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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 or 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:

Mácová 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. 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:

Trefancová A, Mácová A, Kvičerová 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, Mácová 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, Kvičerová 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, Kvičerová 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:

Trefancová A, Kvičerová 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:

Mácová 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 Mácová*

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

Jana Kvičerová

MVDr. Jana Kvičerová, PhD. (for MS2, and Draft 1)

RNDr. Tereza Holicová (for MS3)

RNDr. Eva Myšková (for MS4)

Prof. Dr. rer. nat. Emanuel Heitlinger (for MS5) Jana Kvičerová JANA KVIČEROVÁ

Contents

Attached publications

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 occurence 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 that 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; Filippuci 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 Fst 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 pray, 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 heterogenous 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). Aligments were created by ClustalW algorithm in the programs BioEdit, or MUSCLE in Geneious, and phylogenetic relationships were computed in MrBayes and Phyml.

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.

- one page commenting drafts is excluded

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.

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

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Ms 1:

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

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Rodent

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, preconditions 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, *Austrogoniodes*-penguins, *Polyplax-Apodemus*, or *Ligula*-fish hosts (Demanche et al., 2001; Banks et al., 2006; Štefka and Hypša, 2008; Štefka et al., 2009). These differencies 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 Palaearctic 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; Kvičerová 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 (Kvičerová 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. Consensi 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.; Bellinvia, 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 intertangled 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

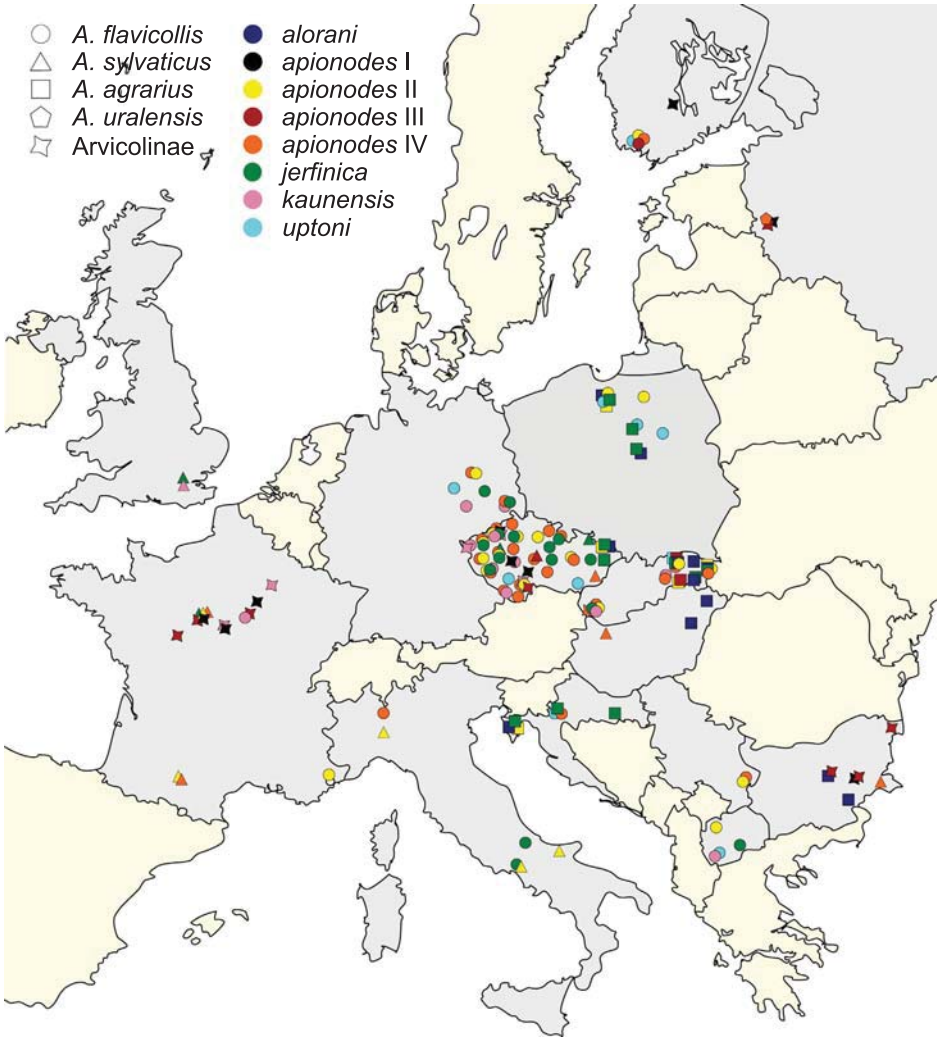

Fig. 1. Distribution of individual *Eimeria* lineages across the sampled localities. Symbols are attributed to the hosts, colours are attributed to the parasites.

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.

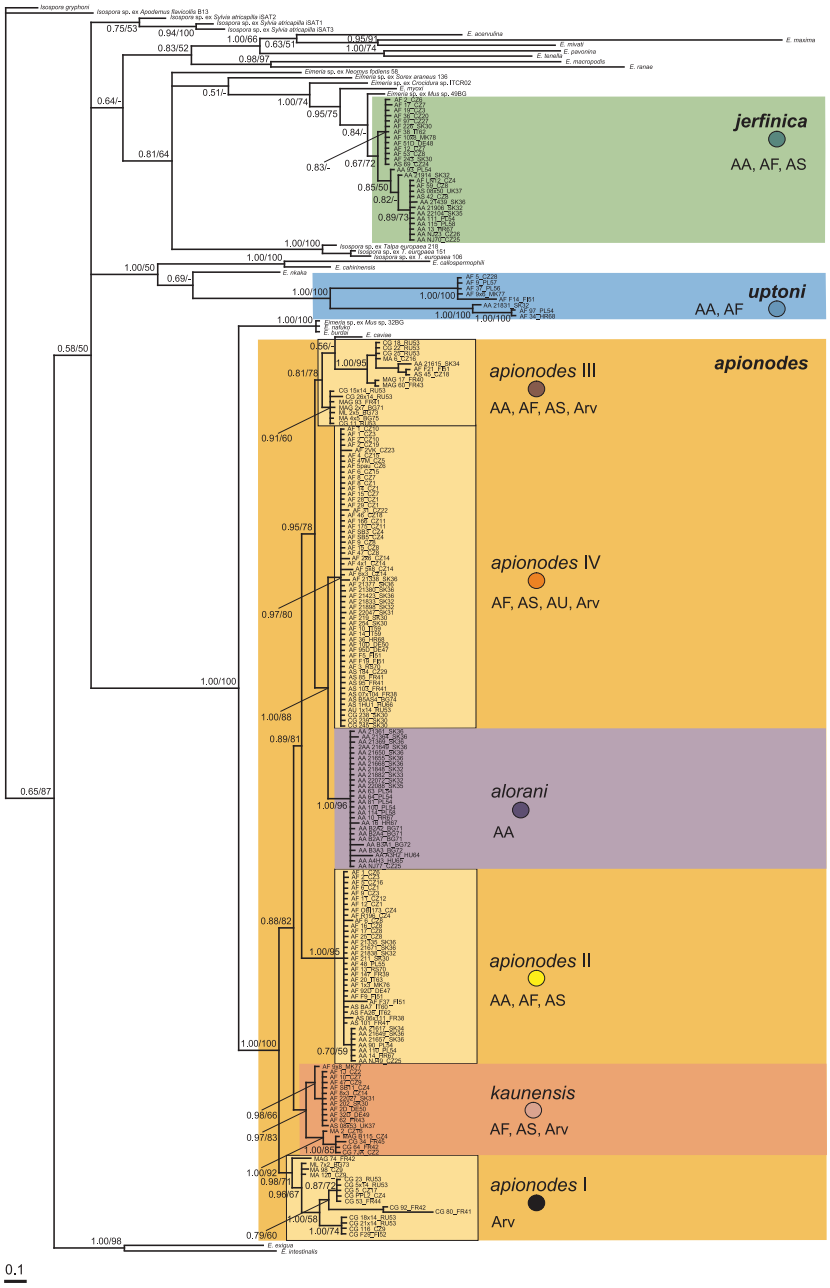

Fig. 2. Phylogenetic relationships of the eimerians inferred by the BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities under the BI analysis/bootstrap values derived from ML analysis. Posterior probabilities and bootstrap supports lower than 0.50 or 50%, respectively, 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.

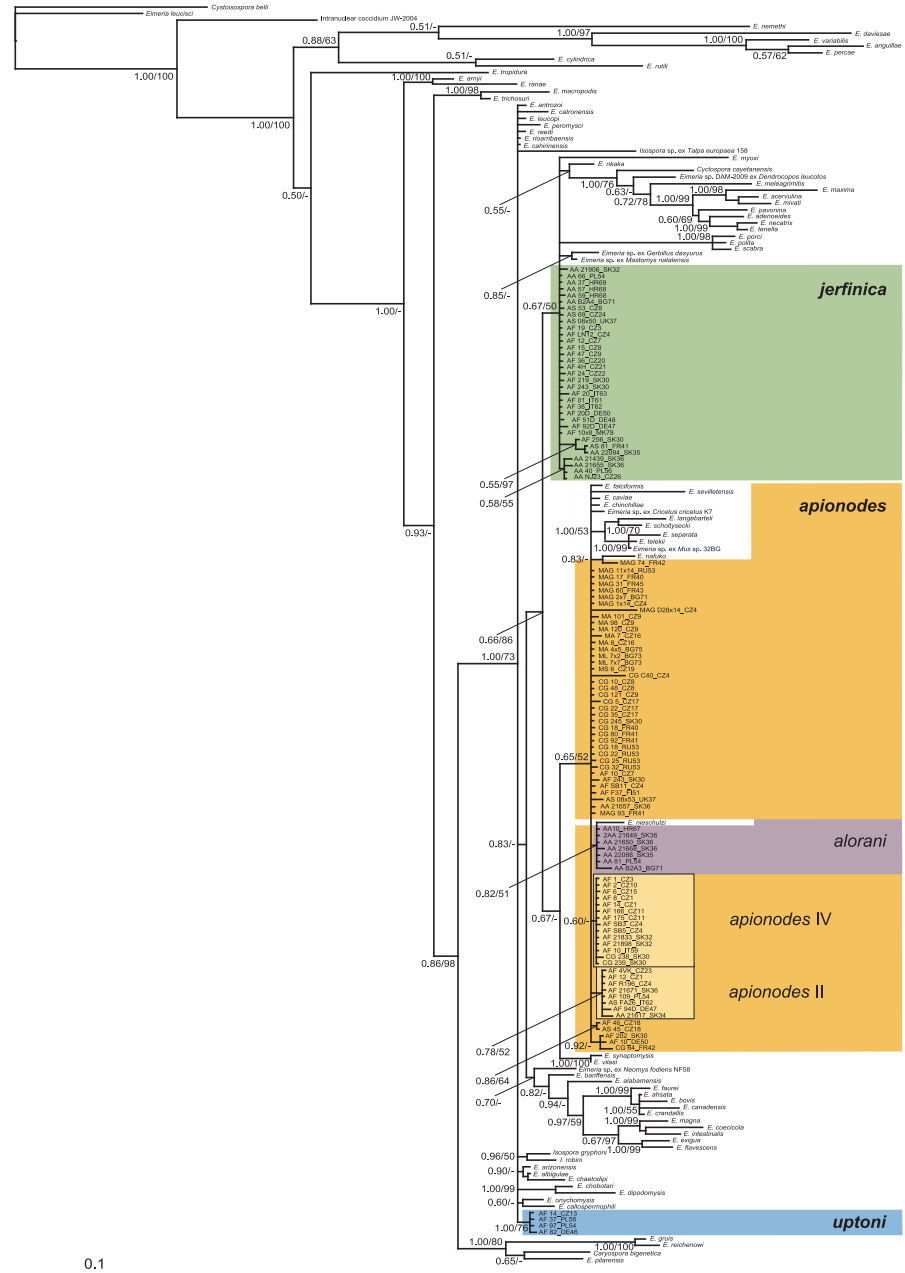

Fig. 3. Phylogenetic relationships of the eimerians inferred by the BI analysis of the 18S rDNA sequences. Numbers at the nodes show posterior probabilities under the BI analysis/bootstrap values derived from ML analysis. Posterior probabilities and bootstrap supports lower than 0.50 or 50%, respectively, 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.

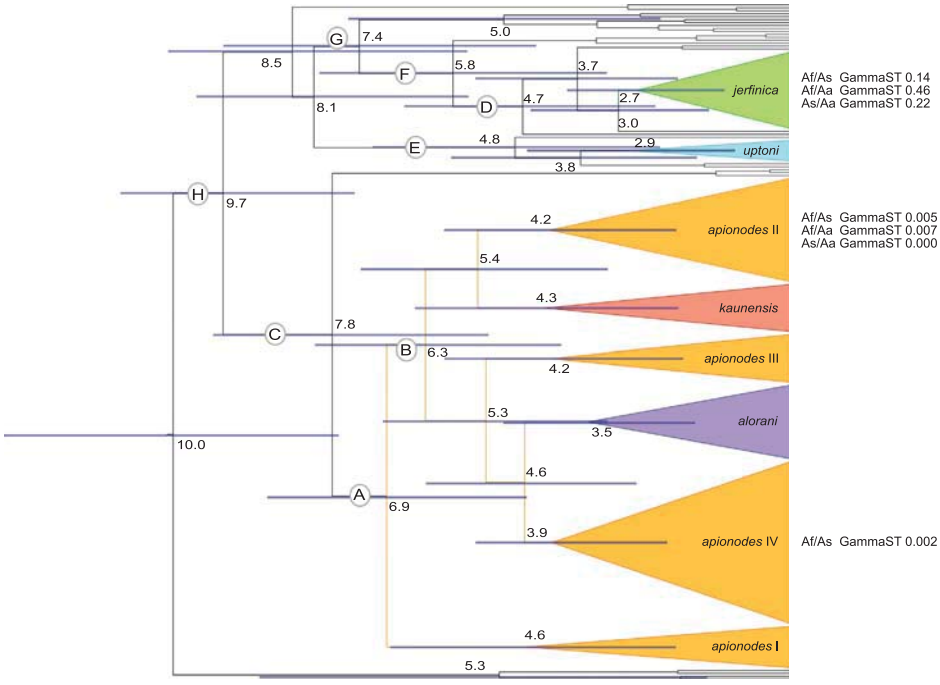

Fig. 4. Divergence times and genetic differentiations calculated by the BEAST package and DnaSP. The numbers at the nodes show relative "ages" of the lineages (see Material and Methods), the bars at the nodes represent posterior probabilities values and 95% credibility intervals.

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

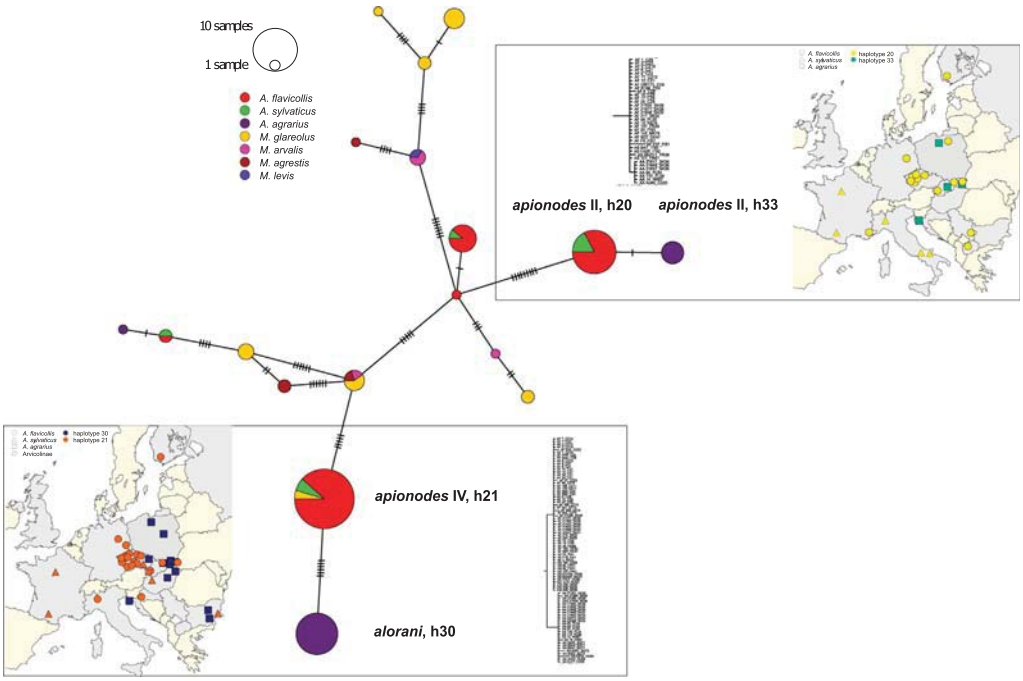

Fig. 5. Haplotype network of the lineage A (as defined in Fig. 4) constructed in PopART. Spatial relationship between the Af/As and Aa haplotypes are shown in the maps.

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 abandoment 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.

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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).

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, monozoic 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 mammalś 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 *M. glareolus*)	2016 (no. of *M. glareolus*)	2017 (no. of *M. glareolus*)	Total number of trapped animals
České Budějovice (South Bohemian Region)	1	10	8	19
Klášterec 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
Tymákov (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 samples were *Isospora*-positive, while 93/338 (27.5%) were *Eimeria*-positive. Most of the *Isospora*-positive samples were obtained in the surroundings of Tymákov (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	♂	Tymákov (Pilsen Region)	March 2016	I+++
57_MG_TYM	♂	Tymákov (Pilsen Region)	March 2016	I+++
60_MG_TYM	♀	Tymákov (Pilsen Region)	March 2016	I++
89_MG_TYM	♂	Tymákov (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	♀	Tymákov (Pilsen Region)	August 2017	I+++
7_MG_TYM	♂	Tymákov (Pilsen Region)	September 2017	I++
13_MG_TYM	♀	Tymákov (Pilsen Region)	September 2017	I+
18_MG_TYM	♀	Tymákov (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	♀	Tymákov (Pilsen Region)	August 2017
12_MG_TYM	♂	Tymákov (Pilsen Region)	September 2017
15_MG_TYM	♀	Tymákov (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 *Capillaria* ++	Coccidia-negative, eggs of *Trichuris*+	Coccidia-negative, eggs of *Trichuris* +++
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, *Capillaria* ++	Coccidia-negative, *Trichuris* +	Coccidia-negative, *Trichuris* +++
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
Isospora anatolicum Sayin, Dincer & Meric, 1977	*Spalax leucodon* (Spalacidae)	Asia	Spherical; 9–11 × 8–9	Ovoid; 6–9 × 4–6	No	No OR or M; smooth pale greenish yellow OW (0.8)
Isospora arvalis Mikeladze, 1973	*Microtus arvalis* (Muridae)	Russia	Subspherical; 10–12 × 8–12	Ovoid; 6–8 × 4–6	No	No OR or M; smooth colorless OW (2)
Isospora assensis Svanbaev, 1979	*Spermophilus fulvus* (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)
Isospora aurangabadensis Kshirsagar, 1980	*Rattus rattus* (Muridae)	India	Spherical or subspherical; 32–44 × 32–40	Ovoid, globose or elongate	Yes	No OR or M; yellowish brown OW (1.5–2)
Isospora batabatica Musaev & Veisov, 1960	*Arvicola amphibius* (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)
Isospora californica Davis, 1967	*Peromyscus californicus* (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)
Isospora calomyscus Musaev & Veisov, 1965	*Calomyscus bailwardi* (Muridae)	Russia	Ellipsoidal; 20–23 × 16–20	Ovoid; 14–17 × 10–15	Yes	No OR or M; smooth, yellow-brown OW (1.5)
Isospora citelli Levine, Ivens & Kruidenier, 1957	*Spermophilus variegatus* (Sciuridae)	North America, Russia	Subspherical; 22–23 × 21–22	Broadly lemon-shaped; about 15 × 10	Yes	No OR or M; smooth brownish OW (1)
Isospora clethrionomydis Golemanski & Yankova, 1973	*Myodes glareolus* (Muridae)	Europe (Bulgaria)	Spherical; 23–27 × 23–27	Ovoid; 21–23 × 11–12	Yes	No OR or M; thin colorless or light yellowish OW
Isospora clethrionomysis Arnastauskiene & Maldzhiunaite, 1981	*M. glareolus* (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
Isospora dawadimiensis Kasim & Al Shawa, 1985	*Jaculus jaculus* (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)
Isospora dryomidis Glebezdin, 1974	*Dryomys nitedula* (Myoxidae)	Russia	Ellipsoidal; 23–26 × 20–23	Ellipsoidal; 12–15 × 9–12	No	No OR or M; smooth, colorless OW
Isospora egypti Prasad, 1960	*Meriones shawi* (Muridae)	Africa	Subspherical; 20–22 × 16–20	Ovoid; 10–12 × 6–8	Yes	No OR or M; smooth light brown OW
Isospora erythrourica Veisov, 1964	*Meriones libycus* (Muridae)	Russia	Spherical or subspherical; about 24–30 × 24-30	Ellipsoidal or spherical; 12–18 × 10–16	Probably not	No OR or M; smooth colorless – yellow–brown OW about (1.5–2.5)
Isospora freundi Yakimoff & Gousseff, 1935	*Cricetus cricetus* (Muridae)	Russia	Spherical or subspherical; 13–27 × 17–24	14 × 8–9	No	No OR or M; smooth double-contoured OW
Isospora golemanskii Levine, 1982	*Apodemus flavicollis* (Muridae)	Europe	Spherical or subspherical; 23–28 × 20-28	Ovoid; 12–16 × 8-11	No record	No OR or M
Isospora hammondi Barnard, Ernst & Stevens, 1971	*Oryzomys palustris* (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)
Isospora hastingsi Davis, 1967	*Peromyscus truei* (Muridae)	North America	Ovoid; 29–33 × 22–24	Lemon-shaped	Yes	No OR or M; smooth tan or yellow-tan OW about (1.2)
Isospora krishnamurthyi Kshirsagar, 1980	*R. rattus* (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)
Isospora laguri Iwanoff-Gobzem, 1934	*Lagurus lagurus* (Muridae)	Russia	Ovoid; 24–32 × 16–22	16–21 × 8–13	No record	OR present; no M; thick OW
*Isospora * masoni* Upton, Lindsay, Current & Ernst, 1985	*Sigmodon hispidus* (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
Isospora mcdowelli Saxe, Levine & Ivens, 1960	*Microtus pennsylvanicus* (Muridae)	North America	Spherical to subspherical; 9–11 × 8–10	Ellipsoidal; 6–8 × 4–5	No record	No OR or M; thin OW
Isospora meriones Musaev & Veisov, 1965	*Meriones vinogradovi* (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)
Isospora mexicanasubsimi Vance & Duszynski, 1985	*Microtus mexicanus subsimus* (Muridae)	Mexico	Spherical; 21–26 × 21–26	Ovoid; 12–16 × 10–12	Yes	No OR or M; smooth OW (1.5)
Isospora ordubadica Musaev & Veisov, 1960	*Meriones persicus* (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)
*Isospora * peromysci* Davis, 1967	*Peromyscus maniculatus* (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)
Isospora ratti Levine & Ivens, 1965	*Rattus norvegicus* (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)
Isospora samsensis Svanbaev, 1979	*Spermophilus fulvus* (Sciuridae)	Russia	Ovoid; 25–32 × 22–26	Ellipsoidal; 11–13 × 8–9	No	No OR or M; smooth greenish OW (1.2–1.5)
Isospora sp.* Barnard, Ernst & Dixon, 1974	*S. hispidus* (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)
Isospora sp. Nukerbaeva & Svanbaev, 1973	*Myocastor coypus* (Myocastoridae)	Russia	Short-oval to spherical; 25–28 × 25–28	About 14 × 11	No record	OR present; no M; OW (1.5)
Isospora sp. Stout & Duszynski, 1983	*Dipodomys agilis* (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
Isospora sp. * Svanbaev, 1963	*Marmota menzbieri* (Sciuridae)	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)
Isospora spermophili Levine, 1984	*Spermophilus maximus* (Sciuridae)	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)
Isospora tamariscini Levine, 1985	*Meriones tamariscinus* (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)
Isospora teres Iwanoff-Gobzem, 1934	*L. lagurus* (Muridae)	Russia	Spherical; 24–36 × 24–36	16–21 × 8–13	No record	No OR or M
Isospora uralicae Svanbaev, 1956	*Apodemus sylvaticus* (Muridae)	Russia	Ovoid; about 26 × 22.5	Ovoid; about 14 × 9	No	No OR or M; smooth greenish double-contoured OW (1.6)
Isospora vanadica Musaev & Veisov, 1965	*Meriones persicus* (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)
Isospora vinogradovi Musaev & Veisov, 1965	*M. vinogradovi* (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, 24.0–29.0 × 19.0–24.0 (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, 14.0–17.2 × 9.0–11.0 (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

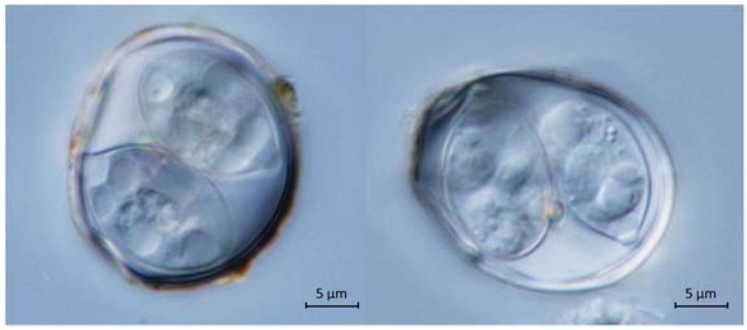

Figure 1. Sporulated oocysts obtained from *M. glareolus* isolate 2_MG_TYM (Tymákov, Pilsen Region, CZ).

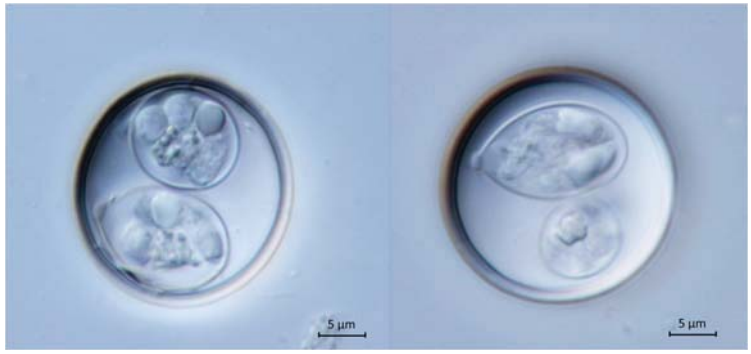

Figure 2. Sporulated oocysts obtained from *M. glareolus* isolate 49_MG_TYM (Tymákov, Pilsen Region, CZ).

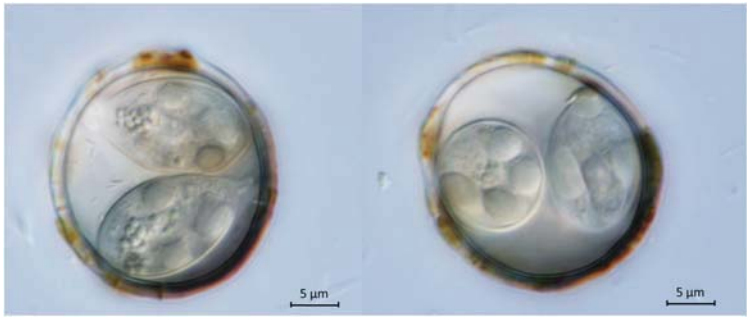

Figure 3. Sporulated oocysts obtained from *M. glareolus* isolate 57_MG_TYM (Tymákov, Pilsen Region, CZ).

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, 23.5–28.3 × 22.6–27.8 (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, 14.3–19.0 × 9.8–11.4 (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, 25.8–29.3 × 24.1–27.0 (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, 13.7–18.7 × 9.4–12.1 (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, 25.0–30.0 × 23.0–24.0 (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, 15.7–18.9 × 10.5–12.0 (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).

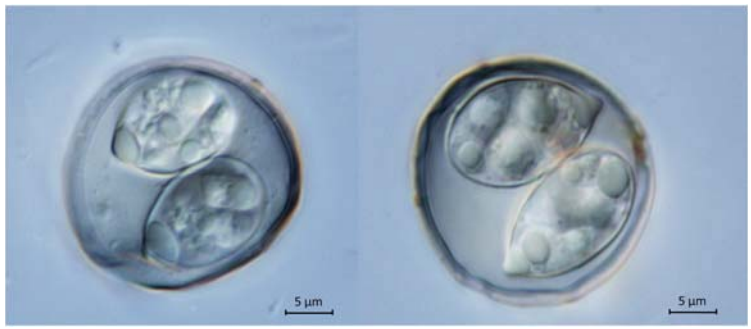

Figure 5. Sporulated oocysts obtained from *M. glareolus* isolate 9_MG_LUZ (Lužnice, South Bohemian Region, CZ).

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

9_MG_LUZ (Fig. 5)

Oocysts were subspherical to ellipsoidal, 24.0–28.4 × 19.0–25.0 (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, 14.0–18.6 × 9.2–12.0 (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

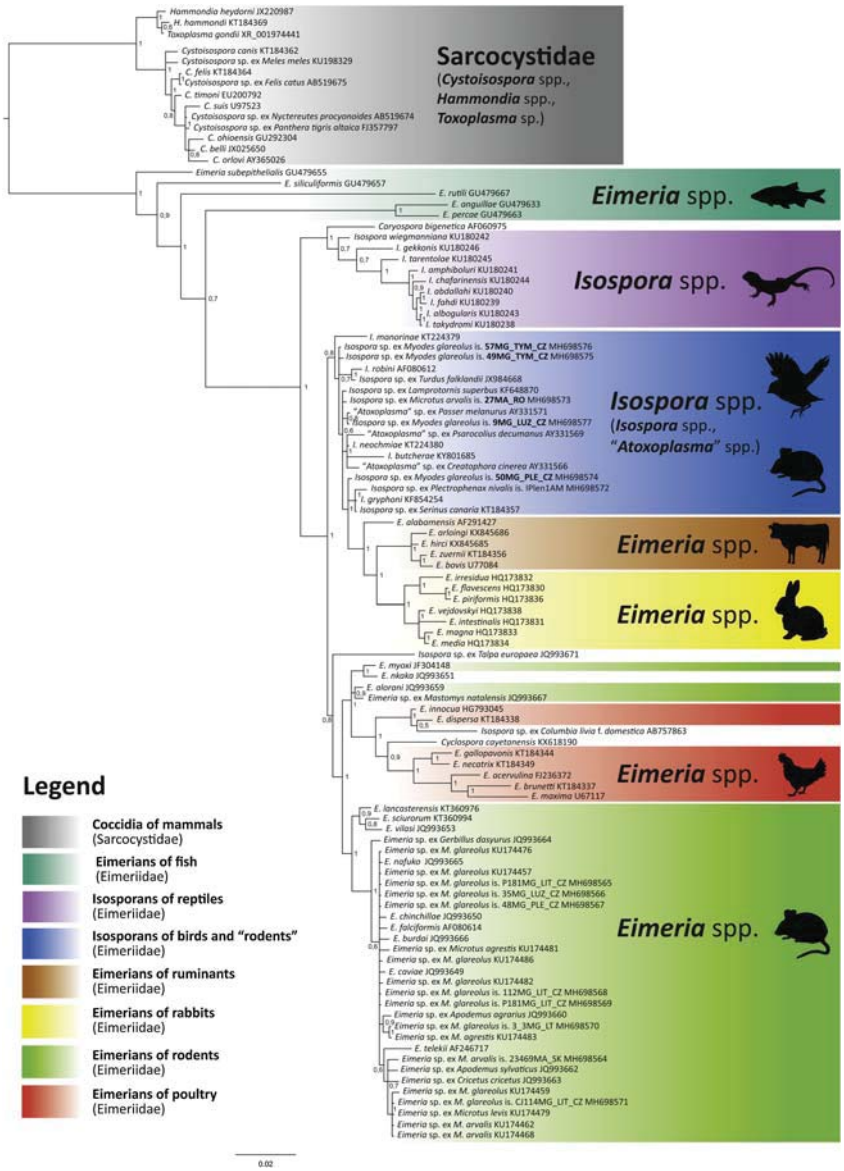

Figure 6. Phylogenetic relationships inferred by the BI analysis of the 18S rRNA sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. The family Sarcocystidae is used as an outgroup.

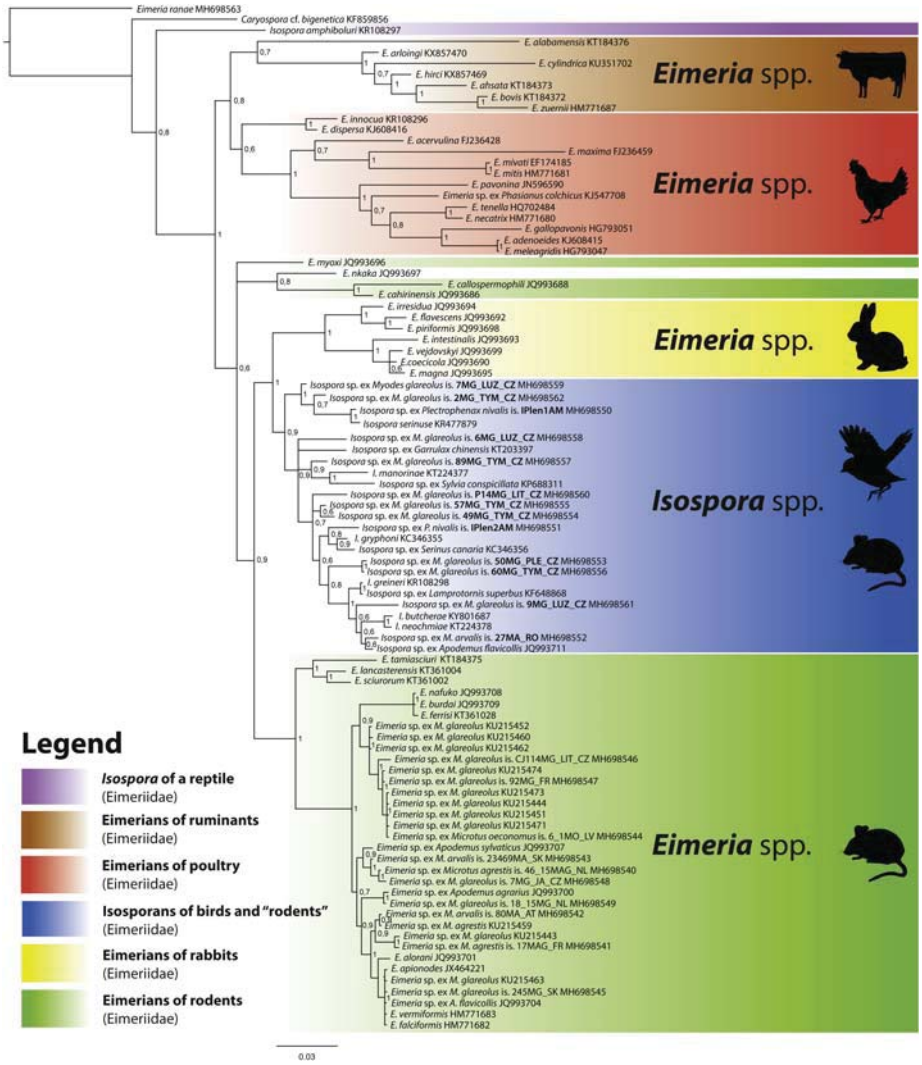

Figure 7. Phylogenetic relationships inferred by the BI analysis of the COI sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Eimeria ranae* is used as an outgroup.

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

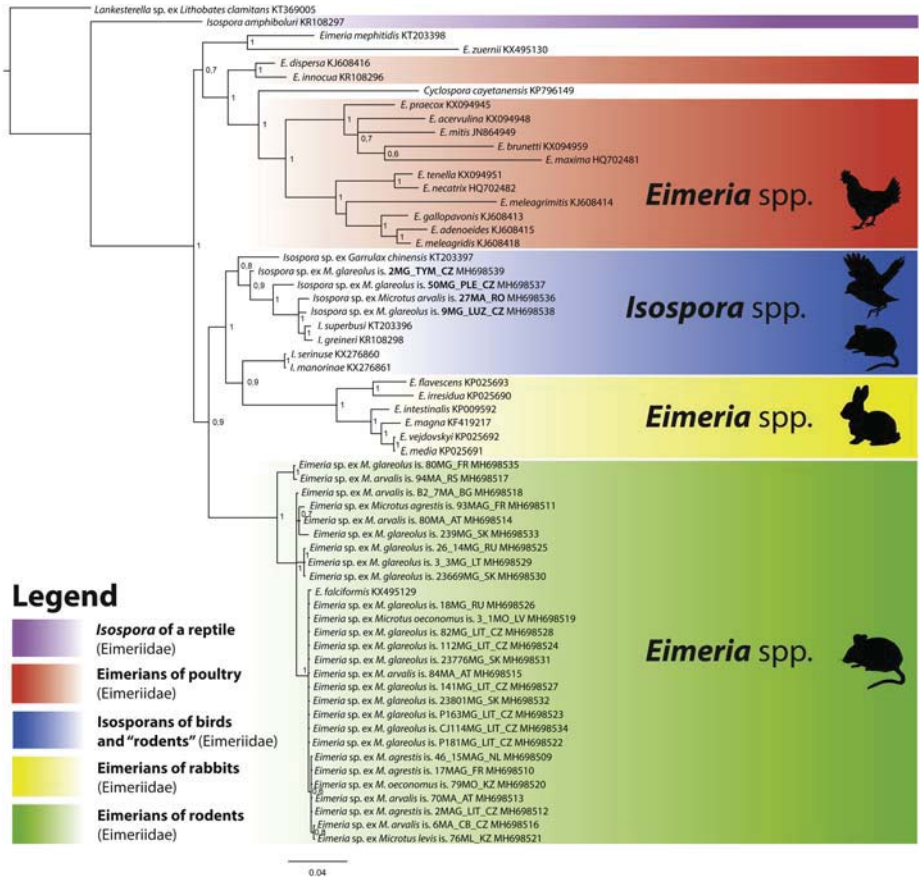

Figure 8. Phylogenetic relationships inferred by the BI analysis of the COIII sequences. Numbers at the nodes show posterior probabilities (PP); major branches are well-supported by high values of PP, and (simultaneously) all the values are higher than 0.5. *Lankesterella* sp. is used as an outgroup.

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-

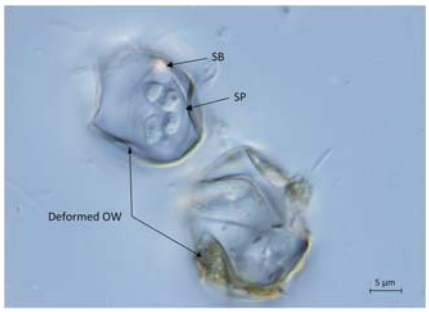

Figure 9. Deformed oocysts that passed through the gastrointestinal tract of bank voles after the administration of isolate of sporulated *Isospora*-type oocysts. OW, oocyst wall; SB, Stieda body; SP, sporocyst.

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 (7–9 × 5–6; 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 passaged 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 known 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, Třísov), Ústí nad Labem Region (Klášterec 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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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. transcaspicus* 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 arvicolines, 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. transcaspicus)* 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 Artic 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 Tougard 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; Tougard 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*: Miesenheim I; Tougard 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; Tougard 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

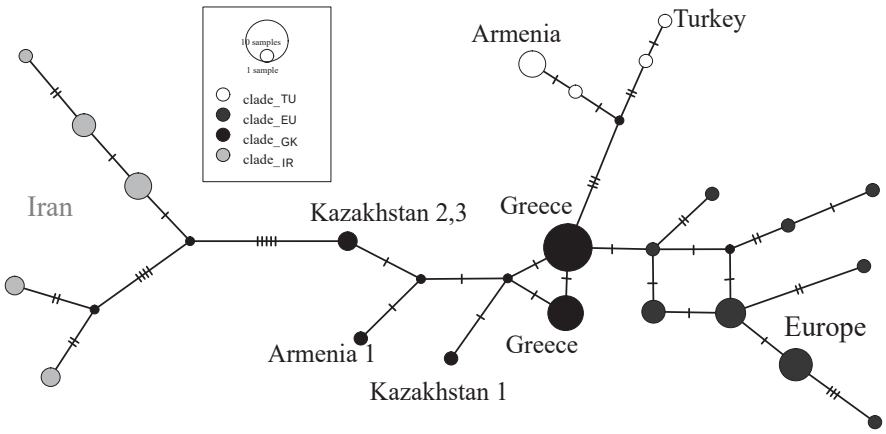

Figure 1. Median Joining Network based on the cyt *b* sequences of *M. mystacinus*.

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. *M. obscurus*	0.067	0.066	0.065	0.062	0.066	0.059	0.068	0.028		
9. *M. arvalis*	0.067	0.057	0.065	0.062	0.066	0.063	0.067	0.059	0.003	
10. *M. transcaspicus*	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. transcaspicus*, 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 $x10^{-7}$ 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 Řičánková 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

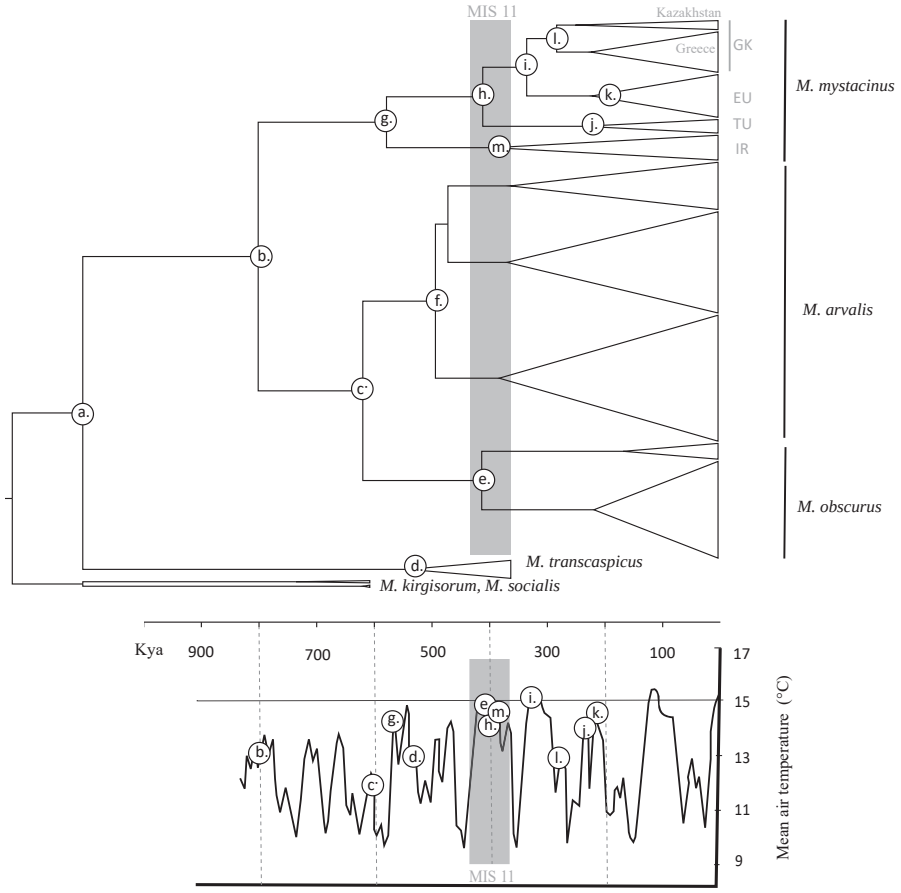

Figure 2. Time of the most recent common ancestor (TMRCA) for *Microtus* species and lineages of *M. mystacinus* using fossil calibrations. Nodes are plotted on a mean air temperature curve in last 800 thousand years (based on Gates 1993). See Table 2 for time estimates.

railway at Zyryanovsk (built after 1930; according to official web page of KTZ – КАЗАКСТАН 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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HELMINTHOLOGY - ORIGINAL PAPER

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 alticolis*, 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; Skírnisson 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 carrions 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. Metacestodes 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 metacestodes, 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 (× 200 and × 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 (GeneAll, 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

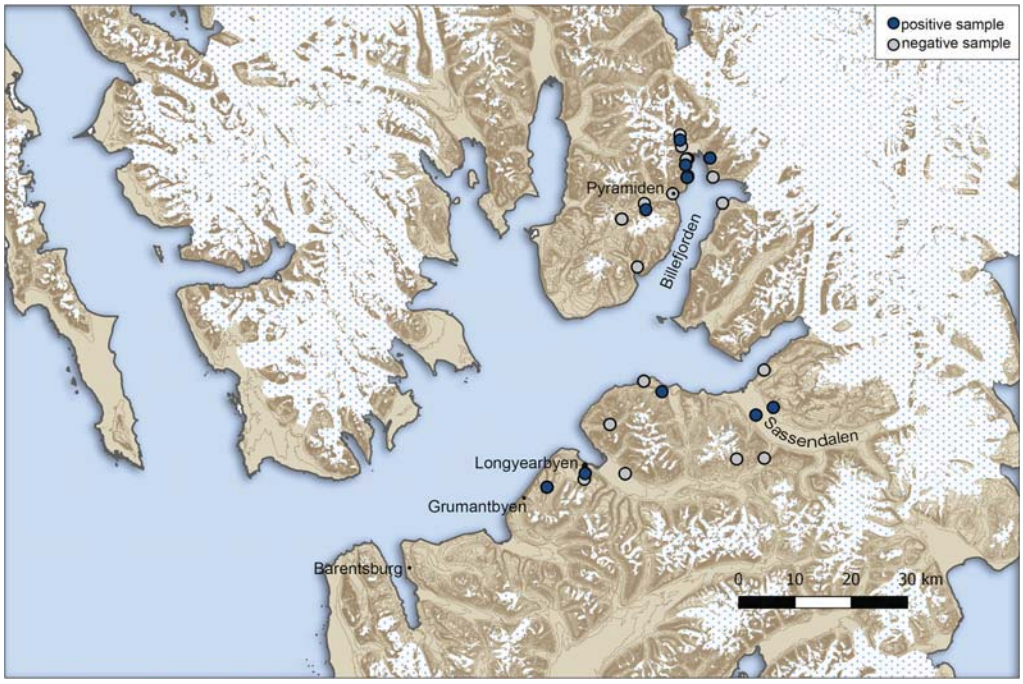

Fig. 1 Map showing the main sites (Longyearbyen, Sassendalen, and the Billefjorden area) of collected arctic fox faeces samples in Spitsbergen, Svalbard. Dark buttons, positive samples; light buttons, negative samples. Map created in QGIS Development Team (2018) QGIS Geographic information system. Open 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 lsrDNA 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 ul 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 Expin^{TM} 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 bieneusi*–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. bieneusi* 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. bieneusi*. 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; Skírnisson 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

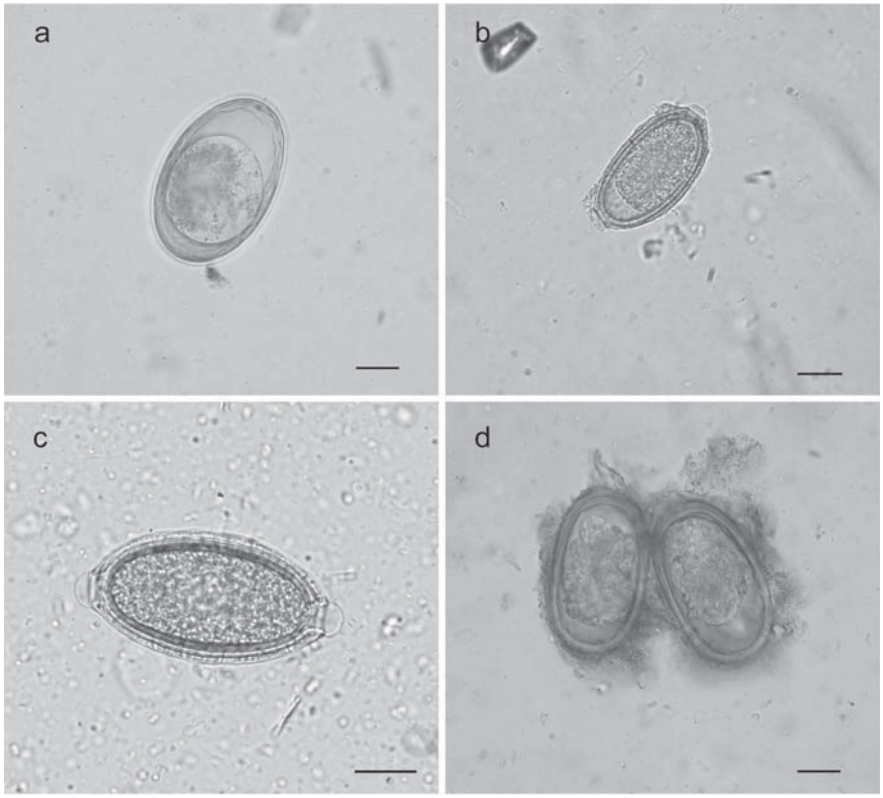

Fig. 2 Nematode eggs observed in the arctic fox faecal samples. **a** *Toxascaris leonina*. **b** *Eucoleus* sp..**c** *Trichuris* sp..**d** *Uncinaria stenocephala*. The scale bar 20 μm

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

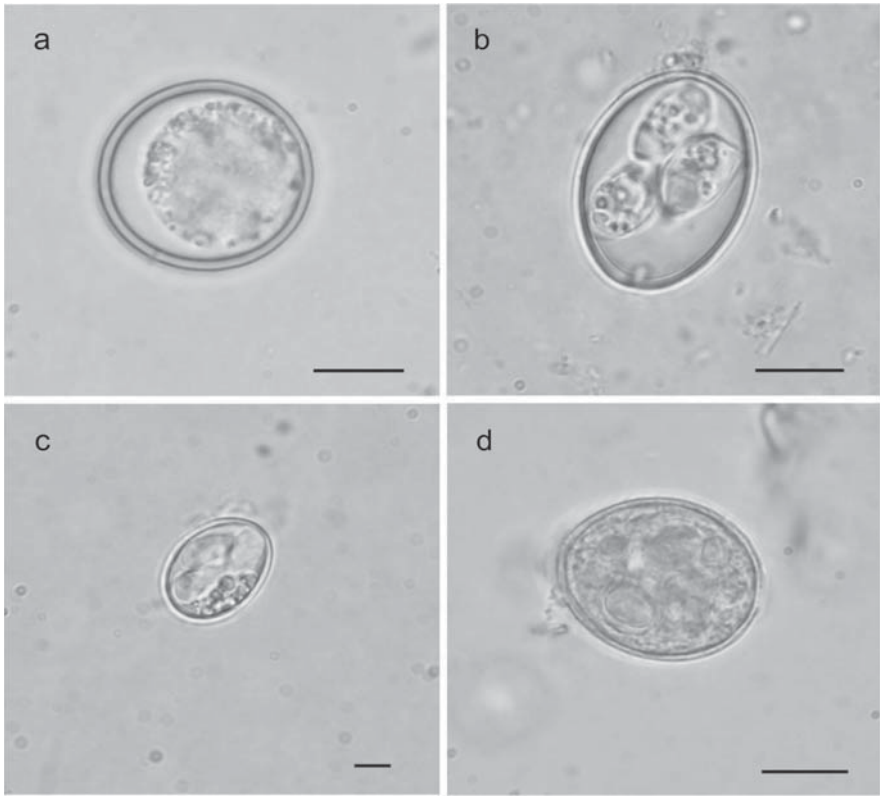

Fig. 3 Different morphotypes of *Eimeria* spp. oocysts observed in the arctic fox faecal samples. **a**, **b** From sample number 153. **c**, **d** From sample number 63A from Billefjorden. In the case of **a** and **d**, these are nonsporulated oocysts. The scale bar 10 μm

(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 (Horčičková 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; Skírnisson 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 and share 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; Skírnisson 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.

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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

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; Kvičerová, 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 (Kvičerová & Hypša, 2013). At shallow nodes of the phylogeny for rodent coccidians, cases of host generalism have been suggested (Mácová 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 (Kvičerová, Pakandl, & Hypša, 2008). Kvičerová and Hypša (2013) suggested that adaptation rather than cospeciation is shaping rodent *Eimeria* cophylogenies. Mácová 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*.

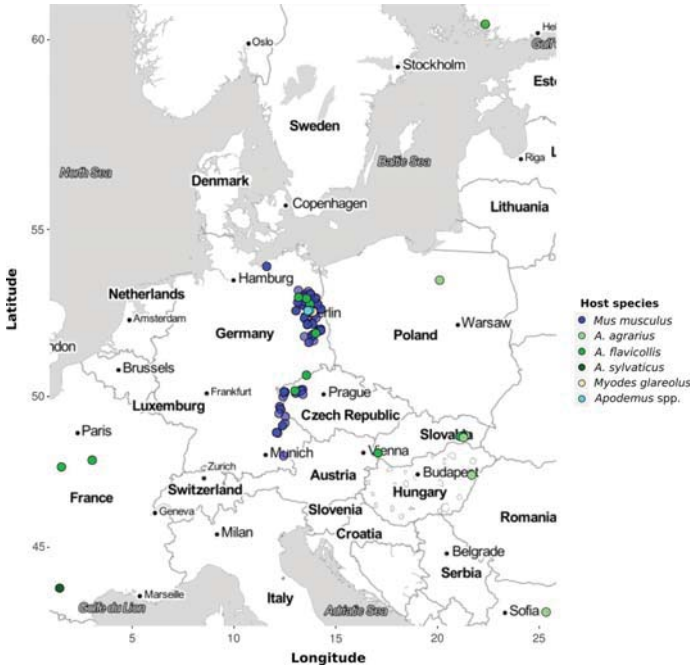

FIGURE 1 Location of rodent samples. *Mus musculus* samples were collected from the German federal states of Mecklenburg-Vorpommern, Bavaria, and Brandenburg and in Bohemia (Czech Republic). Non-*Mus* samples were collected from different countries within Europe. Color in the points indicates the host 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 (Mácová 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 (Ďureje, 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 (ATGTCACTNTCTCCAACCTCAGT) 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$MgCl_2$ 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, MH755302-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 | Multimarker 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 multimarker 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. *Isospora*sp. 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,

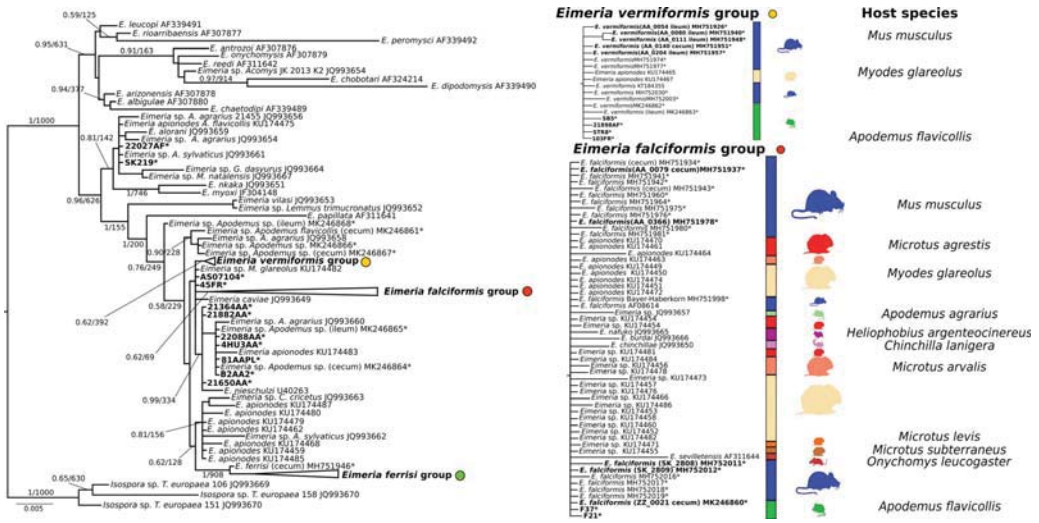

FIGURE 2 Phylogenetic trees inferred from nuclear small ribosomal subunit (18S rDNA). Phylogenetic tree based on 18S rDNA sequences. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* of this 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 multimarker phylogenetic inference

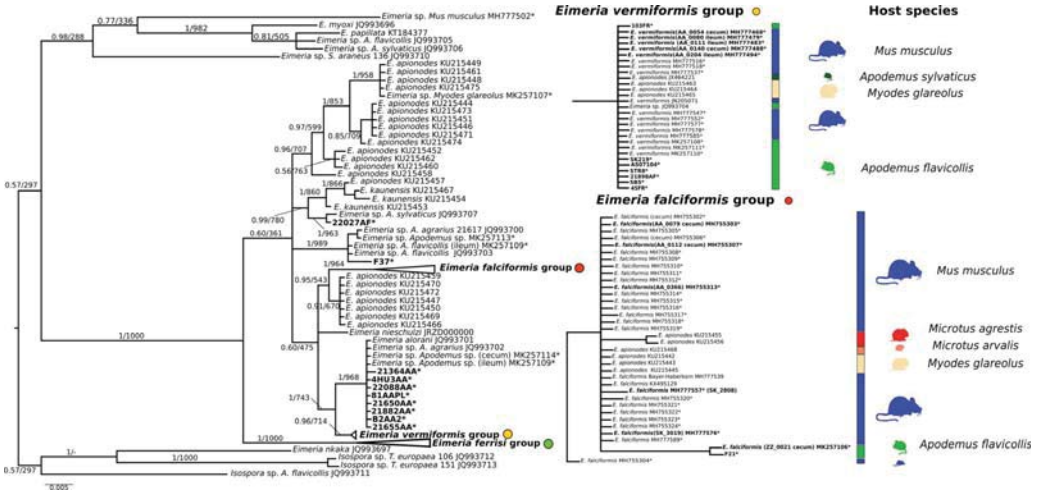

FIGURE 3 Phylogenetic trees inferred from mitochondrial cytochrome c oxidase (COI) sequences. Phylogenetic tree based on COI. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* of this study. 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 multimarker phylogenetic inference

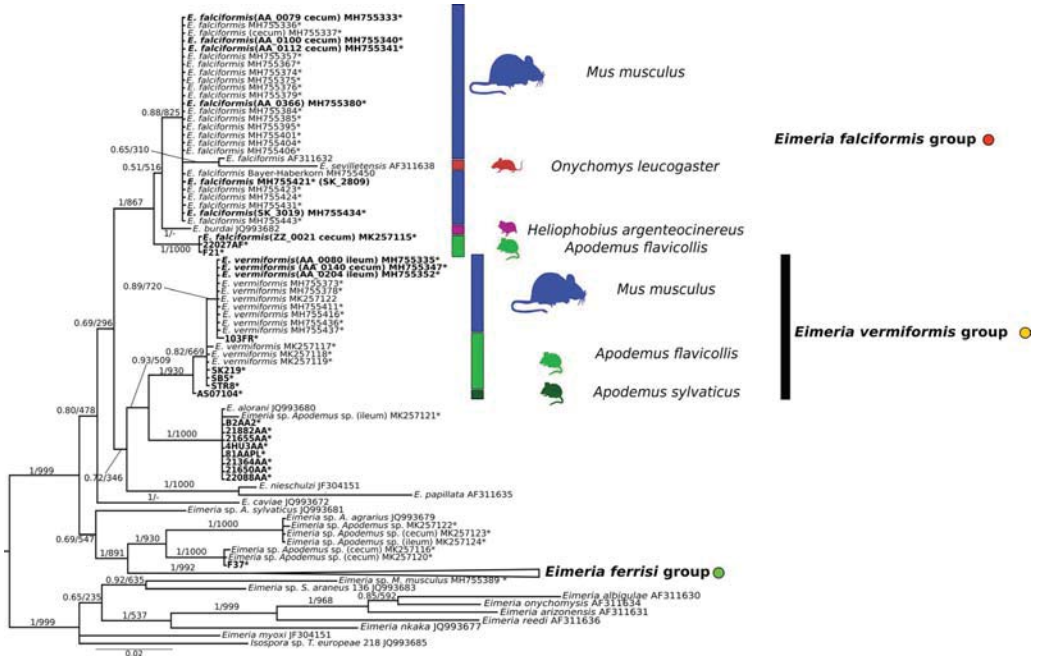

FIGURE 4 Phylogenetic trees inferred from apicoplast open reading frame 470 (ORF470) sequences. Phylogenetic tree based on ORF470 sequences. Numbers in the branches represent Bayesian posterior probability and bootstrap value. The three collapsed groups cluster *Eimeria* sequences from *Mus musculus* 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 multimarker 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

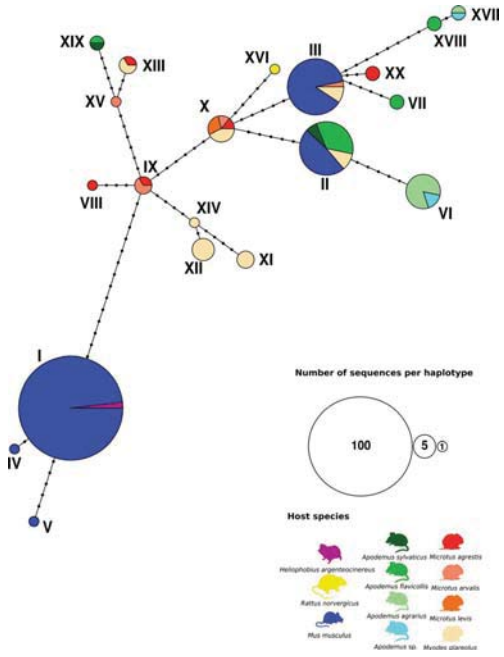

FIGURE 5 Statistical parsimony network of *Eimeria* spp. haplotypes for COI sequences. Network based on a 459 bp region of the gene coding for the mitochondrial cytochrome c oxidase from *Eimeria* isolates detected in rodents (*Mus musculus*, *Apodemus flavicollis*, *A. sylvaticus*, *A. agrarius*) caught 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 *Apodemusagrarius* (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 coinfected 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.

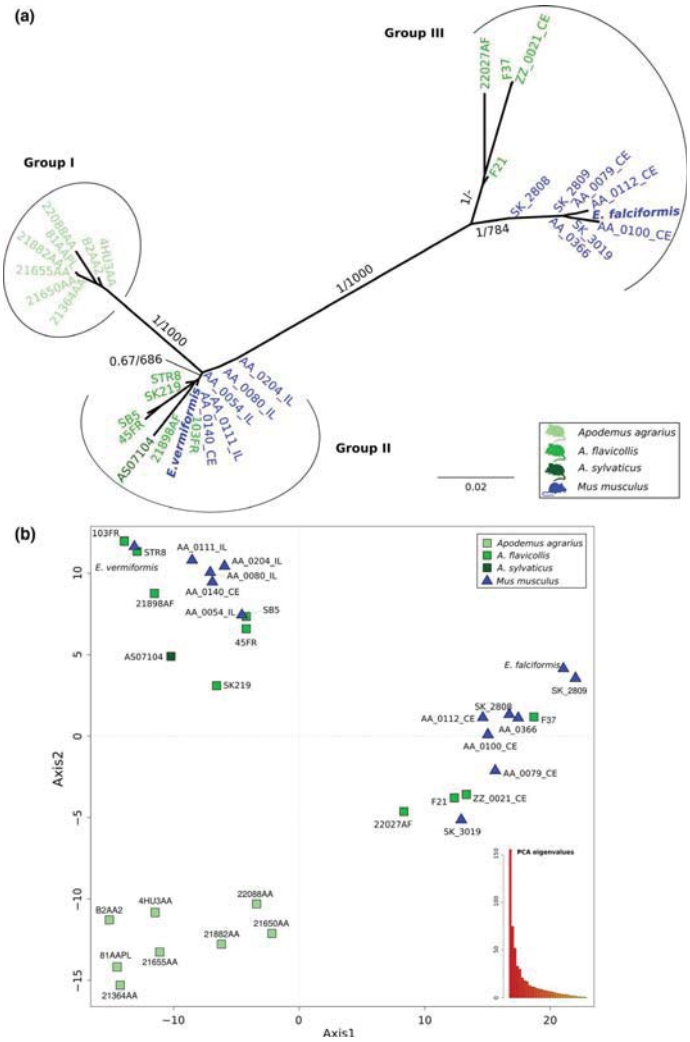

FIGURE 6 Nuclear multilocus genotyping of *Eimeria* isolates from *Mus musculus* and *Apodemus*. (a) The phylogenetic tree was estimated with a multimarker dataset formed with 35 nuclear markers from 31 *Eimeria* isolates derived from wild *Mus musculus* and three species of *Apodemus* (*A. agrarius*, *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 (Kvičerová 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).

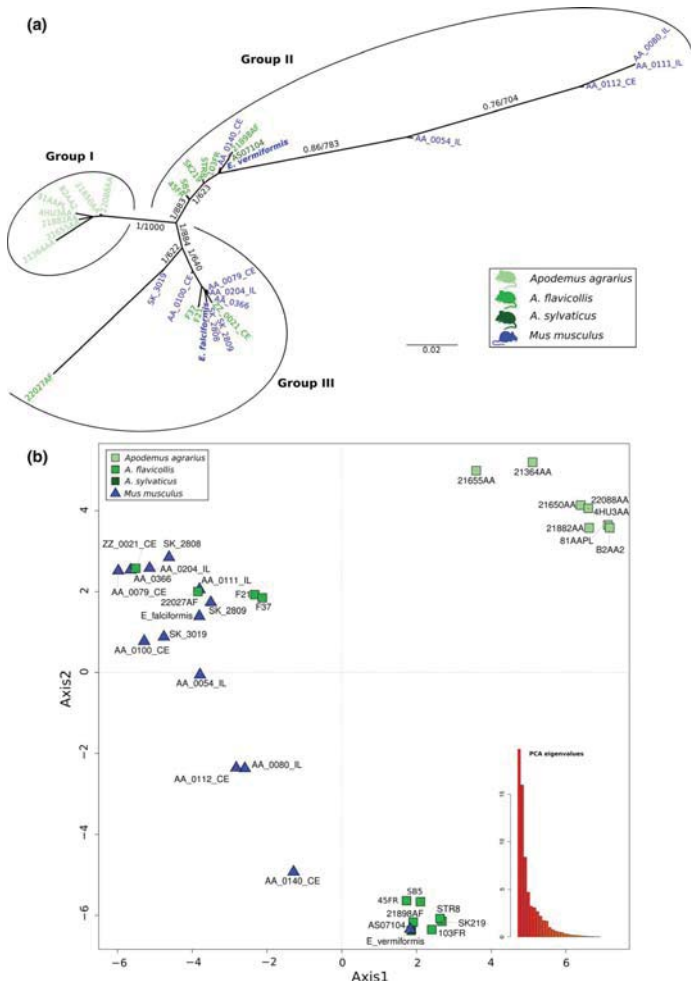

FIGURE 7 Apicoplast multilocus genotyping of *Eimeria* isolates from *Mus musculus* and *Apodemus*. (a) The phylogenetic tree was estimated with a multimarker dataset formed with 5 apicoplast markers from 31 *Eimeria* wild isolates derived from *Mus musculus* and three species of *Apodemus* (*A. agrarius*, *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. flavicolis;* 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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SUPPORTING INFORMATION

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

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

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Teaching and supervision

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

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

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List of publications:

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Jarquín-Díaz VH, Ballard A, Mácová A, Jost J, Roth von Szepeséla T, Berktold K, Tank S, Kvičerová 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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