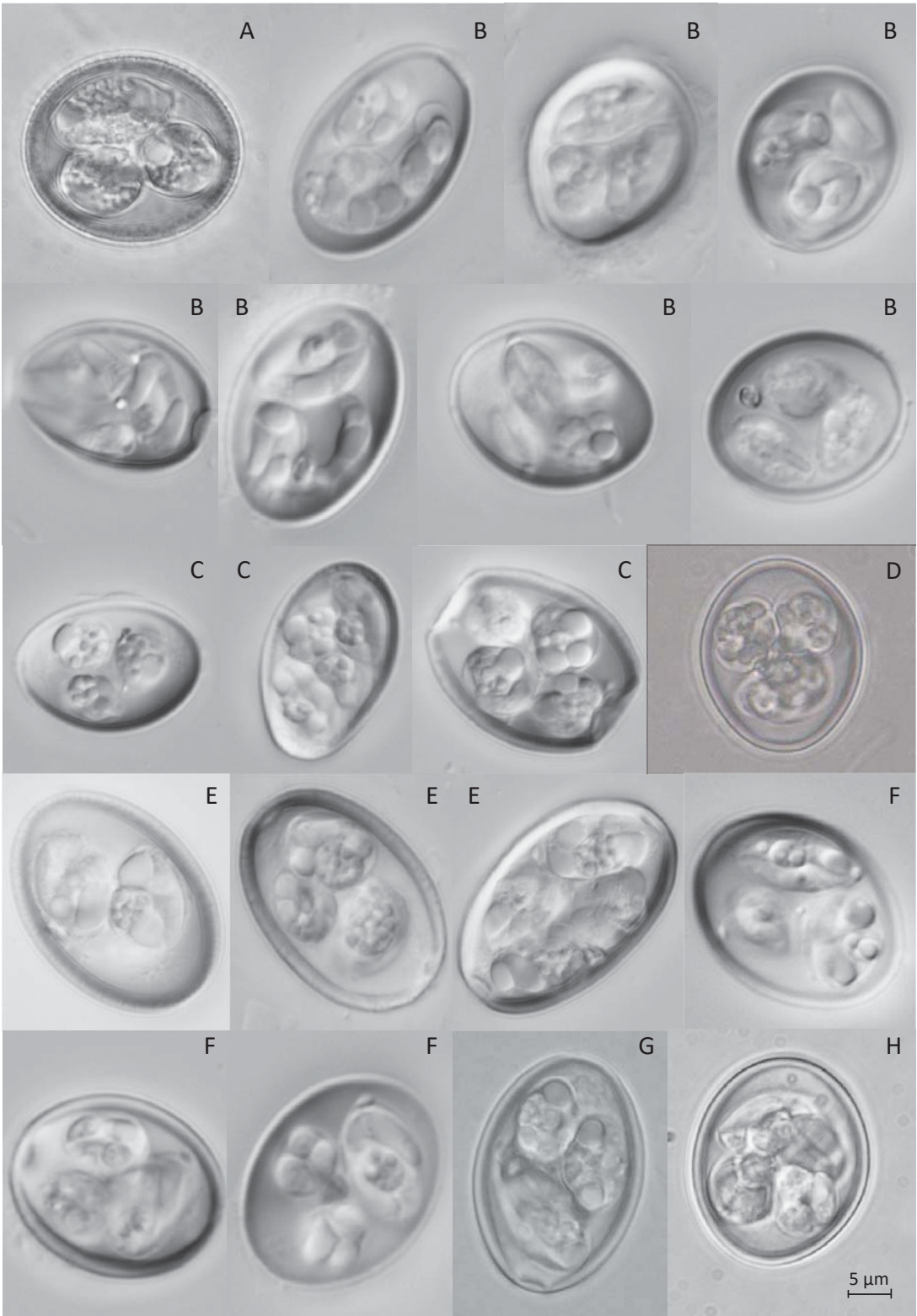
A – *E. jerfinica*; B – *E. apionodes* I; C – *E. kaunensis* II; D – *E. apionodes* II; E – *E. coahuiliensis*; F – *E. apionodes* III; G – *E. alorani*; H – *E. apionodes* IV.

Supplementary data – not included in PhD thesis









Draft 2:

Máková A, Hrdlička R, Kvičerová J: Phylogenetic relationship of coccidian parasites (Eimeriorina, Apicomplexa) infecting shrews: another piece to puzzle. Prepared for submission.

Phylogenetic relationships of coccidian parasites (Eimeriorina, Apicomplexa) infecting shrews: another piece to the puzzle

Abstract

Examination of faecal samples from 135 shrews (Eulipotyphla: genera *Crocidura*, *Neomys*, *Sorex* and *Suncus*) from various localities in Europe (and two localities in Asia) revealed that around one third of the samples was infected with coccidians, however, often in a rather low infection rate. Obtained sequences of three genes (COX1, COX3, and 18S rRNA) revealed the existence of five clusters/morphospecies, four of them being host-specific (two to *Crocidura* sp., one to *Neomys* sp. and one to *Sorex* sp.), and one containing eimerians both from *Crocidura* and *Neomys* hosts. Two sequences of *Isospora* obtained from *Sorex* sp. clustered with isosporans from moles. No obvious geographic pattern was observed. An attempt to determine these coccidia to the species-level was performed.

Introduction

Coccidia are protozoan parasites infecting a broad range of domestic and wild-living hosts. In general, the main focus has centered on those threatening human health (e.g. *Plasmodium*, *Toxoplasma*), or infecting farm animals such as poultry, rabbits, or cattle (mainly genus *Eimeria*) (Barta et al., 1997; Beattie et al., 2001; Pakandl, 2009). The studies have dealt mainly with their pathogenicity, life cycles, or morphology of oocysts (Kreier and Baker, 1987; Duszynski and Upton, 2001; Šlapeta et al., 2001; Berto et al., 2009). In the recent era of molecular biology, studies on evolutionary and host-parasite relationships have begun (Morrison et al., 2004; Kvičerová and Hypša, 2013; Mácová et al., 2018; Trefancová et al., 2019). In the light of molecular genetics, these studies have revealed that not all previously recognized concepts are valid. In eimerians, morphology of oocysts has shown to be a phylogenetically inconsistent trait. The genus *Eimeria* is polyphyletic, and other genera (e.g. *Caryospora*, *Cyclospora*, and some *Isospora* species) cluster within *Eimeria* (Morrison et al., 2004). For a long time, rodent-infecting species of *Eimeria* were thought to form two phylogenetic clusters based on the presence/absence of oocyst residuum (OR) in their oocysts (Zhao and Duszynski, 2001; Power et al., 2009). However, it was later found that *E. myoxi* forms its own, i.e. the third cluster (Kvičerová et al., 2011). Recently, we have revealed that such a clustering may be an artefact depending on number of examined and analyzed samples in the dataset (Mácová et al., 2018). Another objection to the taxonomy based solely on morphological features is posed by changes of oocysts size, and formation of inner structures during the sporulation and

patency (Long and Joyner, 1984; Parker and Duszynski, 1986; Gardner and Duszynski, 1990).

With a few exceptions (Matsubayashi et al., 2005; Kvičerová et al., 2011; Mácová et al., 2018; Trefančová et al., 2019), studies on coccidian phylogeny and parasite-host relationships are rather scarce and neglected in wild-living hosts. In shrews, the situation is dismal. Obtaining samples of these hosts is difficult due to their conservation status. Till now, the vast majority - if not all - of the publications concerning eimerians of insectivores have focused on oocyst morphology, pathogenicity, and life cycles (Yakimoff and Gousseff, 1935; Golemansky and Yankova, 1973; Hertel and Duszynski, 1987; Duszynski and Upton, 2000; Milek and Seville, 2003). Some of the older studies are not much informative since the morphological descriptions are incomplete or simple, and all lack molecular data. Similarly, the basic taxonomical and prevalence data are scarce. Only a fragment of insectivoran species (about 9 % of nearly 500) have been so far screened for the presence of coccidia (Duszynski et al., 2003; Duszynski and Marquardt, 2003; Isaac et al., 2018); approximately 80 species of coccidia have been described and named, of which about 10 from European shrews (Lynch and Duszynski, 2008). Considering the number of coccidians that may potentially infect these hosts, this is only a small amount.

The aim of this study was to analyze the material that we accidentally obtained during the last several years at various European localities, and to explore the phylogenetic relationships between coccidian (mainly eimerian) parasites of insectivores. Elucidation of the relationships in the coccidia-shrew model may represent another fragment into the mosaic of our knowledge on the host-parasite phylogeny.

Materials and Methods

Origin of the hosts and parasites, microscopical examination

Since shrews belong to protected animals, the samples were obtained exclusively from individuals that were found dead on paths and roads at various localities in 14 European and 2 Asian countries during 2007-2018 (Fig. 1). The carcasses were collected and dissected, and the faeces together with a part of the digestive tract were kept in 4% (w/v) aqueous potassium dichromate ($K_2Cr_2O_7$) solution (according to Williams et al., 2010). Faecal samples were processed by density-gradient flotation in Sheather's sucrose solution (specific gravity 1.30) (Zajac and Conboy, 2006; Smith et

al., 2007). Determination of the coccidian species/morphotypes was based on morphology and morphometry of sporulated oocysts (see Fig. 2 and Tab. III) according to guidelines published by Duszynski and Wilber (1997) and Berto et al. (2014). The Olympus BX53 light microscope equipped with a high resolution image cooled digital camera Olympus, and Olympus cellSens Standard 1.13 imaging software was used.

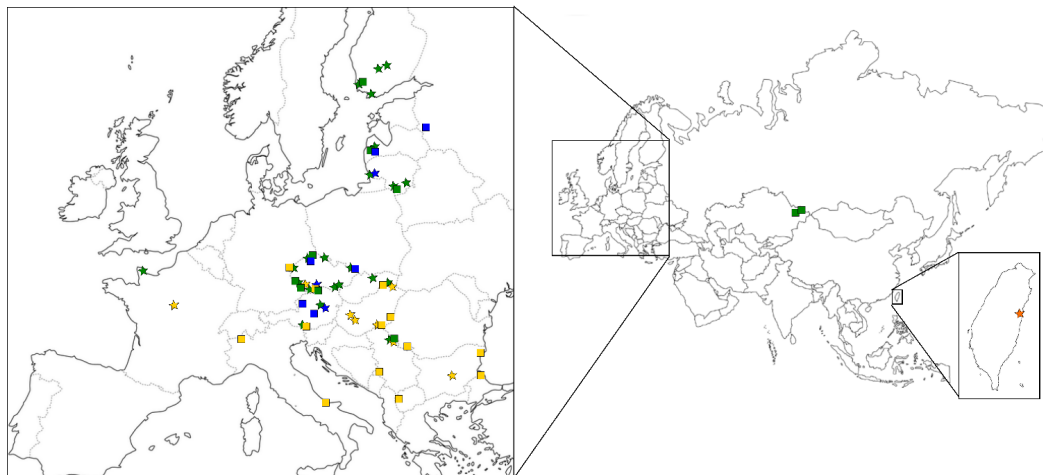


Fig. 1: Schematic map of localities of the sampled shrews. Explanatory notes: square = uninfected, star = infected; yellow = *Crocidura* sp., blue = *Neomys* sp., green = *Sorex* sp., orange = *Suncus* sp.

DNA isolation, PCR amplification, sequencing

Genomic DNA was extracted from the microscopically coccidia-positive faecal samples using the FastDNA® SPIN for Soil Kit (MP Biomedicals, LLC, Santa Ana, California, USA) following the manufacturer's protocol. PCR amplification was performed with Coccidia-specific primers amplifying the gene encoding the small subunit of 18S rRNA (SSU), and mitochondrial genes for cytochrome c oxidase subunit I (COX1) and III (COX3). Primers for 18S rDNA, COX1, and COX3 were adopted from Kvičerová et al. (2008), Schwarz et al. (2009), and Ogedengbe et al. (2015), respectively. HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) was used for all PCR reactions. PCR products were enzymatically purified using the alkaline phosphatase and exonuclease I (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and directly sequenced via the Sanger sequencing method in Macrogen, Inc. (Amsterdam, the Netherlands) on an automated 3730XL DNA analyzer.

Sequence assembling, alignments, phylogenetic analyses

Obtained sequences were examined by the BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), edited and assembled using the Sequence Scanner v.1.0 (Applied Biosystems), EditSeq 5.05 and SeqMan 5.05 (DNASTAR, Inc., Madison, Wisconsin, USA) programs, and deposited in the NCBI GenBank database under the Accession numbers provided in Table S1. The final dataset included our obtained sequences together with relevant sequences from the NCBI GenBank database (Table S2). Alignments were created in Geneious v9.1.3 (<http://www.geneious.com>; Kearse et al., 2012) using the MAFFT v1.3.6 algorithm with default parameters (Kato and Standley, 2013), and then manually adjusted. 18S rDNA sequences were aligned in the nucleotide mode; COX1, and COX3 sequences were aligned in the amino acid mode, then switched to nucleotide mode and used for the analyses. Concatenated matrix was assembled in SeaView (Gouy et al., 2010).

Phylogenetic relationships were analyzed by Bayesian inference (BI), and maximum likelihood (ML) approaches. BI was performed in MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001) with mcmc run for 10 million generations, and with tree sampling every 100 generations; the trees were summarized after removing 25 % burn-in. ML was carried out in PHYML v.3.1 (Guindon and Gascuel, 2003), with bootstrap values calculated by 1,000 replicates. Using jModeltest (Posada, 2008, 2009), the GTR + Γ + I was selected as the best fitting model. In addition, Bayesian analysis of a concatenated matrix was performed under the above described model and parameters. Final trees were visualized and exported in TreeView v1.6.6 (Page, 1996) and Inkscape v.0.91 (<https://inkscape.org>) programs.

Results

Microscopical examination

Altogether, faecal samples were microscopically analyzed from 188 shrew individuals (124 *Sorex* spp., 37 *Crocidura* spp., 24 *Neomys* spp., and 3 *Suncus murinus*). Of them, 45 (24 %) were coccidia-positive. Since the animals were found at various stages of decay, and some were provided by amateurs who could only determine the genus, only a part of the insectivore hosts was determined into species. The lowest prevalence of coccidia was detected in *Sorex*; only 11 of 124 (9 %) were infected, and they mostly contained only few oocysts. A significantly higher prevalence was observed in *Crocidura* where 24 of 40 (60 %) samples were infected with coccidia, with various intensities of infection. Four of the *Crocidura* sp. samples were infected

by more than one (usually 2) eimerian species/morphotypes. A similar situation was in *S. murinus*, where all three samples were infected with *Eimeria* sp. 10 of 24 (42 %) *Neomys* sp. samples contained coccidian oocysts, however, the intensity of infection was very low (Table I).

	<i>Crocidura</i> spp.	<i>Neomys</i> spp.	<i>Sorex</i> spp.
Austria	1/1	3/2	2/0
Bulgaria	4/3	0/0	0/0
Czech Republic	8/2	17/5	90/7
Finland	0/0	0/0	9/1
France	1/0	0/0	1/0
Hungary	10/5	0/0	0/0
Italy	5/5	0/0	0/0
Kazakhstan	0/0	0/0	6/0
Latvia	0/0	2/2	1/1
Lithuania	0/0	1/0	7/1
Macedonia	1/1	0/0	0/0
Romania	1/1	0/0	0/0
Russia	0/0	1/1	0/0
Serbia	2/1	0/0	2/1
Slovakia	4/2	0/0	6/0
Taiwan	3/3 (<i>Suncus</i> sp.)	0/0	0/0
total	40/24	24/10	124/11

Table I: Number of examined/coccidia-positive animals across Europe and in Taiwan.

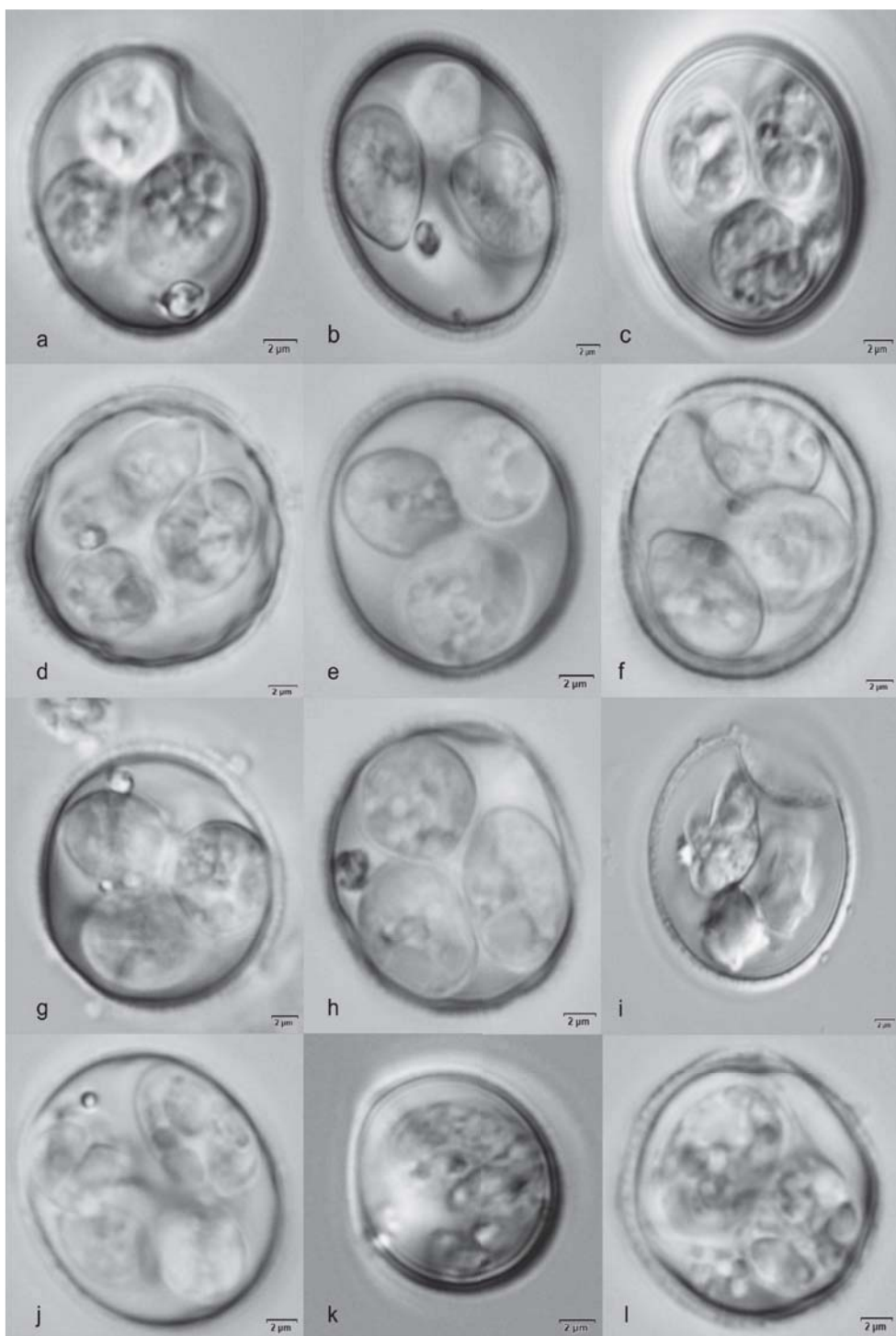


Fig. 2: Photomicrographs of oocysts found in shrews.
a-d: oocysts from *Crociturinae*, group *Croci1*, samples: 23706(a), TW4(b), 19CR(c), 8HB1(d);
e-i: oocysts from *Crociturinae* sp., group *Croci2*, samples: 23696(e), B4B2(f), 54STR(g), 34RUM(h), 6HB3(i);
j-k: oocysts from *Neomys* sp., group *Neomys*, samples NF58(j), and 60R(k);
l: oocyst of *Isospora* sp. from *S. araneus*, sample 21032.

DNA extraction and sequencing

DNA was successfully extracted from 14 samples of *Eimeria* spp. from Crocidurinae (4x *C. leucodon*, 2x *C. suaveolens*, 1x *C. russula*, 5x *Crocidura* spp., and 2x *S. murinus*), 8 samples of *Eimeria* spp. from Soricinae (1x *S. araneus*, 3x *N. fodiens*, and 3x *Neomys* sp.) (Tab. II), and from 2 samples of *Isospora* spp. from *S. araneus*. All these samples contained a single-species infection, with the exception of sample B4B2, where two distinct morphotypes of oocysts were observed. Morphological and morphometrical analyses of oocysts were provided for 16 samples (Table III).

In total, 48 sequences were obtained from these samples (17x COX1, 15x COX3, and 16x 18S rDNA). Of them, sequences for all three markers were obtained for 12 samples (samples 54STR, 6HB1, B4B2, 34RUM, RL, 21032, NF58, SA136, 19CR, 16SM, TW4, and 8HB1), and sequences of two genes were obtained for additional five samples (DK2, 23706, 23696, NF18, and TW1). For two samples (6HB3, 60R), only a single marker (COX1) was available.

Phylogenetic analyses

Phylogenetic analyses of the COX1 and COX3 genes yielded well-resolved trees with a strongly supported inner structure (Figs. 3, 4, 6); the 18S rDNA trees (Fig. 5) were compatible in their main features with the COX1 and COX3 trees, however, they were less resolved. Sequences of eimerians from insectivores formed distinct clusters (Figs. 3-6). One cluster was specific for the genus *Crocidura* (cluster “Croc2”), and was conserved in analyses of all three genes. It consisted of samples 54STR, 34RUM and B4B2 (for COX1 also 6HB3 and 23696). The second cluster was subfamily-specific, including samples from Crocidurinae – *Crocidura* and *Suncus* (samples DK2, 23706, 19CR, TW1, and TW4). The samples from *Neomys* sp. formed the “*Neomys*” cluster (in COX3 consisting of samples NF18, NF58, and 60R; in COX1, only a single sample (NF58) was available).

In 18S rDNA analyses, these samples clustered together with samples from *S. araneus* which were placed in another group called “mix” in both cytochrome

analyses, due to their composition of samples from different host species (including sample SA136 from *S. araneus*, RL from *Neomys* sp., and 6HB1 from *C. leucodon*) (Figs. 3-6).

Sequences of *Isospora* sp. from *Sorex* sp. clustered together with isosporans from moles in the COX1 analyses (Fig. 3). Nevertheless, their clustering differed in the 18S rDNA analyses; the sample 21032 branched within eimerians from *Neomys* sp. and *Sorex* sp. These sequences were, however, on long branches, so it may be an artefact of LBA (long branch attraction) (Fig. 5).



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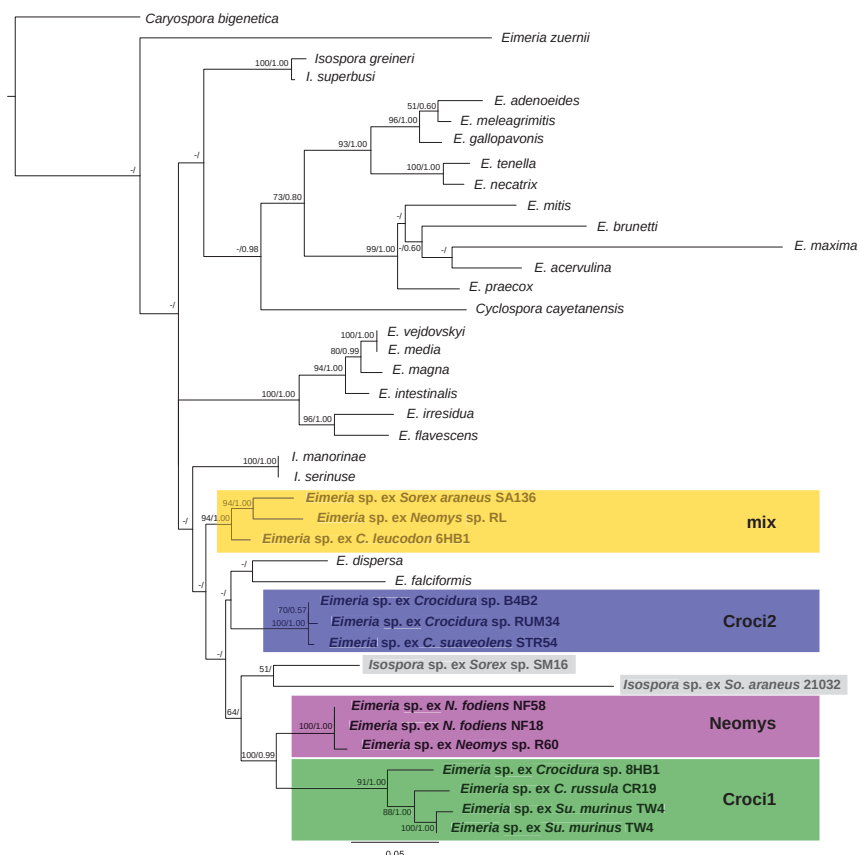


Fig. 4: ML phylogenetic tree inferred from the COX3 sequences. Numbers at the nodes show bootstrap values for ML analysis/posterior probabilities under the BI analysis. Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are not given. Dash indicates a different topology between ML and BI.



Fig. 5. ML phylogene tree inferred from the 18S rDNA sequences. Numbers at the nodes show bootstrap values for ML analysis/posterior probabilities under the BI analysis. Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are not given. Dash indicates a different topology between ML and B

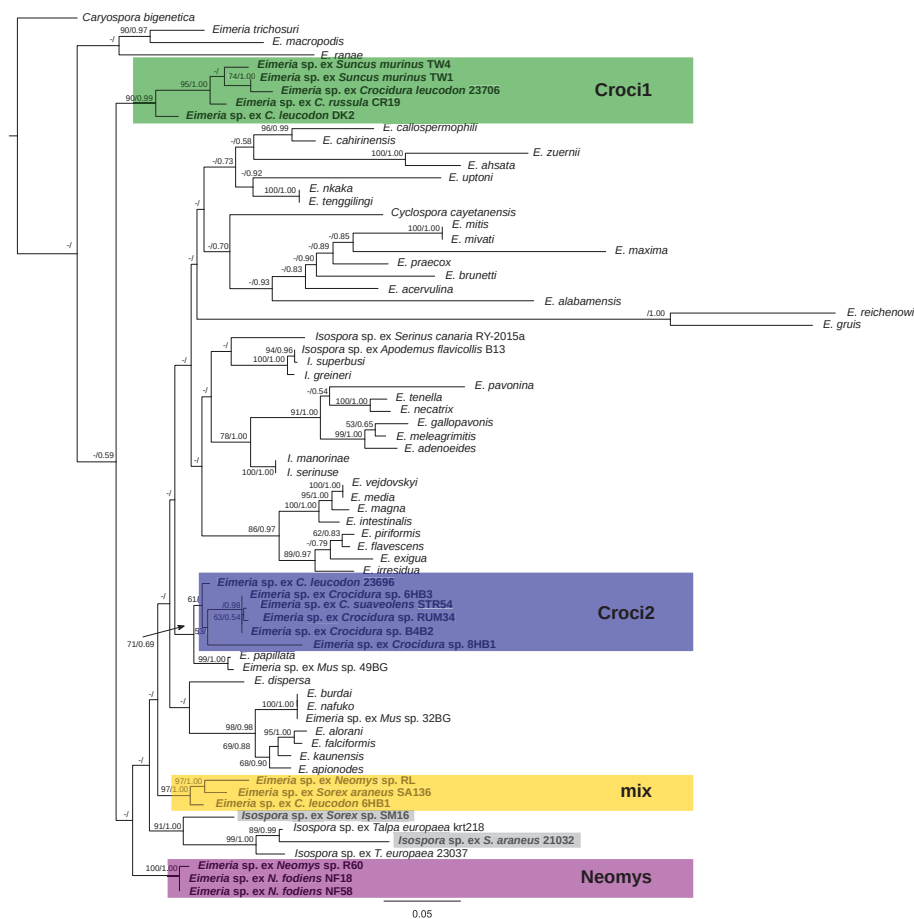


Fig. 6: ML phylogenetic tree inferred from the concatenated alignment of sequences of two genes (COX1, and COX3). Numbers at the nodes show bootstrap values for ML analysis/posterior probabilities under the BI analysis. Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are not given. Dash indicates a different topology between ML and BI.

Discussion

Prevalence of coccidia in shrews

Our findings correspond with the previous data on the prevalence of low and single infections in shrews (Milek and Seville, 2003; Lynch and Duszynski, 2008), who reported the prevalence around 20%. This is in great contrast to another insectivoran representative, the mole (Duszynski and Wattam, 1988). The cause of such a great difference is still unclear (Hertel and Duszynski, 1987; Lynch and Duszynski, 2008).

Morphology and phylogeny

Phylogenetic analyses resulted in presence of four distinct clusters (designated as “Croc1”, “Croc2”, “*Neomys*”, and “mix”).

“Croc1” cluster was formed by eimerians from the *Crocidura* hosts, with addition of two samples from *S. murinus*, a mammal from the same subfamily. The morphological as well as sequential/phylogenetic similarity of these coccidians implied that they represented a single species, *Eimeria chagasi* or *E. neomyi*, which are similar in oocyst inner structure but differ in size, or to *E. leucodontis*, which possess a thicker oocyst wall. The sample 23706 resembled *E. chagasi*, but had a different sporocyst shape index, while 19CR, TW4 and 8HB1 corresponded to *E. neomyi*, but possessed smaller sporocysts, or to *E. leucodontis*, which had a thicker oocyst wall (for more details, see Tab. III). However, such a difference is not so significant because the oocyst and sporocyst size may vary/change during the sporulation process and patency (Cheissin, 1947; Duszynski, 1971; Parker and Duszynski, 1986). The size may also vary within a single *Eimeria* species present in different host species (Reduker et al., 1985). Moreover, the samples DK2 and TW1 were unsporulated, so it was not possible to analyze them morphologically.

“Croc2” consisted of eimerians originating exclusively from the *Crocidura* hosts. Based on morphology and morphometry, this cluster represented a single *Eimeria* species (Fig. 2) corresponding to *Eimeria neomyi* originally de-

scribed by Golemansky (1978) from *N. fodiens* and *N. anomalus* from Bulgaria. However, our samples originated from the genus *Crocidura* collected in Bulgaria, Czech Republic, and Romania. So far, data on the degree of host specificity of insectivore coccidia are scarce. They are usually considered as species- or genus-specific. Nevertheless, several exceptions demonstrating the crossing of the generic boundary have been recorded; e.g. *Isospora brevicauda* whose type host species is *Blarina brevicauda* (Soricomorpha: Soricidae) was also found in *Sorex monticolus*, and later also in *S. caecutiens*, *S. cinereus*, and *S. hoyi* (Milek and Seville, 2003; Lynch and Duszynski, 2008). Taken these facts into account, the eimerians forming this cluster may possibly represent *E. neomyi*. If we consider only the crociduran eimerians, the most closely related described species based on the morphology is *Eimeria ropotomae* described from *C. leucodon* from Bulgaria (Golemansky, 1978). However, it is bigger in oocyst size, and possesses a smaller polar granule compared to the original line drawing (Tabs. III, IV).

The “*Neomys*” cluster contained eimerian sequences obtained from the *Neomys* hosts. The sample NF58 morphologically corresponded to *E. chagasi*. Oocysts of the sample 60R from *Neomys* sp. were peculiar, smaller than in other samples, and were all unsporulated, similarly as NF18.

The “Mix” cluster was more diverse, including the samples from three different host genera belonging to different subfamilies - *Neomys* sp. (RL), *S. araneus* (SA136), and *C. leucodon* (6HB1). This may indicate that at least some of the shrew eimerians are not much host-specific. Unfortunately, the majority of the samples contained unsporulated or damaged oocysts which could be due to their thin oocyst wall.

Two samples of *Isospora* sp. from *Sorex* spp. clustered in the COX1 and concatenated analyses with isosporan samples from the European mole, *Talpa europaea*. The sample 21032 morphologically corresponded to *Isospora araneae*, originally described from *S. araneus* (Golemanski, 1978). The sample 16SM was unsporulated. An additional sequence of *Isospora* isolated from the European mole (23037) was also included in the dataset, and clustered within the isosporans from the European mole. In 18S rDNA analyses, both isosporan se-

quences were placed on long branches with a low support, which indicates that this position is probably not valid.

Host specificity and species plasticity

Obtained phylogenies indicate the presence of several distinct phylogenetic lineages of *Eimeria* in shrews. Eimerians assigned to species in particular clades correspond well to the respective described species in oocyst morphology (Table IV). However, only few sequences of eimerians from shrews are present in the GenBank database, and they are not determined to species. An attempt to make an order in coccidia infecting these hosts was done by Duszynski and Upton (2000) in their comprehensive overview containing 4 eimerian species infecting *Crociodura*, 1 species infecting *Neomys*, and 7 species from *Sorex*.

The original morphological descriptions of oocysts are often insufficient (incomplete and/or of a poor quality), and also the dimensions given in the published descriptions are sometimes not very useful; e.g. the oocyst size of *Eimeria dissimillis*, which ranges between 18-33 x 13-24 μm (Yakimoff and Gousseff, 1935), covers nearly all the samples present in our study. In addition, morphological plasticity of eimerian oocysts may also pose a pitfall. Finally, it may also be possible that some of the observed *Eimeria* represent a yet undescribed species. Therefore, the lack of molecular data is still a challenge.

The confusion with synonyms is quite frequent. Hertel and Duszynski (1987), who summarized the literature on shrew's eimerians, suggested that both *E. ropotomae* and *E. neomyi* (together with *E. komareki*) are junior synonyms of *E. chagasi*. However, in the original description *E. chagasi* had a smooth oocyst wall, did not possess a Stieda body, and the oocysts were smaller than those of *E. ropotomae*. Based on the obtained phylogenies, although it is not possible to determine a particular species with certainty, it is obvious that highly morphologically similar oocysts cluster at different positions in the phylogenetic trees. Therefore, the three previously mentioned *Eimeria* species may represent valid and distinct species, unless it is a polyphyletic taxon. The same opinion about the validity of these species was also given by Duszynski and Upton (2000) who emphasized that without knowledge of host specificity, these species should not be synonymized.

Such a confusion concerning the synonymic names was often caused by an as-

sumption that eimerians are strictly host-specific, and, therefore, the host specificity is a suitable criterion for distinguishing different species (Joyner, 1982). A very strict specificity limited only to a single host species is relatively rare (e. g. *E. agrarii* infecting *Apodemus agrarius*); the most common is the use of several hosts belonging to one genus. On the other hand, species crossing generic (and higher) boundaries have also been described, e. g. *E. apionodes* able to infect either *Apodemus*, *Microtus*, and *Myodes* rodents (Máková et al., 2018), or *E. chinchillae* able to infect even different rodent families (De Vos, 1970). However, the situation in insectivores is unknown. Our findings indicate the possibility of both scenarios – some species may be strictly host-specific (clades “Croc1”, “Croc2” and “Neomys”), while others may have a broader host spectrum (the clade “mix”).

Another point of view on this problem can be given by the oscillation hypothesis (Janz et al., 2006). It assumes the scenario that lineages of a parasite change phases of expansion on hosts with the subsequent retraction. Therefore, the speciation is driven from more general ancestor, with occasional expansions on new hosts. This can explain also the situation in shrews, suggesting that coccidian lineages infecting shrews have undergone such oscillations, therefore, they are so diverse in phylogeny, but similar in morphology (Janz et al., 2016). However, no precise evolutionary conclusions can be done due to the lack of morphological together with corresponding molecular data.

This is the first and complex study on phylogenetic relationships among coccidia of insectivorans, and we believe it will help to resolve some of the previous confusions and questions.

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References

Barta J. R., Martin D. S., Liberator P. A., Dashkevicz M., Anderson J. W., Feighner S. D., Elbrecht A., Perkin-Barrow A., Jenkins M. C., Danforth H. D., Ruff M. D., Profous-Juchelka H, 1997: Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J Parasitol 83: 262-271.

Beattie S. E., Fernando M. A., Barta J. R., 2001: A comparison of sporozoite transport after homologous and heterogenous challenge in chickens imunized with the Guelph strain or the Florida strain of *Eimeria maxima*. Parasitol Res 87: 116-121.

Berto B. P., McIntosh D., Lopes C. W. G., 2014: Studies on coccidian oocysts (Apicomplexa: Eucoccidiorida). Rev Bras Parasitol Vet 23: 1-15.

Cheissin E. M., 1947: Mutability of the oocysts of *Eimeria magna*. Zool Zhurn 26: 17-29. (in Russian)

De Vos A. J., 1970: Studies on the host range of *Eimeria chinchillae* De Vos & Van der Westhuizen, 1968. Onderstepoort J Vet Res 37: 29-36.

Dolnik O., Palinauskas V., Bensch S., 2009: Individual oocysts of *Isospora* (Apicomplexa: Coccidia) parasites from avian feces: from photo to sequences. J Parasitol 95: 169-174.

Duszynski D. W., 1971: Increase in size of *Eimeria separata* oocysts during patency. J Parasitol 57: 948-952.

Duszynski D. W., Eckerlin R. P., McCarthy T. J., 2003: *Eimeria* species from *Cryptotis* shrews (Insectivora: Soricidae) with description of a new species. J Parasitol 89: 974-977.

Duszynski D. W., Marquardt W. C., 2003: Coccidia in the mammary glands of shrews (Order: Insectivora). J Parasitol 89: 609-611.

Duszynski D. W., Upton S. J., 2000: Coccidia (Apicomplexa: Eimeriidae) of the mammalian order Insectivora. Special publication of the Museum of Southwestern Biology No. 4, University of New Mexico Press, Albuquerque, NM, USA. 67 pp.

Duszynski D. W., Upton S. J., 2001: *Cyclospora*, *Eimeria*, *Isospora*, and *Cryptosporidium* spp. In: Samuel W. M., Pybus M. J., Kocan A. A. (Eds.): Parasitic Diseases of wild Mammals, 2nd Edition. Iowa State Press, Iowa City, pp. 416-433.

Duszynski D. W., Wattam A. R., 1988: Coccidian parasites (Apicomplexa: Eimeriidae) from insectivores. IV. Four new species in *Talpa europaea* from England. J Protozool 35: 58-62.

Duszynski D. W., Wilber P. G., 1997: A guideline for the preparation of species descriptions in the Eimeriidae. J Parasitol 83: 333-336.

Franzen C., Müller A., Bialek R., Diehl V., Salzberger B., Fätkenheuer G., 2000: Taxonomic position of the human intestinal protozoan parasite *Isospora belli* as based on ribosomal RNA sequences. Parasitol Res 86: 669-676.

Gardner S. L., Duszynski D. W., 1990: Polymorphism of eimerian oocysts can be a problem in naturally infected hosts: an example from subterranean rodents in Bolivia. J Parasitol 76: 805-811.

Golemansky V., 1978: Description of 9 new species of coccidia (Coccidia Eimeriidae), parasites of small mammals, in Bulgaria. Acta Protozool 17: 261-270.

Golemansky V., Yankova P., 1973: Studies on the species composition and occurrence of coccidia (Sporozoa, Coccidia) in some small mammals in Bulgaria. Bulletin de l'Institut de Zoologie et Musée 37: 5-31.

Gouy M., Guindon S., Gascuel O., 2010: SeaView version 4: a multiplatform

graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27: 221-224.

Guindon S., Gascuel O., 2003: A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52: 696-704.

Hertel L. A., Duszynski D. W., 1987: Coccidian parasites (Apicomplexa: Eimeriidae) from insectivores. III. Seven new species in shrews (Soricidae: Soricinae) from Canada, Japan, and the United States. *J Parasitol* 73: 172-183.

Huelsenbeck J. P., Ronquist F., 2001: MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754-755.

Isaac C., Igbinosa B. I., Ohiolei J. A., Osimen C. E., 2018: Endoparasites of small mammals in Edo state, Nigeria: Public health implications. *Korean J Parasitol* 56: 93-100.

Janz N., Braga M. P., Wahlberg N., Nylin S., 2016: On oscillations and flutterings – A reply to Hamm and Fordyce. *Evolution* 70: 1150-1155.

Janz N., Nylin S., Wahlberg N., 2006: Diversity begets diversity: host expansions and the diversification of plant-feeding insects. *BMC Evolutionary Biology* 6:4.

Joyner L. P., 1982: Host and site specificity. In: Long P. L. (Ed.): *The biology of the coccidia*. University Park Press, Baltimore, pp. 35-62.

Katoh K., Standley D. M., 2013: MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 30: 772-780.

Kearse M., Moir R., Wilson A., Stones-Havas S., Cheung M., Sturrock S., Buxton S., Cooper A., Markowitz S., Duran C., Thierer T., Ashton B., Mentjies P., Drummond A., 2012: Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647-1649.

Kreier J. P., Baker J. R., 1987: Parasitic protozoa. Allen and Unwin. London, United Kingdom of Great Britain and Northern Ireland.

Kvičerová J., Hypša V., 2013: Host-parasite incongruences in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. PLoS One 8: e63601.

Kvičerová J., Mikeš V., Hypša V., 2011: Third lineage of rodent eimerians: morphology, phylogeny and re-description of *Eimeria myoxi* (Apicomplexa: Eimeriidae) from *Eliomys quercinus* (Rodentia: Gliridae). Parasitology 138: 1217-1223.

Kvičerová J., Pakandl M., Hypša V., 2008: Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: Evolutionary significance of biological and morphological features. Parasitology 135: 443-452.

Long P. L., Joyner L. P., 1984: Problems in the identification of species of *Eimeria*. J Protozool 31: 535-541.

Lynch A. J., Duszynski D. W., 2008: Species of coccidia (Apicomplexa: Eimeriidae) in shrews from Alaska, U.S.A., and Northeastern Siberia, Russia, with description of two new species. J Parasitol 94: 883-888.

Máková A., Hoblíková A., Hypša V., Stanko M., Martinů J., Kvičerová J., 2018: Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations. Mol Phyl Evol 127: 179-189.

Matsubayashi M., Takami K., Niichiro A., Kimata I., Tani H., Sasai K., Baba E., 2005: Molecular characterization of crane Coccidia, *Eimeria gruis* and *E. reichenowi*, found in feces of migratory crane. Parasitol Res 97: 80-83.

Milek J. A., Seville S., 2003: Species of *Eimeria* and *Isospora* (Apicomplexa: Eimeriidae) from shrews (Insectivora: Soricidae) in Northwestern Wyoming, U.S.A. Comp Parasitol 70: 72-77.

Morrison D. A., Bornstein S., Thebo P., Wernery U., Kinne J., Mattsson J. G., 2004: The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). *Int J Parasitol* 34: 501-514.

Ogedengbe M. E., Qvarnstrom Y., da Silva A. J., Arrowood M. J., Barta J. R., 2015: A linear mitochondrial genome of *Cyclospora cayetanensis* (Eimeriidae, Eucoccidiorida, Coccidiasina, Apicomplexa) suggests the ancestral start position within mitochondrial genomes of eimeriid coccidia. *Int J Parasitol* 45: 361-365.

Page R. D. M., 1996: TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Applic Biosci* 12: 357-358.

Pakandl M, 2009: Coccidia of rabbit: a review. *Folia Parasitol* 56: 153-166.

Parker B. B., Duszynski D. W., 1986: Polymorphism of eimerian oocysts: A dilemma posed by working with some naturally infected hosts. *J Parasitol* 72: 602-604.

Posada D., 2008: jModelTest: phylogenetic model averaging. *Mol Biol Evol* 25: 1253-1256.

Posada D., 2009: Selection of models of DNA evolution with jModelTest. *Methods Mol Biol* 537: 93-112.

Power M. L., Richter C., Emery S., Hufschmid J., Gillings M. R., 2009: *Eimeria trichosuri*: Phylogenetic position of a marsupial coccidium, based on 18S rDNA sequences. *Exp Parasitol* 122: 165-168.

Reduker D. W., Hertel L., Duszynski D. W., 1985: *Eimeria* species (Apicomplexa: Eimeriidae) infecting *Peromyscus* rodents in the Southwestern United States and Northern Mexico with description of a new species. *J Parasitol* 71: 604-613.

Schwarz R. S., Jenkins M. C., Klopp S., Miska K. B., 2009: Genomic analysis of *Eimeria* spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. J Parasitol 95: 871-880.

Smith P. H., Seklau B. S., Wiles E., Malone J. B., Monahan M. C., 2007: Collection, Preservation, and Diagnostic Methods. In: Baker D. G. (Ed.): Flynn's Parasites of Laboratory Animals, 2nd Edition. Wiley-Blackwell, Iowa, pp. 1-13.

Šlapeta J. Modrý D., Votýpka J., Jirků M., Oborník M., Lukeš J. Koudela B., 2001: *Eimeria telekii* n. sp. (Apicomplexa: Coccidia) from *Lemniscomys striatus* (Rodentia: Muridae): morphology, pathology and phylogeny. Parasitology 122: 133-143.

Trefančová A., Máčová A., Kvičerová J., 2018: Isosporan oocysts in the faeces of bank voles (*Myodes glareolus*; Arvicolinae, Rodentia): Real parasites, or pseudoparasites? Protist 170: 104-120.

Wilber P. G., Duszynski D. W., Upton S. J., Seville R. S., Corliss J. O., 1998: A revision of the taxonomy and nomenclature of the *Eimeria* spp. (Apicomplexa: Eimeriidae) from rodents in the Tribe Marmotini (Sciuridae). Syst Parasitol 39: 113-135.

Williams R. B., Thebo P., Marshall R. N., Marshall J. A., 2010: Coccidian oocysts as type-specimens: long-term storage in aqueous potassium dichromate solution preserves DNA. Syst Parasitol 76: 69-76.

Yakimoff W. L., Gousseff W. F., 1935: On the coccidia of shrews, grass-snakes, and lizards. J R Microsc Soc 55: 170-173.

Zajac A. M., Conboy G. A., 2006: Veterinary Clinical Parasitology. Blackwell Publishing, Iowa, USA.

Zhao X., Duszynski D. W., 2001: Phylogenetic relationships among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. Int J Parasitol 31: 715-719.

Sample code	Host species	Locality and date of collection
DK2	<i>C. leucodon</i>	CZ, Třisov/Dívčí Kámen, 11.9.2015
6HB1	<i>C. leucodon</i>	HU, Medgyesegyháza, 18.9.2013
23696	<i>C. leucodon</i>	SK, Hrhov, 29.9.2016
23706	<i>C. leucodon</i>	SK, Hrhov, 29.9.2016
19CR	<i>C. russula</i>	ES, La Roca del Valles, 26.9.2009
54STR	<i>C. suaveolens</i>	CZ, Křečov, 20.11.2016
B4B2	<i>Crocidura</i> sp.	BG, Kamen Brjag, 30.8.2014
8HB1	<i>Crocidura</i> sp.	HU, 20.9.2013
34RUM	<i>Crocidura</i> sp.	RO, Caraşova, 26.8.2016
6HB3	<i>Crocidura</i> sp.	HU, Medgyesegyháza, 18.9.2013
NF18	<i>N. fodiens</i>	CZ, Boršov nad Vltavou, 10.8.2007
NF58	<i>N. fodiens</i>	CZ, České Budějovice, 26.8.2007
60R	<i>Neomys</i> sp.	AT, Aigen, 18.9.2015
RL	<i>Neomys</i> sp.	CZ, Litvínov, 13.10.2012
SA136	<i>So. araneus</i>	CZ, Boršov nad Vltavou – Březí, 15.9.2008
21032	<i>So. araneus</i>	SK, Bušince, 11.6.2008
16SM	<i>So. araneus</i>	CZ, Mořičov, 23.11.2018
TW1	<i>Su. murinus</i>	TW, Gancheng, 8.11.2018
TW4	<i>Su. murinus</i>	TW, Gancheng, 9.11.2018

Table II: Information on sequenced samples from shrews.

Localities: AT = Austria, BG = Bulgaria, CZ = Czech Republic, ES = Spain, HU = Hungary, IT = Italy, RO = Romania, RU = Russia, SK = Slovakia, TW = Taiwan.

sample code	oocyst size [μm]	range of the OO size [μm]	L/W	OW	sporocyst size [μm]	range of the SC size [μm]	SB	PG	S R	R B	note
60R	13.9 × 11.8	13.6-14.3 × 11.5-12.2	1.2		7.7 × 5.9	7.2-8.2 × 5.7-6.1					PS
23706	15.9 × 14.5	15.2-16.3 × 13.8-15.0	1.1	0.7	8.2 × 7.0	7.8-8.8 × 6.1-7.4	?	1	1?	?	PS
RL	16.8 × 14.6	16.4-17.3 × 14.2-14.9	0.4								US
23696	16.8 × 15.3	15.4-19.9 × 13.9-16.9	1.1	0.8	8.8 × 6.5	6.9-10.7 × 5.4-7.6	+	1	+	+	
21032	17.3 × 15.2	16.1-19.3 × 12.9-16.4	1.1	1.0	7.7 × 5.2	7.3-10.8 × 4.7-9.2	?	1	?	?	
NF58	17.4 × 15.5	16.3-19.1 × 14.2-16.6	1.1	0.7	8.7 × 5.9	6.6-9.9 × 4.7-7.0	+	>1?	++	++	
19CR	17.9 × 16.8	15.7-19.9 × 15.1-18.9	1.1	0.7	8.3 × 5.9	7.1-10.0 × 5.5-6.8	+F	1		+	
34RUM	18.1 × 16.5	16.6-19.0 × 15.8-17.7	1.1	0.9	10.1 × 6.8	8.6-12.1 × 6.3-7.7	+	1	+	+	
54STR	18.3 × 17.8	17.3-19.8 × 16.1-19.7	1.0	1.0	8.5 × 7.1	6.3-10.8 × 6.2-8.3	+	1	++	+	PS
SA136	19.3 × 17.8	18.1-20.5 × 16.5-20.0	1.1	0.8	?	?	?	?	?	?	PS
TW4	20.0 × 17.7	17.0-22.9 × 15.7-19.3	1.1	1.0	9.4 × 6.8	7.6-10.9 × 5.7-8.1	+P	+	?	+	
8HB1	20.9 × 18.8	19.1-22.9 × 15.9-20.9	1.1	1.2	9.4 × 6.5	6.6-11.9 × 5.0-8.5	+	1 (-2?)	++	++	
TW1	21.6 × 21.6		1.0	1.1	9.4 × 7.3		?	+	?	?	US
6HB3	21.8 × 21.1	21.4-22.5 × 20.8-21.5	1.1	0.8	11.1 × 7.0	9.6-14.7 × 5.7-7.8					
B4B2	22.1 × 19.4	20.9-22.7 × 18.7-20.1	1.1	1.2	10.4 × 7.7	8.1-11.8 × 5.6-9.4	+	>1?	?	?	PS
6HB1	23.2 × 19.5	21.7-24.9 × 18.3-21.0	1.2	1.1	?	?	?	?	?	?	PS

Tab. III: Morphological features of the samples. Explanatory notes: F= flat, L/W = length/width ratio, OO = oocyst, OW = oocyst wall, P = pointed, PG = polar granule, PS = partially sporulated, RB = refractile body, SB = Stieda body, SC = sporocyst, SR = sporocyst residuum, US = unsporulated, + = present, ? = unclear.

	hosts	localities of origin	oocyst		sporocyst							
			shape	size (µm)	SI	wall (µm)	P G	shape	size (µm)	SI	SB	SR
<i>E. chagasi</i> ¹	SA, SM	BG, CZ, RU	SPH	14.5 x 14.5 (13-19 x 12-15)	1.0-1.1	1	+		8-9 x 4-5		0	+
<i>E. neomyi</i> ²	NA, NF	BG	SPH - SUB	18-20 x 18-20 (for SPH); 18.3 x 16.5 (16-20.5 x 15.2-17.8 for SUB)	1.0; 1.1	BL; 1	+		12 x 7.5	1.6	+HP	+
<i>E. soricis</i> ³	SA, SM	LI, RU, (USA)	SUB, OVO, ELI	19 x 14 (19-22 x 13-14)	1.4	<1	+	ELO - ELI	?	?	0	+?
<i>E. leucodontis</i> ⁴	CL, CS	BG, (AZ)	SPH	21 x 21 (18-24 x 18-24)	1.0	BL; 2.5	0		8.6 x 6.4 (8-10 x 6-8)	1.3	0	+
<i>E. ropotomae</i> ²	CL	BG		22-25 x 20-22.5	1.1	BL?; SC;>1	+	OVO	12 x 7.6	1.6	+HP	+
<i>E. dissimilis</i> ¹	SA, SM	BG, LI, RU	ELO, OVO, PT	28 x 19.4 (18-33 x 13-24)	1.4	1	+	ELI	15 x 10		0	0?
<i>I. araneae</i> ²	SA	BG	SPH - SUB	17 x 17 (for SPH); 16-18.5 x 15-18 (for SUB)	1.0	BL; ~1	+	OVO	12.5 x 9.5	1.3	0?	+
<i>I. neomyi</i> ²	NA, NF	BG	SPH	12-16 x 12-16	1.0	BL; ~1	+	OVO	8-11 x 5-6.5		0	+
<i>I. soricis</i> ^{5*}	SA	BG	ELI	24 x 17 (23-25 x 16-18)	1.5	<1	+	SPH	10.5 x 10.5	1.0	0	+

Tab. IV: *Eimeria* and *Isospora* spp. described from european shrews, valid species without micropyle and oocyst residuum

Explanatory notes: PG = polar granule, SB = Stieda body, SI = shape index, SR = sporocyst residuum; CL = *Crocidura leucodon*, CS = *C. suaveolens*, NA = *Neomys anomalus*, NF = *N. fodiens*, SA = *Sorex araneus*, SM = *S. minutus*;

AZ = Azerbaijan, BG = Bulgaria, CZ = Czech Republic, RU = Russia, LI = Lithuania, USA = United States of America;

BL = bilayered, ELI = ellipsoidal, ELO = elongated, HP = hyaline plug, OVO = ovoidal, PT = pointed, SC = sculptured, SPH = spheroidal, SUB = subspheroidal,

* = this species has not been recorded since its description, and was found only in a single individual

¹ Yakimoff and Gousseff, 1935; ² Golemansky, 1978; ³ Henry, 1932; ⁴ Musaev & Veisov, 1961; ⁵ Golemansky & Yankova, 1973

Curriculum Vitae

Mgr. Anna Mácová

Date and place of birth: 3rd August, 1988, Tábor

Education:

2003 – 2007	High School of Ecology, subject Protection of the environment
2007 – 2011	Bachelor study of Biology, University of South Bohemia, Faculty of Science, České Budějovice
2011 – 2013	Master study of Parasitology, University of South Bohemia, Faculty of Science, České Budějovice
2013 – present	Ph.D. study of Parasitology, University of South Bohemia, Faculty of Science, České Budějovice

Professional experience, employment:

2011 – 2018	Biology Centre CAS, Institute of Parasitology; Research Assistant
2012 – 2019	University of South Bohemia, Faculty of Science, Department of Parasitology; Research Assistant
2019 – present	Biology Centre CAS, Institute of Entomology; Research Assistant

Conferences:

26.-30.9. 2014, Dresden (Germany): International Coccidiosis Conference. Phylogenetic relationships and population structure of *Eimeria* species parasitizing field mice of the genus *Apodemus*. (presentation)

25.-30.5. 2014, Stará Lesná (Slovakia): V4 Parasitological Meeting. *Eimeria* and *Apodemus* – phylogenetic relationships and population structure. (poster)

27.-31.10. 2014, Nové Hradky (Czech Republic): NGS workshop and conference MouseGene2014.

Apodemus vs. *Eimeria*: Evolutionary factors of speciation and genomic diversification in host-parasite system. (presentation)

20.-24.7. 2016, Turku (Finland): European Multicolloquium of Parasitology (EMOP). Interesting evolutionary patterns in rodent-coccidia associations: host specificity, diversification and host-switching. (presentation)
Coccidian parasites infecting snow bunting (*Plectrophenax nivalis*) in Arctic. (poster)

8.-9.6. 2017, Riga (Latvia): Conference of Scandinavian-Baltic Society for Parasitology. Phylogenetic relationships of eimerian parasites infecting shrews: another piece to the puzzle. (poster)

9.-11.10. 2019, Copenhagen (Denmark): Conference of Scandinavian-Baltic Society for Parasitology.

Present state of rodent populations in Svalbard. (poster)

Study stays:

March – June 2015, Berlin (Germany): 4 months in the Department of Biology, Humboldt University Berlin. Laboratory of Ecology and Evolution of molecular Parasite-Host Interactions (Prof. Emanuel Heitlinger).

July 2016, Berlin (Germany): 1 month in the Leibnitz Institute for Zoo and Wildlife Research. Laboratory of Parasite-Host Interactions (Prof. Emanuel Heitlinger).

Teaching and supervision

Field practice „Vomáčka“. Rodent trapping, demonstration of hosts and parasites, dissections. Autumn 2014.

Biology of parasitic arthropods. Teaching assistant, 2016-2017.

Summer camp „Letní tábor s přírodovědou“. Dissections of rodents. Summer 2018.

Šárka Schmidtmayerová: Phylogeny of coccidia parasitizing field mice at various localities in Europe. Supervision of the High School Scientific Work, 2015-2017.

Klára Charvátová: Variability of the TLR2 receptor in arvicoline rodents (Arvicolidae) across Europe, depending on parasitation of the host. Supervision of the Bachelor Thesis, 2016-2018.

Veronika Žánová : Intestinal parasites of birds on Svalbard. Co-supervision of the Bachelor Thesis, 2018-2021.

List of publications:

Máková A, Hoblíková A, Hypša V, Stanko M, Martinů J, Kvičarová J, 2018: Mysteries of host switching: Diversification and host specificity in rodent-coccidia associations. *Molecular Phylogenetics and Evolution* 127: 179-189. doi: 10.1016/j.ympev.2018.05.009.

Trefančová A, Máková A, Kvičarová J, 2018: Isosporan oocysts in the faeces of bank voles (*Myodes glareolus*; Arvicolinae, Rodentia): Real parasites, or pseudoparasites? *Protist* 170: 104-120. doi: 10.1016/j.protis.2018.12.002.

Holicová T, Sedláček F, Máková A, Vlček J, Robovský J, 2018: New record of *Microtus mystacinus* in eastern Kazakhstan: phylogeographic consideration. *Zookeys* 781: 67-80. doi: 10.3897/zookeys.781.25359.

Myšková E, Brož M, Fuglei E, Kvičarová J, Máková A, Sak B, Kváč M, Ditrich O, 2019: Gastrointestinal parasites of arctic foxes (*Vulpes lagopus*) and sibling voles (*Microtus levis*) in Spitzbergen, Svalbard. *Parasitology Research* 118: 3409-3418. doi: 10.1007/s00436-019-06502-8.

Jarquín-Díaz VH, Ballard A, Máková A, Jost J, Roth von Szepeséla T, Berkold K, Tank S, Kvičarová J, Heitlinger E, 2020: Generalist *Eimeria* species in rodents: Multilocus analyses indicate inadequate resolution of established markers. *Ecology and Evolution* 10: 1378-1389. doi: 10.1002/ece3.5992.

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