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Phylogeny of coccidia and coevolution with their hosts

Ph.D. Thesis

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Annotation

The relationship among morphology, host specificity, geography and phylogeny has been one of the long-standing and frequently discussed issues in the field of parasitology. Since the morphological descriptions of parasites are often brief and incomplete and the degree of host specificity may be influenced by numerous factors, such analyses are methodologically difficult and require modern molecular methods. The presented study addresses several questions related to evolutionary relationships within a large and important group of apicomplexan parasites, coccidia, particularly *Eimeria* and *Isospora* species from various groups of small mammal hosts. At a population level, the pattern of intraspecific structure, genetic variability and genealogy in the populations of *Eimeria* spp. infecting field mice of the genus *Apodemus* is investigated with respect to host specificity and geographic distribution.

Declaration [in Czech]

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Jana Kvičerová

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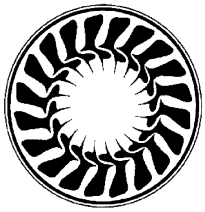


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List of papers and author's contribution

The thesis is based on the following papers:

I. **Kvičerová J**, Pakandl M, Hypša V, 2008. Phylogenetic relationships among *Eimeria* spp. (Apicomplexa: Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. Parasitology 135 (4), 443-452 (IF = 2.522).
Jana Kvičerová was responsible for DNA extraction, PCR, cloning, sequence assembling, phylogenetic analyses, and writing the manuscript.

II. **Kvičerová J**, Mikeš V, Hypša V, 2011. Third lineage of rodent eimerians: morphology, phylogeny and re-description of *Eimeria myoxi* (Apicomplexa: Eimeriidae) from *Eliomys quercinus* (Rodentia: Gliridae). Parasitology 138 (10), 1217-1223 (IF = 2.522).
Jana Kvičerová was responsible for microscopic examination of obtained faecal samples, morphological and morphometrical evaluation of coccidian oocysts, DNA extraction, PCR, cloning, sequence assembling, phylogenetic analyses, and writing the manuscript.

III. **Kvičerová J**, Ptáčková P, Modrý D, 2007. Endogenous development, pathogenicity and host specificity of *Eimeria cahirinensis* Couch, Blaustein, Duszynski, Shenbrot, and Nevo, 1997 (Apicomplexa: Eimeriidae) from *Acomys dimidiatus* (Cretzschmar, 1826) (Rodentia: Muridae) from the Near East. Parasitology Research 100 (2), 219-226 (IF = 1.812).
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IV. Jirků M, **Kvičerová J**, Modrý D, Hypša V, 2012. Phenotypic plasticity in coccidia (Apicomplexa) – striking morphological convergence in unrelated coccidia from related hosts: phylogeny of *Eimeria* spp. from African and Asian pangolins (Mammalia: Pholidota). Manuscript in preparation.
Jana Kvičerová participated in microscopic examination of faecal samples and histological sections, DNA extraction, PCR, cloning, sequence assembling, phylogenetic analyses, and revising the manuscript.

V. **Kvičerová J,** Hypša V, 2012. Extended set of *Eimeria* spp. indicates that eimerian host specificity is conserved due to adaptive rather than cophylogenetic processess. Manuscript in preparation.
Jana Kvičerová was responsible for obtaining samples, microscopic examination of faecal samples, morphological and morphometrical evaluation of coccidian oocysts, DNA extraction, PCR, cloning, sequence assembling, phylogenetic analyses, and writing the manuscript.

VI. Preliminary results for the population structure, host specificity and biogeography in *Apodemus* and *Eimeria*. Draft: **Kvičerová J**, Mácová A, Hypša V, 2012.
Jana Kvičerová was responsible for the study design, field sampling, microscopic examination of obtained faecal samples, morphological and morphometrical evaluation of coccidian oocysts, DNA extraction, PCR, sequence assembling, phylogenetic analyses, and writing the draft.

Contents

1. INTRODUCTION

1.1. Molecular insight into phylogenetic relationships, host specificity and morphology

Similar to other organisms, most features and processes in parasites can only be understood and interpreted in a phylogenetic/evolutionary context. While this approach had long been hampered by the lack of characters suitable for phylogenetic and genealogical reconstruction, an immense amount of molecular data has been accumulated during the last two decades. Based on the analyses of these data, many previously inaccessible questions in parasitology could be addressed or even solved, and some traditional views had to be changed or completely abandoned. For example, several parasite phyla or genera were relegated to different taxonomic groups (e.g. Microsporidia and *Pneumocystis* from protozoa to Fungi, Myxozoa from protozoa to Metazoa), relationships between some parasites and free-living, non-parasitic organisms were discovered (e.g. Apicomplexa and the photosynthetic alga *Chromera*, or Acanthocephala and Rotifera), or some artificially established parasite assemblages containing unrelated taxa were revealed (e.g. in eucestodes or protozoa) (Edman et al. 1988, Smothers et al. 1994, Winnepenninckx et al. 1995, Keeling and McFadden 1998, Kodedová et al. 2000, Miquelis et al. 2000, Brabec et al. 2006, Moore et al. 2008).

Early parasitological studies using methods of molecular biology were based predominantly on PCR detection of individual parasite species or simple phylogenetic analyses of a single gene, usually nuclear-encoded ribosomal RNA (Clark and Cross 1988, McCutchan et al. 1988, Jaureguiberry et al. 1990, Cai et al. 1992, Weiss et al. 1992, Putland et al. 1993, Awad-el-Kariem et al. 1994, Ellis et al. 1995). Since this region was eventually found to be quite conserved (and the phylogenetic information limited), other genes with higher degree of variability (mitochondrial, plastid, various protein-coding genes, or a combination of these) were adopted in such analyses. However, phylogenies based on a single gene may not match the correct phylogeny. The inclusion of more sequences/taxa into the data set, or less effectively, increasing the length of the sequences, can improve the accuracy and robustness of phylogenetic inference (Cao et al. 1994, Graybeal 1998, Whelan et al. 2001, Noda et al. 2012). Recently, combined (concatenated) analyses based on a set of several

different genes have been shown to provide more reliable information on evolutionary history and the genetic structure of parasites (Whelan et al. 2001, Gill and Fast 2006, Bartošová et al. 2009, Knapp et al. 2011). However, even the concatenation approach is sensitive to serious artifacts due to different evolutionary histories of individual genes. This problem, sometimes called "gene trees vs. species trees", is particularly critical at the low phylogenetic or even population level. It can be solved by adding multiple genes together with multiple individuals per species into the data set and analysing by modern MCMC-based Bayesian methods (programs BEAST or *BEAST) (Heled and Drummond 2010, Drummond et al. 2011).

In addition, results of such molecular analyses have also revealed significant incongruencies between morphology and phylogeny. Coccidia and Myxozoa provide typical examples of this trend. Whereas their genera and species are described based on oocyst/spore morphology, their phylogenetic relationships do not often reflect such classification (Smothers et al. 1994, Relman et al. 1996, Pieniazek and Herwaldt 1997, Andree et al. 1999, Kent et al. 2001, Modrý et al. 2004, Barta et al. 2005, Fiala 2006). This phenomenon could be caused either by incorrect phylogenetic reconstruction (when the obtained phylogeny does not reflect the true phylogeny due to various artifacts mentioned above and in section 1.4.), or by the homoplasy of morphological characters. The same problem of phylogenetic incongruency applies to some other biological traits. Among them, the host specificity (i.e. distribution of a parasite in a restricted taxonomic set of hosts) belongs to the most important and often discussed. This leads to the question of how host specificity in various parasite groups originates, evolves and is maintained, and also what are its main causes and consequences.

1.2. Host specificity

Host-parasite-environment interactions lead to the development of either susceptibility or resistance of a host species to a particular parasite taxa. This situation results in a characteristic pattern of parasite distribution in a more or less restricted group of hosts, generally called "host specificity". It is a complex interplay of at least 4 components that overlap each other (Duszynski 1986, Poulin 2007): 1) The parasite, its viability, fecundity, factors and modes of transmission (e.g. physical contact vs. ingestion). 2) The host and its attributes

(e.g. age, sex, body size, nutritional state, immune status, genetic constitution, social behaviour). 3) The ecosystem with its biotic & abiotic, geographic & ecological factors. 4) The coevolutionary process with its macro- and microevolutionary patterns.

An array of concepts and methods for expressing host specificity has been developed, based on a number of parasite individuals in a particular host species (Rohde 1980, Rohde and Rohde 2005), usage and availability of individual hosts (Lymbery 1989), phylogenetic relationships among hosts (Poulin and Mouillot 2003) or a combination of both ecologic and evolutionary aspects (Poulin and Mouillot 2005).

When exploring host specificity, we must be vigilant since this phenomenon poses several problems. It is generally known that some parasites are reported to be "highly/strictly host-specific", i.e. restricted to a single host species, whereas others are more flexible in their host requirements (on the genus-, family- or even class- level) (de Vos 1970, Pellérdy 1974, Duszynski 1986, Duszynski and Upton 2001, Hůrková et al. 2005, Poulin 2007). However, the observed degree of host specificity is influenced by numerous factors. First, high host specificity can be an artifact caused by inadequate sampling (Klompen et al. 1996, Poulin 1992, 1997) that depends on the frequency of the collection of a particular species. Second, a parasite with broader host range, able to exploit several species, may be adapted only to locally available hosts (and thus appears more "host-specific" since the host range is limited by their availability). Third, the incorrect identification of parasite species may also play an important role in assessing host specificity; in particular, many descriptions of parasite species were based merely on their host and a parasite found in a new host was often designated as a new species. Many of these "species" were eventually found to be conspecific; coccidia and helminths belong to the most typical examples (Pellérdy 1974, Higgs and Nowell 1991, Seville and Stanton 1993a, Wilber et al. 1998, Dallas et al. 2001, Bell et al. 2002, Hůrková et al. 2005).

In coccidia, another problem can arise due to their passive ingestion by a non-susceptible host. When a coccidium is found to occur in faeces of a particular host, two possible hypotheses should be taken into account: 1) this coccidium represents a real parasite of the host species, 2) it is just an occurrence of a random "passage" through the host; such a phenomenon is

typical for predators (a passage of coccidia of the prey item through the intestinal tract of their predators) and for geographically syntopic hosts with similar nutritional requirements (Duszynski 1986, Wilber et al. 1998, Zhao and Duszynski 2001a, Golemansky and Koshev 2009).

Moreover, some degree of "resistance to reinfection" exists in host organisms, mostly cell-mediated and correlated with host age. Coinfections and interactions with other microorganisms (bacteria, viruses, parasites), leading sometimes to cross-immunity, also play an indisputable role in the host specificity phenomenon (Desowitz 1957, Duszynski 1986, Behnke et al. 2005, Hang et al. 2010, Noland et al. 2010).

One of the methods allowing the assessment of the degree of host specificity is the transfer of a parasite to a new host under laboratory conditions (an experimental cross-transmission study). However, such an artificial process poses significant problems; the success rate of cross-transmission studies relies on many factors, e.g. the origin, strain, age/viability of the used parasite and above mentioned host attributes (section 2 of this chapter). The possibility that some negative results of these experimental studies can be due to adverse laboratory conditions must always be taken into account (Duszynski 1986). However, the most reliable current methods of studying host specificity and parasite distribution in different host taxa are based on molecular techniques combined with phylogenetic and population genetic data. These approaches have been successfully applied within all major groups of parasites, i.e. arthropods (Štefka and Hypša 2008), helminths (Nieberding et al. 2004, 2005, Brouat et al. 2011) and protists (Jenkins and Owens 2011, Rougeron et al. 2011, Salim et al. 2011).

1.3. Host - Parasite cophylogeny

Host-parasite associations represent suitable model systems for studying coevolutionary processes, when host and parasite lineages evolve and adapt together over a length of time (Price 1980, Brooks and McLennan 1993, Thompson 1994, Johnson and Clayton 2001, Timothy and Littlewood 2003, Poulin 2007). The main question in cospeciation studies is the extent to which cladogeneses of the two counterparts, host and parasite, are correlated (Brooks and McLennan 1991). An identity of host and parasite phylogenies often serves as a null hypothesis for evaluation of host-parasite coevolution. In reality, most

host and parasite phylogenies are more or less incongruent, so they mirror each other only imperfectly (Paterson and Banks 2001, Clayton et al. 2004). These incongruencies may be caused by the complex interplay of cophylogenetic events, such as cospeciation, host switching, sorting events and duplication (Clay 1949, Page 1994, 1995, 1996a, Paterson and Gray 1997, Paterson and Banks 2001).

Several methods of analysing cospeciation have been developed (e.g. Brooks´ parsimony analysis, reconciliation analysis, maximum likelihood methods, rates of evolution, molecular clock). These methods are topology-based and rely on topologies being robust enough and accurate (Brooks 1988, Page 1991, 1993, 1994, Huelsenbeck et al. 1997, Charleston 1998, Huelsenbeck et al. 2000).

Within last two decades, many studies of different cophylogenetic associations were carried out in this area. For example, associations between pocket gophers and their chewing lice (Page 1996a), birds and tapeworms (Hoberg et al. 1997), birds and feather mites (Dabert et al. 2001), ascourarid mites and megapodes (Proctor 1999), trematods and teleost fish (Jousson et al. 2000), field mice (*Apodemus sylvaticus*) and its nematode *Heligmosomoides polygurus* (Nieberding et al. 2004, 2005). In particular, much of the recent progress has been made in studies of lice and their hosts (Hafner and Nadler 1988, Barker 1994, Page 1996a, Page et al. 1996, Page et al. 1998, Paterson et al. 1999, Johnson and Clayton 2001, Banks et al. 2006, Š tefka and Hypša 2008). Interestingly, all possible scenarios of cophylogenetic events were described in lice-vertebrate hosts associations. For example, a strict cospeciation pattern was revealed for chewing lice and geomyid rodents (Hafner and Nadler 1988, 1990), while frequent host switches were strongly suggested in lice infecting rock wallabies in Australia (Barker 1991). The accidental occurrence ("straggling") of lice on an atypical host species was described by Ròzsa (1993) and Whiteman et al. (2004). The duplication event probably occurred in *Polyplax serrata* infecting field mice of the genus *Apodemus* (Štefka and Hypša 2008). In lice parasitizing birds, cospeciation, host switching and "missing the boat" represent the most common events (Paterson et al. 1993, 1999, Clayton et al. 1996, Johnson et al. 2002a, Weckstein 2004, Whiteman et al. 2004). On the contrary, host switching is

supposed to play a crucial role in the evolution of helminth parasites (Brant and Gardner 2000, Carney and Dick 2000, Hoberg et al. 2001, Perlman et al. 2003).

Compared to all of these studies, surprisingly small attention has been given to the coevolutionary relationships of protistan parasites. Possible cospeciation was proposed between microsporidians and their insect hosts (Baker et al. 1998). In apicomplexans, only a few studies have so far been published in this area, dealing with cophylogenetic associations within haemosporoid parasites and their hosts (Escalante and Ayala 1995, Escalante et al. 1995, Carreno et al. 1997, Ricklefs and Fallon 2002, Ricklefs et al. 2004), and between *Sarcocystis* and reptiles (Doležel et al. 1999, Šlapeta et al. 2003).

1.4. Inter- and intra- specific variability in parasites

Although most coevolutionary studies are based on phylogenetic-level analyses (for references, see section 1.3.), the genealogy and population structure of parasites appear to represent the key determinants in the coevolutionary, speciation and diversification processes (Nadler 1990, Brooks and McLennan 1993, Page and Holmes 1998, Banks and Paterson 2005, Brooks and Ferrao 2005, Leo et al. 2005).

The degree of host specificity may significantly influence intraspecific genetic structure (Johnson et al. 2002b). It is generally known that a high degree of polymorphism exists in parasite populations. Many parasites reported as polyxenous form assemblages of morphologically indistinguishable but genetically distinct species/strains (Jousson et al. 2000, Demanche et al. 2001, Štefka and Hypša 2008). A model of neutral evolution, depending only on the frequency of new mutations and probability of their fixation, serves as a null hypothesis for assessing genetic variability within a population. The real degree of polymorphism within a population varies due to the mutational rate and population size. However, closely related species can share a polymorphism that was inherited from a common ancestor – such phenomenon is called "ancestral polymorphism" and significantly influences the genealogical relationships within species.

Several methods are commonly used for analysing parasite relationships at a population level; classic methods of molecular phylogeny based on analyses of a single or multiple genes possessing a higher degree of variability (see section 1.1.), fragment length polymorphism-based methods (AFLP,

RFLP) or methods of population genetics (haplotype networks). However, each method offers both advantages and drawbacks.

Nuclear ribosomal DNA (rDNA) is the most commonly used marker for reconstructing phylogenies among and within many organisms (Hillis and Dixon 1991, Buckler et al. 1997, Avise 2004). Although intragenomic rDNA diversity is generally low due to the concerted evolution within ribosomal loci, divergent paralogues, pseudogenes and recombinants can sometimes emerge in a single genome. These phenomena influence phylogenetic analyses and can result in erroneous phylogenies (Sanderson and Doyle 1992, Buckler et al. 1997). For example, divergent rDNA paralogues and pseudogenes are common within internal transcribed spacers (ITS), so these regions are not suitable for reconstructing meaningful phylogenies or examining genetic diversity (Buckler et al. 1997, Alasaad et al. 2009). AFLP (Amplified Fragment Length Polymorphism) is a highly sensitive method for detecting polymorphisms in DNA, using restriction enzymes to digest genomic DNA and subsequently amplify and analyse selected fragments. However, it requires ultrapure parasite samples. The parsimony-based methods using haplotypes describe reticular relationships (networks, "star patterns") among individual sequences. Such an arrangement reflects both natural (recombinations) and methodical (uncertainty of reconstruction, difficulty with rooting) problems.

1.5. Model organisms

In this study, I use the largest genus of the phylum Apicomplexa, *Eimeria*, as a model group for addressing various questions connected to parasite speciation, host specificity and phylogeny. I focus mainly on the eimerian taxa associated with small mammals (especially rodents) since they represent easily obtainable hosts with relatively high prevalences of coccidia.

1.5.1. Apicomplexa: Eucoccidiorida

The protistan phylum Apicomplexa Levine, 1970 (Chromalveolata: Alveolata) is well-adapted to a parasitic strategy. Its members possess complicated life-cycles, usually formed by combination of both asexual and sexual reproduction. At least one of their developmental stages contain an

apical complex, the unique assemblage of organelles evolved for penetration into the tissues and cells of host organism.

Within Apicomplexa, members of the family Eimeriidae Minchin, 1903 (Conoidasida: Coccidiasina: Eucoccidiorida), comprising 17 genera, belong to the most abundant. They are usually homoxenous, excystating via Stieda bodies. The second largest family, Sarcocystidae Poche, 1913, is represented by heteroxenous coccidia excystating via 4 plates, and comprises 6 genera (Perkins et al. 2000). Since the last summarizing taxonomic review (Perkins et al. 2000), several genera have been cancelled or synonymized with others (e.g. *Atoxoplasma* became the junior objective synonym of *Isospora*; *Frenkelia* was proposed to be cancelled and synonymized with *Sarcocystis*), or new genera have been established or revived (e.g. *Acroeimeria*, *Choleoeimeria*, *Cystoisospora*, *Epieimeria*, *Goussia*) (Frenkel 1977, Dyková and Lom 1981, Overstreet et al. 1984, Carreno et al. 1998, Votýpka et al. 1998, Lainson and Paperna 1999, Mugridge et al. 1999, Franzen et al. 2000, Modrý et al. 2004, Barta et al. 2005).

The definition of coccidian genera is based on the morphology of infectious stages - sporulated oocysts, containing a fixed number of sporocysts, each possessing a constant number of sporozoites. However, this rigid definition is often in contradiction to results from molecular phylogeny (Relman et al. 1996, Pieniazek and Herwaldt 1997, Eberhard et al. 1999, Franzen et al. 2000, Jirků et al. 2002, Barta et al. 2005, Li et al. 2007).

The genus *Eimeria* Schneider, 1875 is the largest genus within coccidia, with more than 1700 described species. A majority of them parasitize the gastrointestinal tract of vertebrates. Several species (e.g. *Eimeria intestinalis*, *E. necatrix*, *E. stiedai*, *E. tenella, E. zuernii*) are important parasites of domestic animals that cause serious diseases with high morbidity and mortality. Sporulated oocyst contains 4 sporocysts, each filled with 2 sporozoites (Pellérdy 1974, Levine and Ivens 1990, Perkins et al. 2000).

1.5.2. Taxonomic pitfalls in coccidiology

The identification of *Eimeria* and *Isospora* species is based merely on the morphology and morphometry of sporulated oocysts (oocyst and sporocyst shapes and sizes, character and thickness of oocyst wall, presence/absence of oocyst and sporocyst structures - micropyle, micropyle cap, oocyst residuum,

polar granule/s, sporocyst residuum, Stieda body) (Pellérdy 1974, Levine and Ivens 1990, Duszynski and Wilber 1997). However, such a description and classification of coccidian species is insufficient and suffers significant pitfalls. Older descriptions (ca. 1890s-1960s) especially are often brief and inadequate, lacking important details of oocyst inner structures; in most cases, line drawings or photomicrographs of oocysts are absent (for examples, see Pellérdy 1974). Therefore, the validity of such descriptions remains debatable; many of these species have never been reported again and within the revisions, it turned out that species described from one host are identical with species described from another host - so they were united into a single species (Lewis and Ball 1983, Higgs and Nowell 1991, Seville and Stanton 1993a, Wilber et al. 1998, Hůrková et al. 2005). Second, the oocyst/sporocyst sizes vary within a single species during the patency (Duszynski 1971, Joyner 1982, Parker and Duszynski 1986, Gardner and Duszynski 1990, Upton et al. 1992, Seville and Stanton 1993b), thus do not represent a reliable discriminative trait. The oocyst size within a single species usually fluctuates 5-7 μm and 2-3 μm in sporocysts (for examples, see Pellérdy 1974, Wilber et al. 1998, Š lapeta et al. 2001, Hůrková et al. 2005, Golemansky and Koshev 2007). Therefore, it is often difficult to judge on the species identity of *Eimeria* oocysts present in the examined samples.

In several studies, however, other features (site of endogenous development, morphology of endogenous stages, sporulation time, prepatent and patent periods, pathogenicity and host specificity) were also utilized for taxonomy (Kartchner and Becker 1930, de Vos 1970, Pellérdy 1974, Long and Joyner 1984, Koudela et al. 2000, Šlapeta et al. 2001, Kvičerová et al. 2007).

Only a few species (mostly coccidia infecting rodents and domestic animals) have also been characterized using modern methods of molecular biology (Barta et al. 1997, Carreno et al. 1998, Hnida and Duszynski 1999a, b, Franzen et al. 2000, Ruttkowski et al. 2001, Š lapeta et al. 2001, Zhao and Duszynski 2001a, b, Kvičerová et al. 2008, 2011, Motriuk-Smith et al. 2009, Miska et al. 2010). These studies have shown that many morphological traits do not correlate with molecular phylogeny. For example, the genus *Isospora* is undoubtedly polyphyletic, scattered among *Eimeria* species (mammal-associated isosporan species on the base of eimerian topology/related to Sarcocystidae, bird-associated species split into 2 lineages, one scattered among

rodent eimerians and one related to fowl-*Eimeria* spp.) (Franzen et al. 2000, Jirků et al. 2002, 2009, Samarasinghe et al. 2008, Dolnik et al. 2009). Sporulated oocysts of *Isospora* spp. are morphologically quite uniform (2 sporocysts containing 4 sporozoites, usually spherical oocyst shape, smooth, thin and delicate oocyst wall, absent oocyst residuum) (Pellérdy 1974, Duszynski and Upton 2000). Nevertheless, the genus *Isospora* was divided into 2 separate genera according to phylogeny, host specificity and presence/absence of a Stieda body (SB): bird-associated *Isospora* (former *Atoxoplasma*) with SB belonging to Eimeriidae, and mammal-associated *Cystoisospora*, lacking SB belonging to Sarcocystidae (Carreno et al. 1998, Franzen et al. 2000, Barta et al. 2005). Recently, it seems that such a division is not entirely correct: several *Isospora* ("*Cystoisospora*") species described from insectivores (i.e. mammals) possess distinct SB (Duszynski and Upton 2000) – however, none have been sequenced yet. Sequences from these species could potentially bring new, surprising insight into isosporan phylogeny.

A similar taxonomic problem has emerged within eimerians. While the genus *Eimeria* is evidently polyphyletic, members of the so far monophyletic genus *Cyclospora* cluster within fowl-associated *Eimeria* (Relman et al. 1996, Pieniazek and Herwaldt 1997, Eberhard et al. 1999, Lopez et al. 1999, Li et al. 2007). However, only data on *Cyclospora* spp. from man, primates and dairy cattle are currently available, while inclusion of additional *Cyclospora* species from other hosts (e.g. insectivores or reptiles) may bring more surprises.

This situation poses a serious problem for future reclassification of this species-rich group of parasites. Undoubtedly, more samples and studies are essential for better understanding the diversity of coccidian parasites and their evolutionary history. A combination of molecular methods with data on morphology, host specificity and geographic distribution seems to represent the most reliable approach both for species determination and analysing the evolutionary relationships within coccidia.

1.5.3. Coccidia associated with rodents

Eimeria together with *Isospora* are the most frequent and common coccidian genera parasitizing rodent hosts (Pellérdy 1974, Levine and Ivens 1990). According to the latest review by Duszynski and Upton (2001), 415 *Eimeria* spp. and 40 *Isospora* spp. were reported from 280 rodent species (out

of >2200 extant species), which is certainly only a fragment of the real diversity within these hosts. Laboratory animals (namely *Mus musculus* and *Rattus norvegicus*) represent the best studied rodents for *Eimeria* species, with *Eimeria falciformis* (originally described from *Mus musculus* in 1870) as the type species of the genus *Eimeria*.

The epidemiological role of coccidian parasites still remains unknown in small mammals. Some species are highly pathogenic to their hosts (e.g. *E. chinchillae*), while others are not (e.g. *E. cahirinensis*) (de Vos and van der Westhuizen 1968, Kvičerová et al. 2007). Wild-living rodents are often infected with several species concurrently (so called multi-species infections) (Wash et al. 1985, Duszynski 1986, Hůrková et al. 2005, Golemansky and Koshev 2007). Host specificity restricted to certain species (*E. caviae*, *E. gundii*, *E. micromydis*), genus (*E. apodemi*, *E. saxei*, *E. scholtysecki*) or family (*E. beecheyi*, *E. callospermophili*, *E. larimerensis*) was reported in rodent *Eimeria* species; rarely, even the familial boundaries are crossed (*E. chinchillae*) (Todd and Hammond 1968a, b, de Vos 1970, Pellérdy 1974, Wilber et al. 1998, Čížkovská 2003, Hůrková et al. 2005).

As in many other groups of organisms, molecular techniques have brought new significant insight into the phylogeny, taxonomy and evolution of eimerian species. Such a first attempt to include molecular data into eimerian phylogeny was a study by Reduker et al. (1987), based on cladistic and phenetic analyses of isozyme banding patterns, sporulated oocyst morphology and life history traits. He realized that *Eimeria* species from the same rodent host, but with different oocyst morphology, were grouped into two separate lineages. Similar results, based on phylogenetic analyses of nuclear ITS1 sequences and riboprinting data, were recorded by Hnida and Duszynski (1999a, b). Subsequent analyses within a broader phylogenetic context proved that most of the biological and morphological features used to classify these parasites are phylogenetically inconsitent and taxonomically irrelevant (Eberhard et al. 1999, Zhao and Duszynski 2001a, Morrison et al. 2004, Matsubayashi et al. 2005, Kvičerová et al. 2008).

Interestingly, the presence or absence of an oocyst residuum (OR) in sporulated oocysts of *Eimeria* from rodent hosts corresponds well to the phylogenetic relationships among rodent *Eimeria* species (Zhao and Duszynski 2001a, b). Phylogenetic analyses based on plastid ORF 470 and nuclear

18S rDNA sequences placed 10 studied *Eimeria* species from rodents into 2 major lineages, corresponding to the morphology of their sporulated oocysts. Species in lineage A had spherical to subspherical oocysts, that did not differ much in size, and possessed OR. In contrast, eimerian species in lineage B were ovoidal or ellipsoidal, differed greatly in size, and lacked OR (Zhao and Duszynski 2001b). From data obtained from these studies, it seems that the morphological similarity of sporulated oocysts of *Eimeria* is more significant in reflecting evolutionary relationships than is host specificity.

However, further analyses of eimerian 18S rDNA sequences from more host taxa suggest that *Eimeria* spp. tend to form lineages specific to their host taxa (e.g. the fowl-, rabbit-, livestock-, porcine- and rodent- lineages) (Morrison et al. 2004, Matsubayashi et al. 2005, Kvičerová et al. 2008, Power et al. 2009). Interestingly, unlike other host-specific lineages, rodent *Eimeria* species were described to cluster into 2 (Zhao and Duszynski 2001a, b, Power et al. 2009), and recently appended to 3 (Kvičerová et al. 2011), distinct lineages. Since only a few of the described *Eimeria* species infecting rodents have been sequenced, it can be assumed that the real number of the rodent-specific *Eimeria* lineages may be even higher.

1.5.4. Mammalia: Rodentia

The diversification of mammals and evolutionary relationships among their major taxonomic groups (encompassing ~5400 living species described) have been a subject of exciting debates for decades. Fierce battles were waged among proponents of morphological, paleontological/fossil and molecular approaches (Catzeflis 1993, Graur 1993a, b, Novacek 1993). In the latest review, Meredith et al. (2011) seem to resolve the long-term puzzle regarding the relationships among mammalian families; a study based on 164 mammals, 5 outgroups and 26 gene fragments has yielded a well-resolved phylogeny, representing the first molecular phylogeny that incorporates all living mammalian families.

Rodents (Mammalia: Rodentia), encompassing more than 2200 extant species in 33 families, are the most diverse order among placental mammals (Wilson and Reeder 2005). Numerous studies have been published regarding rodent phylogeny and the position of the "rodent root", unfortunately without success. According to various morphological approaches (dentition, masticatory

apparatus, angle of the jaw, fetal membranes, middle ear features, arterial pattern), rodents were divided into suborders and families and the monophyly of order Rodentia was strongly supported (Bugge 1985, Lavocat and Parent 1985, Luckett 1993, Luckett and Hartenberger 1993, McKenna and Bell 1997). Based on the position of masseter muscles, rodents were split into 3 suborders: Hystricomorpha, Myomorpha and Sciuromorpha. Since this feature was found to be homoplasic, a new system was proposed, clustering rodents into 2 suborders – Hystricognathi and Sciurognathi - according to the position of incisors and the angle of the jaw. However, this classification did not reflect evolutionary relationships; moreover, several analyses revealed the paraphyly of the order Rodentia, whereas others supported their monophyly (Graur 1993a, b, D´Erchia et al. 1996, Adkins et al. 2001, Huchon and Douzery 2001, DeBry 2003, Montgelard et al. 2008). It is thus evident that morphological features alone are not sufficient for resolving rodent relationships and that the results of molecular phylogeny are determined by the range of taxon sampling and evolutionary model used (Luckett and Hartenberger 1993, Sullivan and Swofford 1997, Montgelard et al. 2008, Blanga-Kanfi et al. 2009). The latest review by Blanga-Kanfi et al. (2009), based on analyses of six genes and 41 rodent species, strongly supports the division of Rodentia into 3 clades: a squirrel-related clade (Sciuroidea and Gliridae), a mouse-related clade (Myodonta, Anomaluromorpha and Castorimorpha), and Ctenohystrica (Ctenodactylidae and Hystricognathi).

In coccidia, most of sequences and molecular studies on *Eimeria* infecting rodents are available from the mouse-related clade, namely Myodonta. Eimerians from the Ctenohystrica are completely lacking in GenBank, and only a few *Eimeria* sequences representing the squirrel-related host clade (namely the genera *Cynomys*, *Marmota*, *Sciurus*, *Urocitellus* and *Eliomys*) are available to date (www.ncbi.nlm.nih.gov).

2. OBJECTIVES

The main goal of this study is to investigate phylogenetic and genealogical relationships among various *Eimeria* species on both the interspecific and intraspecific levels, to assess the evolutionary history of eimerians as well as the intraspecific variability and population structure traits of the selected model system. The project combines techniques of field and laboratory parasitology/zoology, together with molecular, phylogenetic and coevolutionary approaches. The results will be used as the basis for evaluating the coevolution between coccidia and their hosts and the influence of host specificity on coccidian parasites.

The specific objectives are the following:

1. To extend the data set of *Eimeria* species for molecular and phylogenetic studies with species parasitizing different rodent families and other small mammal hosts (insectivores, rabbits, tree pangolin) and reconstruct their evolutionary relationships.
2. To evaluate the intraspecific variability and population structure of *Eimeria* species from field mice of the genus *Apodemus* (*Eimeria* - *Apodemus* model).
3. To compare morphological traits of sporulated oocysts of *Eimeria* species with results of molecular phylogeny (topology) and specify the features of phylogenetic and taxonomic significance.
4. To interpret observed patterns with respect to biology and evolutionary history of the hosts.

3. METHODOLOGY

Coccidian oocysts were obtained from fresh faeces or the gut content of host organisms. Rodents were trapped in the field using the Sherman live-traps or classic wooden traps, with official permissions. Faecal samples from insectivores, mole-rats and tree pangolin were obtained from already deceased animals.

Faecal samples were placed into 4% (w/v) potassium dichromate solution ($K_2Cr_2O_7$), allowed to sporulate on air for several days, and then stored at 4 ^oC. Oocysts of coccidian parasites were detected by the standard flotation technique with Sheather´s sucrose solution (sp.gr. 1.30) and light microscopy. An Olympus BX51 microscope equipped with the Olympus Camedia C-5060W camera and Quick Photo Pro v. 2.0 PC software was used for species-specific identification of oocysts. Morphological and morphometrical features were evaluated according to criteria suggested by Duszynski and Wilber (1997).

Genomic DNA of coccidia was extracted using commercial kits (Qiagen or MP Biomedicals). PCR reactions were performed at a 25 μl volume with HotStarTaq DNA polymerase (Qiagen). In total, 3 different genes were selected as suitable for amplification, sequencing and phylogenetic analyses: nuclear 18S rRNA (~1500 bp), plastid ORF 470 (~700 bp) and mitochondrial COI (~700 bp). Primers and PCR protocols were designed manually (18S rDNA) or adopted from publications by Zhao and Duszynski (2001b) (ORF 470) and Schwarz et al. (2009) (COI). PCR products were enzymatically purified and cloned into the pGEM–T Easy Vector (Promega). Plasmids were extracted by the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Sequencing of selected genes was performed by Macrogen, Inc. (Korea). Obtained sequences were identified by BLAST analysis (www.ncbi.nlm.nih.gov), manually adjusted using the SequenceScanner (Applied Biosystems), EditSeq and SeqMan (DNASTAR Inc.) programs, and deposited in the GenBank database (NCBI).

Alignments were created in MAFFT (Katoh et al. 2002, 2005) and BioEdit (Hall 1999) programs. Phylogenetic relationships were analysed using 3 principal approaches - maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), employing 3 different phylogenetic programs - PAUP (Swofford 2001), Phyml (Guindon and Gascuel 2003) and MrBayes (Huelsenbeck and Ronquist 2001). The most suitable evolution models were selected with the jModeltest program (Posada 2008, 2009). The trees were

visualized using TreeView (Page 1996b) and adjusted in Adobe Illustrator (Adobe Systems Inc.). The genealogy of eimerians from field mice was evaluated using the TCS program (Clement et al. 2000). More detailed descriptions of the methods and parameters are available in the individual publications.

4. RESULTS AND DISCUSSION

4.1. Coccidia in small mammals as model organisms and the current state of their phylogeny

Within coccidia, *Eimeria* species are among the best represented not only in morphological, but also in phylogenetic and evolutionary studies. Molecular data currently available in GenBank (www.ncbi.nlm.nih.gov) cover 98 *Eimeria* spp. infecting 41 host genera (including 1 environmental sample from wastewater).

Within the scope of this doctoral thesis, 3 different genes were selected as suitable markers for inference of phylogenetic relationships among coccidian parasites: nuclear 18S rRNA (commonly used in most of the available studies and reported from 83 of 98 *Eimeria* spp. deposited in GenBank), plastid ORF 470 and mitochondrial COI.

Over 1500 faecal samples from various host organisms (rodents, lagomorphs, insectivores, dogs, cats, horses, reptiles, birds) were examined by the standard flotation technique. In addition, I also aimed to enlarge the coccidian sequence data set with some rare samples, and samples from endemites (*Calotriton arnoldi*, *Castor fiber*, *Chaetophractus villosus*, *Eulemur albocollaris*, *Lagurus lagurus*, *Mus spretus*, *Pleurodeles waltl*, *Procavia capensis*), but unfortunately all of the obtained specimens were negative for coccidia. I managed to extend the molecular data with 79 coccidia spp. from 23 small mammal hosts from worldwide sampling and 125 sequences of selected genes. The following section summarizes the results and conclusions of the five studies.

Phylogenetic relationships among all valid eimerians (11 species) infecting rabbits (*Oryctolagus cuniculus*) are described in **Manuscript (MS) no. 1**. Despite the fact that the problematics of rabbit coccidia is quite "popular" since they are important pathogens, most of the studies deal with descriptions of endogenous life-cycles or precocious strains for vaccine development (Pakandl et al. 1996a, b, c, Pakandl and Jelínková 2006). Only sporadic data focused on molecular methods, namely PCR identification of individual *Eimeria* species (Ceré et al. 1995, 1996, 1997, Oliveira et al. 2011). However, it was never clear whether the rabbit-specific *Eimeria* species originated from different eimerian

groups by switching to rabbit or differentiatied only after their ancestor established an association with the rabbit (and perhaps a few related) hosts.

Surprisingly, MS no. 1 represents the first study published on rabbit coccidia in the field of molecular phylogeny and evolution. Based on analyses of nuclear 18S rDNA sequences, we have proved that all rabbit *Eimeria* species are monophyletic. This finding indicates that the speciation of rabbit coccidia occurred in a single host, or several closely related species. This monophyletic group, most related to bovine- and ovine- *Eimeria* spp., is further formed by 2 distinct lineages (MS no. 1, Fig. 1). An interesting aspect of the study is a lack of congruence between phylogeny and the bionomical traits of rabbit eimerians. A thorough comparison of phylogenetic relationships with morphological and biological traits (MS no. 1, Table 3) indicates that this two-lineage-clustering of rabbit coccidia clearly correlates only with the presence/absence of the OR (MS no. 1, Fig. 1). The inner arrangement of the OR+ lineage does not further reflect any morphological OR trait (e.g. compact globule vs. scattered granules vs. vacuoles). This finding is in contradiction to the conclusions of Barta et al. (1997), who observed a relatively high degree of phylogenetic congruence for some bionomical features (oocyst shape and size, site of infection and degree of pathogenicity) within a monophyletic lineage of fowl *Eimeria* species.

Interestingly, *Eimeria stiedai* evinces several remarkable peculiarities, both from the molecular and bionomical point of view: its 18S rDNA sequence (1345 bp) forms a relatively long branch with a ca. 100 bp long deletion, its endogenous development is located extraintestinally (in the bile ducts), it possesses an unusual OR structure and is able to also infect a different host genus (hares of the genus *Lepus*) (Pellérdy and Dürr 1970, Pellérdy 1974, Varga 1976, Entzeroth and Scholtyseck 1977, Scholtyseck et al. 1979, Eckert et al. 1995).

The original description of *E. myoxi* (Galli-Valerio 1940) does not provide the morphology of oocyst inner structures and is merely based on the oocyst shape and size. Therefore, subsequent findings of eimerian oocysts in glirid hosts were difficult to assign to particular species, causing many confusions and misinterpretations (Pellérdy 1974, Golemansky and Darwish 1993, Bertolino and Canestri-Trotti 2005).

Hence, the aim of the next study described in **Manuscript no. 2** was twofold: 1) to provide a detailed description (re-description) of *Eimeria myoxi*; 2) more importantly, to extend the available set of rodent-associated *Eimeria* species with coccidium from the squirrel-related clade, until now missing for reliable molecular analyses. We obtained and examined 54 faecal samples of *Eliomys quercinus* (garden dormouse), a critically endangered species in the Czech Republic. In 46 samples (85.2 %), a single coccidium species, morphologically similar to *Eimeria myoxi* and also sequentially quite uniform, was detected and characterized both morphologically and molecularly (MS no. 2, Figs. 1, 2, 3, 4).

Phylogenetic analyses based on nuclear 18S rRNA and plastid ORF 470 genes revealed an unstable position of *E. myoxi* within other eimerians. However, this instability was not due to the general lack of phylogenetic signal; other *Eimeria* species in the data set clearly clustered according to their host group (the rabbit-, poultry- and rodent- derived lineages), corresponding to previous studies by Morrison et al. (2004) and Matsubayashi et al. (2005). The most suprising finding was that *E. myoxi* (a coccidium lacking OR) did not fall into any of the 2 rodent-specific lineages. Moreover, it formed its own, independent lineage, representing a "third lineage" of rodent eimerians (MS no. 2, Figs. 3, 4). Since *E. myoxi* is the first representative of the "squirrel-related host clade" (Blanga-Kanfi et al. 2009), for which suitable phylogenetic data are now available, we propose that additional species from phylogenetically unexplored host taxa might lead to surprising results and undermine the concept of host-specific lineages within *Eimeria* species, as suggested by Relman et al. (1996), Pieniazek and Herwaldt (1997) or Zhao et al. (2001) (*Eimeria* species from bats clustering inside the "rodent-clade" and *Cyclospora* spp. inside the "fowl-clade").

A comprehensive species description, including detailed oocyst morphology, photomicrographs, prepatent and patent periods, sporulation time, complete endogenous development, pathogenicity and host specificity pattern, is reported in **Manuscript no. 3** on *Eimeria cahirinensis* from *Acomys dimidiatus* (Sinai Spiny Mouse). Oocysts of *E. cahirinensis*, previously described by Couch et al. (1997), were obtained from 3 different localities in

the Near East: south- and north- facing slopes (SFS and NFS) of "Evolution Canyon" in Israel, and Wadi Ramm (WR) in Jordan.

E. cahirinensis, infecting the duodenal and jejunal villi of spiny mice, seems to be only mildly pathogenic to its hosts. Even when infected by a large amount of sporulated oocysts (~300 000), no clinical signs of coccidiosis were observed, and only inflammatory infiltrate in the jejunal mucosa appeared in histological sections (MS no. 3, Fig. 3). This observation correlates with previous conclusions that *Eimeria* spp. developing within the intestinal villi are less pathogenic than those located within the crypts of enterocytes or in the cells of the lamina propria mucosae (Mesfin et al. 1978, Duszynski and Upton 2001, Šlapeta et al. 2001).

To assess the degree of host specificity, experimental cross-transmission studies were performed. *E. cahirinensis* was successfully transmitted to all 6 tested *Acomys* species, even those geographically (*A. cahirinus*, *A. cilicicus*, *A. wilsoni*) or phylogenetically (*A. russatus*) distant from the original host species. However, attempts to infect other rodent genera (*Apodemus*, *Gerbillus*, *Lemniscomys*, *Mastomys*, *Meriones*, *Mus*) or immunocompromised hosts (SCID mice) failed. This observation indicates that *E. cahirinensis* is likely to represent a genus-specific species.

Since this study does not include any molecular data, because these were obtained only after publication of the results, I added a **"Supplement to the MS no. 3"**, containing phylogenetic analyses based on nuclear 18S rDNA and mitochondrial COI sequences. All analyses placed *E. cahirinensis*, possessing OR, to the rodent-derived *Eimeria* lineage with OR (Supplement to the MS no. 3, Figs. 1, 2). These results further support the conclusions by Zhao and Duszynski (2001a, b) about the unexplained importance of OR in coccidian phylogeny. Compared to the original description by Couch et al. (1997), we found a difference in OR morphology in our isolates of *E. cahirinensis*; two distinct OR forms, a globule consisting of many small granules versus several smooth vacuoles, were observed. Interestingly, we noticed that the first OR type is typical for "young" oocysts (up to 15 days after faeces collection), whereas vacuoles only occur in "older" oocysts. Furthermore, we also revealed that several coccidia species (e.g. *E. citelli* from *Spermophilus citellus* and/or *Eimeria* n. sp. from *Habromys lophurus*) possess OR when young, but it

entirely disappears when older. Such a weird and unknown pattern may play a crucial role in the evolution of coccidia.

Two morphologically similar, but phylogenetically unrelated *Eimeria* species from ancient mammals (the Tree Pangolin *Phataginus tricuspis* and the Sunda Pangolin *Manis javanica*; Pholidota: Manidae), originating from two distant geographic areas (Africa, Angola and Asia, Singapore), are described and compared in **Manuscript no. 4**. Moreover, the eimerian found in *P. tricuspis* is designated here as a new species, *Eimeria nkaka* n. sp.

Members of the family Manidae, inhabiting forests of Central and Southern Africa and Southern Asia, represent a lineage of ancient placental mammals, most closely related to Carnivora (Meredith et al. 2011). They are on the brink of extinction due to hunting for both subsistence and commercial purposes. Only a single coccidian species has been so far reported from the entire order Pholidota, namely *Eimeria tenggilingi* described from *Manis javanica* by Else and Colley (1976). Thus, *E. nkaka* is a second described (and the first sequenced) coccidium from this host order and its detailed oocyst morphology together with phylogenetic relationships are provided in MS no. 4.

In MS no. 4, the oocysts of *E.* cf. *tenggilingi* and *E. nkaka* share a similar morphological feature, a relatively thick oocyst wall with rough and yellowish/brownish outer layer (MS no. 4, Fig. 1. A-D). The thick oocyst wall may represent an adaptation allowing for the high resistance of oocysts to severe environmental conditions in the tropics and their long-term viability outside the host.

Phylogenetic analyses based on 18S rDNA, ORF 470 and COI sequences yielded an unstable position of *E. nkaka*. In all analyses, *E. nkaka* clusters with *E. myoxi* from the garden dormouse as a sister lineage to fowl-associated eimerians, however always with low bootstrap support (MS no. 4, Figs. 2-4). Only 18S rDNA and COI sequences were successfully obtained from *E.* cf. *tenggilingi*, clearly unrelated to *E. nkaka*. In the 18S rDNA analyses, *E.* cf. *tenggilingi* clusters most closely to *E. pilarensis* described from the vespertilionid bat *Myotis ciliolabrum* (with low bootstrap support, Fig. 2). In the COI analyses, *E.* cf. *tenggilingi* clusters with the fowl *E. tenella* and *E. necatrix*, most probably due to the lack of other representative taxa in the COI

data set (only bird-associated *Isospora* species and *Eimeria* spp. from rabbits, fowl and 3 rodents are available, Fig. 3).

This study demonstrates a typical issue of the importance of representative sampling – while several host groups (e.g. rodents, poultry, livestock) are relatively well-studied in the field of coccidian phylogeny, other hosts (e.g. carnivore families except for Canidae and Felidae, or ancient mammals such as Xenarthra) are entirely missing in the data sets. Enrichment of the existing/present data set by *Eimeria* sequences from the closest relatives of Manidae (members of families Canidae, Eupleridae, Felidae, Herpestidae, Mephitidae, Mustelidae, Nandiniidae, Procyonidae, Ursidae and Viverridae) would certainly provide more insight into coccidian phylogeny. However, no coccidium has yet been described from some of these families (namely Eupleridae, Nandiniidae and Prionodontidae).

A rigorous study of the evolutionary relationships among phylogeny, host specificity and morphology is presented in **Manuscript no. 5.** It contains 86 new coccidian sequences (27 eimeriids from various rodent groups, involving 11 rodent genera from 8 families). Sequence data for another 81 specimens were retrieved from GenBank and incorporated into the analyses (MS no. 5, Table 1).

Recently published phylogenetic studies on coccidia suggest that the genus *Eimeria* is not monophyletic. A majority of *Eimeria* species tend to form several paraphyletic lineages, clustering according to their host organism (Morrison et al. 2004, Matsubayashi et al. 2005, Yabsley and Gibbs 2006, Kvičerová et al. 2008, Power et al. 2009). Rodent *Eimeria* species were supposed for a long time to be divided into 2 monophyletic but distinct lineages: the OR possessing and OR lacking lineages (Zhao and Duszynski 2001a, b). However, these samples only represented 3 rodent families - Cricetidae, Heteromyidae and Muridae – and were all collected on the North American continent (mostly USA). Nevertheless, a similar phenomenon regarding OR distribution was also observed in rabbit-associated *Eimeria* species (MS no. 1, Fig. 1). Thus, the discovery of a third rodent lineage formed by a single *E. myoxi* from the garden dormouse indicates that the situation might be much more complex (MS no. 2).

In all analyses provided in MS no. 5, the rodent *Eimeria* species are divided into several paraphyletic lineages, corresponding with the trait of possessing or lacking OR. In contrast to the study of Barta et al. (1997) - who found some correlations between topology, oocyst shape and size plus the site of infection in *Eimeria* spp. from domestic fowl - but similar to other studies (Zhao and Duszynski 2001a, b, MS no. 1), other morphological criteria do not fully correlate with the obtained phylogenies.

In MS no. 5, the pattern revealed by individual analyses of 18S rRNA, ORF 470 and COI genes was compatible with results obtained by analysis of a concatenated data set and yielded well-resolved phylogenies. This study confirms previous suggestions that eimerians are not a monophyletic group, and indicates that the host specificity plays a much weaker role in eimerian phylogeny than has been believed so far. It seems that with an increasing number of available taxa, phylogenetic relationships become less host-dependent. Similarly, the geographic origin of samples included in the analyses did not show any phylogenetically consistent pattern. However, the concatenated tree also demonstrates the issue of insufficient sampling; there are still several taxa lacking a robust phylogenetic position (e.g. eimerians from the tree pangolin, garden dormouse, ferret or marsupials) (MS no. 5, Fig. 2).

The sporocyst excystation structures (Stieda body vs. plates) and presence/absence of OR were recently reevaluated by phylogenetic methods as taxonomic markers for clustering of coccidian species (Zhao and Duszynski 2001a, b, Jirků et al. 2002, MS no. 1). However, this pattern is not absolute. For instance, mammal *Isospora* species were reported to lack SB (excysting via 4 plates) and to be phylogenetically related to family Sarcocystidae, therefore transferred to a separate genus *Cystoisospora* (Jirků et al. 2002, Barta et al. 2005). It is pertinent to stress that so far, only 10 *Isospora*/*Cystoisospora* species from mammals (mainly cats and dogs) out of >130 described species have been sequenced. However, according to comprehensive descriptions including photomicrographs, several *Isospora* species infecting mammals (namely *I. brevicauda*, *I. condylurae*, *I. cristatae*, *I. lamoillensis*, *I. neurotrichi* and *I. palustris* – parasites of moles and shrews) evidently possess conspicuous SB (Duszynski and Upton 2000). In MS no. 5, some new sequences of mammal isosporans (namely *Isospora* sp. from *Apodemus flavicollis* and *Isospora* spp. from *Talpa europaea*) cluster clearly within the family Eimeriidae, not

Sarcocystidae. Therefore, it is evident that adding more sequences of *Isospora* spp. from other hosts could potentially bring new, surprising insight into isosporan phylogeny.

Another counterexample is represented by *Eimeria rioarribaensis* from bats, always clustering within the uniform lineage of OR+ rodent eimerians, but clearly lacking this structure (Duszynski et al. 1999). Since it appears that OR can be present in the oocyst of certain coccidium species but changes its structure or completely disappears after time (Kartchner and Becker 1930, MS. no. 3), this phenomenon may apply also to this species. In fact, virtually nothing is known about the importance and function of this curious structure, which may play a significant role in coccidian evolution.

4.2. *Eimeria - Apodemus* model

A taxonomically and methodologically suitable complex host-parasite system was designed for the following study. It is represented by rodents of the genus *Apodemus* (field mice) and a protistan parasite of the genus *Eimeria*.

Altogether, 44 coccidia samples (43 *Eimeria* and 1 *Isospora*) from *Apodemus* spp. were gathered for the analyses of population structure (**Draft no. 1**). Analysed material was retrieved from 3 host species (*Apodemus agrarius*, *A. flavicollis* and *A. sylvaticus*) sampled across Europe (Czech Republic, England, France, Germany, Italy, Macedonia and Slovak Republic) (Draft no. 1, Fig. 1, Table 1). These 3 species often live in sympatry. The mitochondrial gene for cytochrome c oxidase subunit I (COI) was selected as the most suitable genetic marker for such an analysis in coccidia. This gene has previously been successfully applied to resolve intraspecific variability within fowl *Eimeria* species (Schwarz et al. 2009). Analyses were performed using both phylogenetic approaches and methods of population genetics.

The current state of knowledge on coccidia provides only limited information on intraspecific structure and the significance of both host-preference and geography. For example, Hnida and Duszynski (1999b) did not find any intraspecific variability within multiple isolates of 4 rodent *Eimeria* species of different geographic origin. On the contrary, a notable genetic variation between strains of chicken *Eimeria* species was described by Barta et al. (1998), Lew et al. (2003) and Blake et al. (2004).

Indication of a possible intraspecific pattern was already noted in MS no. 5. Against expectation, the more detailed analyses revealed great phylogenetic diversity of 11 *Eimeria* samples obtained from the genus *Apodemus*. While the exact taxonomic status of the analysed samples and their precise position could not be entirely clear from the available topologies, they evidently clustered at least at 4 different places in the tree and covered quite a large phylogenetic span (MS no. 5, Fig. 2).

In Draft no. 1, phylogenetic analyses of the COI gene of 43 *Eimeria* specimens from field mice reveal 6 previously unrecognized lineages, differing strikingly in their host distribution, degree of host specificity, and population sizes (Draft no. 1, Fig. 2). In contrast, only 4 *Eimeria* species (*E. alorani*, *E. apionodes*, *E. jerfinica* and *E. kaunensis*) (Musaev and Veisov 1965, Pellérdy 1974, Arnastauskiene et al. 1978, Hůrková et al. 2005) are distinguishable based on morphological and morphometrical features of sporulated oocysts (Draft no. 1, Figs. 1, 2, Table 2). Populations of *Eimeria* spp. from field mice are structured only according to one of the studied components, the host species. The geographic origin of individual isolates does not seem to play a significant role (Draft no. 1, Fig. 3, Table 1). As expected, the single sequence of *Isospora* sp. formed a distant, separate branch in both phylogenetic tree and TCS haplotype network.

Draft no. 1 represents the first study at a population level on *Eimeria* spp. infecting hosts in the wild and may have important epidemiological and evolutionary implications.

5. CONCLUSIONS AND FUTURE PROSPECTS

The availability of detailed morphological descriptions together with informative molecular data on a representative set of species is a prerequisite for any meaningful analysis of coccidian diversity and evolution. However, while hundreds of coccidia species have so far been described from various taxonomic groups of mammals (Pellérdy 1974, Levine and Ivens 1990), their descriptions as well as subsequent reports are often incomplete and do not allow for their comparison. Thus, it is extremely difficult to decide on the identity of individual coccidian species (whether two morphologically similar or even indistinguishable coccidian oocysts really represent two distinct species) and the degree of their host specificity. Apparently, except for the availability of a representative taxonomic sample of the host, another serious problem rests in the knowledge of the eimerian diversity within a single host genus or species. Taken together, "there is an enormous lack of information regarding the occurrence of coccidia in most host groups, not because they are not there, but because we have not made a concerted effort to look for them." (Duszynski et al. 2007).

Results based on molecular techniques (namely DNA extraction and PCR) may be influenced by numerous factors; e.g. base composition (GC content), secondary structure, amplicon size, copy numbers, involvement of potential inhibitors, but also by such factors as PCR reagents (polymerase, buffers) and parameters of the PCR reaction (temperatures and times). In phylogeny, results of the analyses depend on the gene/s selected for the study and its/their informativeness, alignment parameters and adjustment, phylogenetic approach used (e.g. MP vs. ML vs. BI) and evolutionary model selected (Buckler et al. 1997, Whelan et al. 2001). Therefore, the results of molecular phylogeny may vary considerably according to the above mentioned factors and approaches used, and thus should be interpreted with caution.

Nowadays, population-genetics, genomics and proteomics approaches (microsatellites/STRs, minisatellites/VNTRs, AFLPs, SNPs, ESTs) in parasitology have rapidly developed, allowing even more comprehensive analyses (Su and Wellems 1996, Cacciò et al. 2000, Chigagure et al. 2000, Shirley et al. 2004, Elsheikha et al. 2006, Höglund et al. 2006, Simo et al. 2008, Blaxter et al. 2011, Caballero et al. 2011, Freitas et al. 2011, Xie et al. 2011, Liu et al. 2012). However, some of these methods (e.g. AFLP) cannot be

applied for coccidians, since they require ultrapure parasite samples. Unlike helminths or arthropods, which are macroscopic and easily collected, life-cycle stages of microscopic unicellular coccidia occur in host faeces or tissues, so it is almost impossible to obtain ultrapure material.

Recently, an advanced molecular technique, real-time PCR (qPCR) has started to be used in "coccidiology" (Blake et al. 2008, Morgan et al. 2009). It is a sensitive assay enabling both quantification and identification of different *Eimeria* species present in hosts with mixed-species infections, irrespective of the life-cycle stage or the presence of other pathogens. This method could therefore represent an advance over traditional microscopic techniques. It was successfully applied to pure strains of chicken *Eimeria* species (Blake et al. 2006, 2008, Swinkels et al. 2006, 2007, Morgan et al. 2009). However, the qPCR method requires large amounts of sporulated oocysts/DNA, so it might be difficult to employ it for coccidia of wild-living or even endangered host species.

Despite the above mentioned difficulties, coccidia represent easily available material, obtained by non-invasive techniques (oocysts are present/discharged in host faeces), and are therefore suitable model organisms for scientific research.

For the future, I intent to enlarge the COI sequence data set for population studies of *Eimeria* spp. from *Apodemus* species with more samples throughout Europe (~ 50 additional samples) and compare the genealogical structures between *Eimeria* spp. and their hosts. A similar study evaluating the intraspecific variability and population structure of coccidia will be performed also on the *Eimeria* - *Microtus* model. Patterns obtained within *Eimeria*-rodent host systems will be compared with results obtained by analyzing "lower apicomplexans", haemogregarines, namely the intracellular blood parasites *Hemolivia mauritanica*, infecting tortoises of the genus *Testudo*.

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Manuscript No. 1:

Kvičerová J, Pakandl M, Hypša V (2008) Phylogenetic relationships among *Eimeria* spp. (Apicomplexa: Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. *Parasitology* 135 (4): 443-452.

Abstract

Monophyly of all 11 valid *Eimeria* species from rabbits (*Oryctolagus cuniculus* Linnaeus, 1758) was revealed based on nuclear 18S rDNA sequence data. This finding implies that these species, which vary considerably in terms of their morphology and biology, diversified on a single host or several closely related species. Phylogenetic analysis divided rabbit *Eimeria* species into 2 sister lineages, corresponding to the presence/absence of the oocyst residuum. Other morphological or biological traits (oocyst shape and size, presence/absence of oocyst inner structures, pathogenicity, infection site, pre-patent and patent periods, sporulation time, and number of asexual generations) do not explicitly correlate with the phylogeny of rabbit coccidia.

Manuscript No. 2:

Kvičerová J, Mikeš V, Hypša V (2011) Third lineage of rodent eimerians: morphology, phylogeny and re-description of *Eimeria myoxi* (Apicomplexa: Eimeriidae) from *Eliomys quercinus* (Rodentia: Gliridae). *Parasitology* 138 (10): 1217-1223.

Abstract

Coccidian oocysts from feces of 46 individuals of the garden dormouse, *Eliomys quercinus* (Rodentia: Gliridae), were morphologically and molecularly characterized. Both morphological and sequence data (18S rDNA and ORF 470) showed low variability, indicating that all samples represent a single species. By comparison with published morphological descriptions of coccidia from glirid rodents, we determined that the samples represent *Eimeria myoxi*. Molecular data suggest that this species does not fall within the 2 known rodent-specific groups but branches as a third independent lineage. However, its exact position in respect to other eimerian clusters could not be established due to the lack of phylogenetic information at this taxonomic level for the 18S rRNA and ORF 470 genes. Based on these results, we provide a redescription of *Eimeria myoxi*, which contains morphological and molecular characteristics sufficient for its further unequivocal identification.

Manuscript No. 3:

Kvičerová J, Ptáčková P, Modrý D (2007) Endogenous development, pathogenicity and host specificity of *Eimeria cahirinensis* Couch, Blaustein, Duszynski, Shenbrot, and Nevo, 1997 (Apicomplexa: Eimeriidae) from *Acomys dimidiatus* (Cretzschmar, 1826) (Rodentia: Muridae) from the Near East. *Parasitology Research* 100 (2): 219-226.

Abstract

Eimeria cahirinensis Couch et al. 1997 was found in faecal samples of *Acomys dimidiatus* from three different localities in the Near East. Twenty-two of 104 (21 %) *A. dimidiatus* trapped on both the south- and northfacing slopes of "Evolution Canyon", Lower Nahal Oren, Mt. Carmel, Israel in August 2001 and 2002 were infected with *E. cahirinensis*. Oocysts were also obtained from a single individual of *A. dimidiatus* trapped in Wadi Ramm, Jordan in the summer of 1999. Laboratory-reared spiny mice (*Acomys* spp.) were inoculated to determine the prepatent and patent period, sporulation time, site of infection, immunogenicity, pathogenicity, pathology and morphology of endogenous stages of *E. cahirinensis*. Both asexual and sexual stages were localised in the apical part of duodenal and jejunal villi. An experimental inoculation of representatives of several rodent genera revealed the host range of *E. cahirinensis* to be limited to the genus *Acomys*.

Supplement to the MS no. 3

Additional molecular data were obtained for the taxa studied in the Manuscript no. 3 after the MS was accepted for publication. Here I attach summary of the new data and analyses as a Supplement. The 18S rDNA tree is shown below in Fig. 1, the tree obtained by analysis of a concatenated matrix is a part of the MS no. 5 (Fig. 2).

Materials and methods

Molecular approaches and phylogenetic analyses

Genomic DNA of *E. cahirinensis* (samples from all 3 localities - NFS, SFS and WR) was extracted using the standard phenol-chloroform technique. PCR reactions were performed at a 25 µl volume with HotStarTaq DNA polymerase (Qiagen). Two different genes were amplified and sequenced: nuclear 18S rRNA (~1500 bp) and mitochondrial COI (~700 bp). Primers and PCR conditions were designed manually (18S rDNA) or adopted from a publication by Schwarz et al. (2009) (COI). PCR products were enzymatically purified and cloned into the pGEM–T Easy Vector (Promega). Plasmids were extracted by the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Sequencing of selected genes was performed by Macrogen, Inc. (Korea). Obtained sequences were identified by BLAST analysis (www.ncbi.nlm.nih.gov) and manually adjusted using the SequenceScanner (Applied Biosystems), EditSeq and SeqMan (DNASTAR Inc.) programs.

Alignments were created in MAFFT and BioEdit programs (Hall 1999, Katoh et al. 2002, 2005). Phylogenetic relationships were analysed using 3 principal approaches - maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI), employing 3 different phylogenetic programs – PAUP v. 4.0b10 (Swofford 2001), Phyml v. 2.4.3 (Guindon and Gascuel 2003) and MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Most suitable evolution models were selected with jModeltest program (Posada 2008, 2009). The trees were visualized using TreeView v. 1.6.6 (Page 1996b) and adjusted in the Adobe Illustrator CS5 v. 15.0 (Adobe Systems Inc.).

Since the single-gene COI analyses did not provide reliable phylogenies due to the unsufficient sampling, we used a concatenated (18S rDNA + COI) matrix to include the COI information into the phylogenetic analysis. Therefore, two phylogenetic trees (18S rDNA and a concatenated tree) are provided in this Supplement (Figs. 1, 2).

Results

Molecular characterization of sequences of *Eimeria cahirinensis*.

Partial sequences of two genes were obtained for this eimerian species from each locality.

E. cahirinensis NFS:
Nuclear 18S rDNA: total length 1517 bp, GC content of 47 %.
Mitochondrial COI: total length 755 bp, GC content of 36 %, 251 amino acids.

E. cahirinensis SFS:
Nuclear 18S rDNA: total length 1500 bp, GC content of 47 %.
Mitochondrial COI: could not be amplified.

E. cahirinensis WR:
Nuclear 18S rDNA: total length 1426 bp, GC content of 47 %.
Mitochondrial COI: total length 679 bp, GC content of 35 %, 226 amino acids.

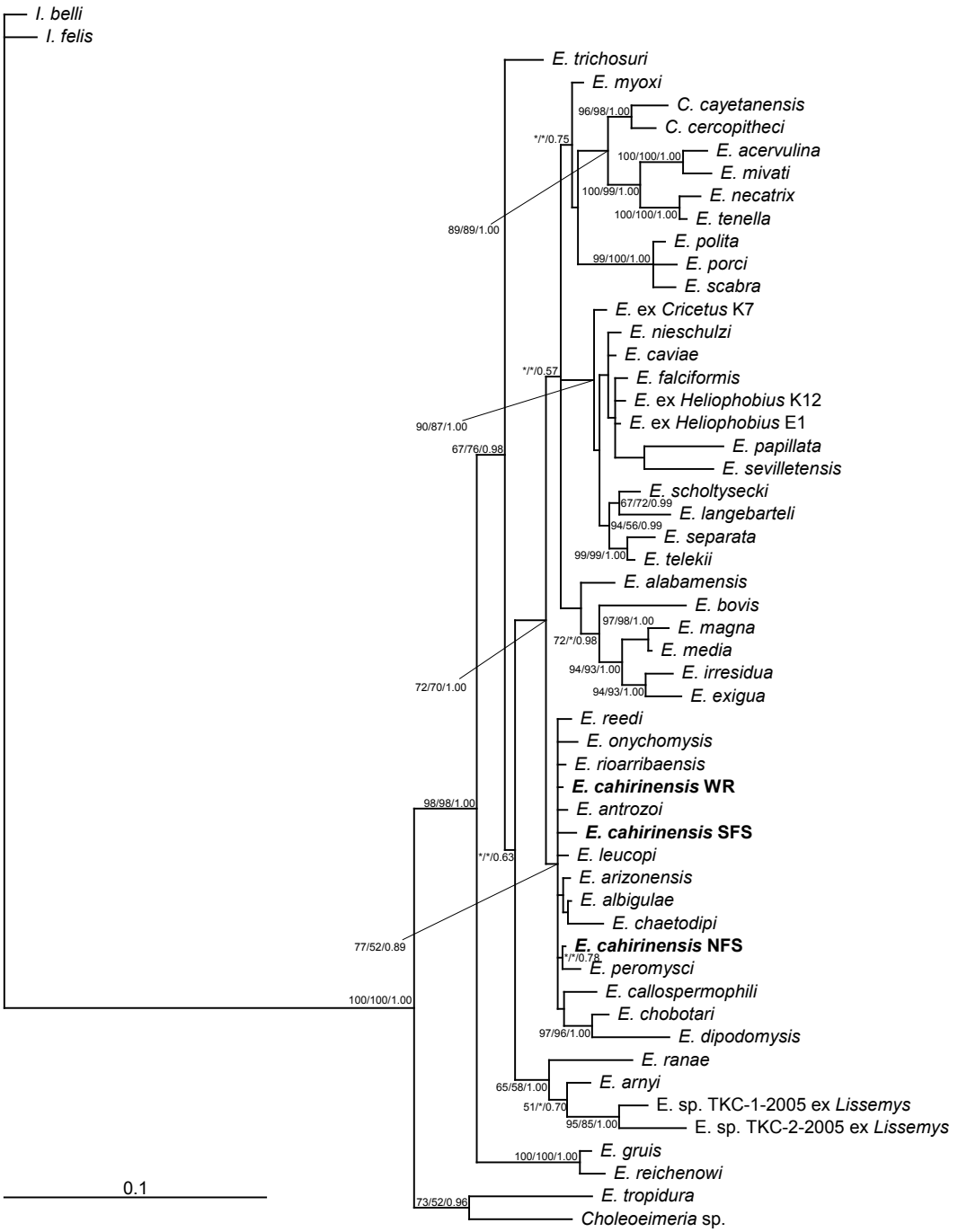

Fig. 1. Phylogenetic tree of the 18S rDNA obtained by BI. The tree is rooted with *Isospora belli* and *I. felis*. Numbers at the nodes show bootstrap values for ML and MP, and posterior probability under BI (the values are provided only for the nodes also present in ML and MP trees). Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are marked with asterisk (*).

Manuscript No. 4:

Jirků M, **Kvičerová J**, Modrý D, Hypša V (2012) Phenotypic plasticity in coccidia (Apicomplexa) - striking morphological convergence in unrelated coccidia from related hosts: phylogeny of *Eimeria* spp. from African and Asian pangolins (Mammalia: Pholidota). In preparation.

Phenotypic plasticity in coccidia (Apicomplexa) - striking morphological convergence in unrelated coccidia from related hosts: phylogeny of *Eimeria* spp. from African and Asian pangolins (Mammalia: Pholidota)

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Abstract

Two morphologically similar, but phylogenetically unrelated *Eimeria* species from ancient mammals (the Tree Pangolin *Phataginus tricuspis* and the Sunda Pangolin *Manis javanica*; Pholidota: Manidae), from two distant geographic areas (Africa, Angola and Asia, Singapore), are characterized and compared both morphologically and molecularly. The evolutionary relationships of these coccidia in respect to other eimerian groups are discussed. Phylogenetic analyses suggest the unstable topology of both *Eimeria* species within other eimerians. While their precise position can not be established from the available topologies due to the lack of related taxa, it is evident that both *Eimeria* species do not fall into any of the so far recognized eimerian lineages. Moreover, an eimerian found in *P. tricuspis* is described here as a new species, *Eimeria nkaka* n. sp.

Key words: coccidia, *Eimeria*, pangolin, oocyst morphology, geographic origin, phylogenetic relationships.

Introduction

Within the apicomplexan subclass Coccidiasina, the taxonomically most diverse order Eucoccidiorida (commonly known as "coccidia") includes plethora of families and genera with unclear relationships. This situation is reflected by numerous taxonomic misinterpretations and rearrangements that occur throughout the literature (Tenter et al. 2002). Due to a coincidence of high diversity and limited data available for molecular analyses, surprisingly little is known about phylogenetic relationships among coccidians. One of the few patterns in coccidian phylogeny, recognized by several authors but seldom discussed (e.g. Morrison et al. 2004), is a monophyly of coccidians possessing unique structure in their sporocyst wall, the Stieda body (SB) (Jirků et al. 2009a, b). In all analyses, the SB-bearing taxa appear as a bulk of homoxenous coccidia infecting mostly homeothermic vertebrates. They include several genera traditionally characterized by number of sporocysts and sporozoites per oocyst. In all analyses encompassing sufficiently wide array of taxa, the most speciose genus *Eimeria* seems paraphyletic with SB-bearing members of several other genera (*Caryospora*, *Cyclospora* and *Isospora* from birds, i.e. *Atoxoplasma*) clustering among *Eimeria* species (Morrison et al. 2004, Matsubayashi et al. 2005, Jirků et al. 2009b). Although such pattern calls for taxonomic rearrangements, the relatively small size and marked bias of available data sets make any taxonomic changes premature. For example, over 860 *Eimeria* species have been described from mammalian hosts (Duszynski and Upton 2001), whereas only 56 nuclear 18S rDNA sequences and even fewer sequences of other genes (e.g. 23S rRNA, ORF 470, ITS, COI, Hsp 90) are available in the GenBank database (NCBI). Due to their medical and veterinary importance, coccidia parasitizing man (and other primates) and domestic animals (mainly rabbit and chicken) received main attention. The other relatively well-sampled host groups are only rodents and bats. As a result, phylogenetic knowledge on the most diverse apicomplexan order is based on very incomplete sampling.

Several morpho- and biologically peculiar lineages of homeothermic vertebrates are particularly interesting from the evolutionary point of view. These various groups mostly share a relatively low diversity of extant forms with rather restricted distributions and a status of surviving representatives of

ancestral taxa that were much more widespread and diversified in the more or less distant geological past. Among mammals, such lineages include for example marsupials, anteaters, sloths, tenrecs, sirens, pangolins, elephant shrews and other groups, which are rather marginal in terms of diversity. Importantly, these groups often represent either unique radiations, such as marsupials, or ancestral sister lineages of speciose extant taxa - such a relationship is for example between pangolins (Pholidota) and Carnivora (Meredith et al. 2011). Coccidia parasitizing these distinct hosts are of a special interest from the phylogenetic point of view. Their molecular characteristics may provide missing information allowing for better resolution among SB-bearing lineages as well as better understanding to their diversity and evolution. To date, this issue has only been addressed by Power et al. (2009), who suggested coevolution of SB-coccidia with higher-level taxa of hosts by the analysis of a marsupial coccidium.

Eight extant species of pangolins or scaly anteaters (Pholidota: Manidae) represent unique ancestral Laurasian lineage of mammals, forming a sister group of the Carnivora. All extant representatives of Pholidota are restricted to the Old World tropics (Arnason et al. 2002, Amrin-Madsen et al. 2003, Springer et al. 2004, Gaudin et al. 2009, Agnarsson et al. 2010, Yu et al. 2011). Four species representing two genera (*Phataginus*, *Smutsia*) occur in sub-Saharan Africa, while another four species belonging to the genus *Manis* occur in Oriental realm. All species of Pholidota are progressively getting rare due to the large-scale hunting for both subsistence and commercial purposes, and are therefore listed in appendix II by CITES. Only a single coccidian species is known from the entire group, namely *Eimeria tenggilingi* Else et Colley, 1976, described from Sunda Pangolins *Manis javanica* from Malay Peninsula.

In the present work, we provide phylogenetic analyses of two morphologically similar *Eimeria* species from African and Asian pangolins using three molecular markers. We show striking morphological convergence of unrelated coccidia from phylogenetically and biologically close, but biogeographically distant hosts. In addition, we describe the African species as new to science.

Materials and Methods

Sample collections and treatment, oocyst morphology

Samples of intestinal contents and tissues were obtained from a single adult African Tree Pangolin or African White-bellied Pangolin *Phataginus tricuspis* (Rafinesque, 1821), snared by local people. The animal originated from environs of the village Kungutadi in Mayombe forest in Cabinda province, Angola, 4°42'31.76"S, 13° 0'52.14"E. A sample of colon contents was preserved in 2.5% (w/v) potassium dichromate solution ($K_2Cr_2O_7$) and tissue samples from the stomach, duodenum, jejunum and colon were fixed in 10% buffered formalin. For histology, the formalin-preserved tissues were embedded in paraffin, sectioned at 6 μm, stained with haematoxylin-eosin (H&E) and mounted in Canada balsam.

Comparative material of *Eimeria* cf. *tenggilingi* was obtained from faecal samples of captive, wild-originating Sunda Pangolins *Manis javanica* Desmarest, 1822, generously provided by Wildlife Reserves Singapore. Oocysts were detected in huge numbers in samples from 1 out of 5 examined animals. Although only unsporulated oocysts were available for the study, their dimensions (18.0-20.0 × 17.5-19.5 μm) and typical character of the oocyst wall (Fig. 1. C, D) suggest this coccidium to represent *E. tenggilingi*. Oocysts of *E.* cf. *tenggilingi* used in this study are preserved in absolute ethanol and deposited at the protistological collection of the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, under accession number IPASCR ProtColl 18.

Oocysts concentrated by flotation and histological sections were examined by light microscopy using an Olympus AX70 microscope equipped with Nomarski interference-contrast optics (NIC, used for oocysts only). Morphological and morphometrical features were evaluated according to Duszynski and Wilber (1997).

Molecular techniques and phylogenetic analyses

Genomic DNA of coccidia was extracted from oocysts isolated from the intestinal content by the standard phenol-chloroform procedure. Nuclear 18S rRNA (~1400 bp), plastid ORF 470 (~450 bp) and mitochondrial cytochrome c oxidase subunit I (COI; ~770 bp) genes were amplified by PCR using specific

primers and protocols described by Zhao and Duszynski (2001), Kvičerová et al. (2008), and Schwarz et al. (2009), and sequenced on an automatic 3730XL DNA analyzer (Macrogen Inc., Korea). Sequences were identified by BLAST analysis, adjusted using the DNASTAR program package (DNASTAR Inc.) and deposited in the GenBank database (NCBI) under the Accession numbers xx-xx. Alignment of 18S rDNA was created with the MAFFT v. 6 program (Katoh et al. 2002, 2005) using the G-INS-i algorithm with default parameters, and then manually adjusted in the BioEdit program (Hall 1999). Sequences of the ORF 470 and COI genes were aligned and manually adjusted in the BioEdit program (Hall 1999) in the aminoacid mode. The alignments were then switched to nucleotide mode and used for analyses. Three different phylogenetic approaches were employed for analyses – maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) – using the programs PAUP v. 4.0b10 (Swofford, 2001), Phyml v. 2.4.3 (Guindon and Gascuel, 2003) and MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). MP was performed by heuristic search with TBR swapping algorithm and the clade support was assessed with 1000 bootstrap replicates. ML was computed using the GTR+Γ+I evolutionary model and the clade support with bootstrap analysis of 1000 replicates. BI was performed with parameters (rates=invgamma, nst=6, ncat=4) corresponding to the model estimated (GTR+Γ+I). The MCMC was run for 10 million generations and tree sampling every 100 generations. The program AWTY (Nylander et al. 2008) was used to check the MCMC convergence and determine burn-in. A possible effect of LBA (long branch attraction) artifact was tested by several methods (removing and adding of taxa, LogDet analyses). The trees were visualized and exported using TreeView v. 1.6.6 (Page 1996) and adjusted in the Adobe Illustrator CS5 v. 15.0 (Adobe Systems Inc.). More detailed descriptions of the methods and parameters are provided in Table 1.

Results

Huge numbers of coccidian oocysts containing four dizoic sporocysts were found in intestinal contents of the single *P. tricuspis* examined by the flotation method. According to a presence of SB in sporocysts, the coccidian

was assigned to the genus *Eimeria*. In addition, coccidian endogenous developmental stages were detected in histological sections of both small and large intestine. Due to the presence of exceptionally high numbers of oocysts representing a single *Eimeria* sp. in intestinal contents and numerous endogenous stages in intestinal epithelial cells, the oocysts and endogenous stages are considered conspecific. Comparison with other mammal-host *Eimeria* spp. shows that our material represents a new species, the description of which is provided below.

Oocyst morphology of *Eimeria nkaka* n. sp. Fully sporulated oocysts (Fig. 1.A) are variable both in shape and size, spherical to broadly elliptical with mean length/width ratio 1.1 (range 1.0-1.3), measuring 17.5 (14.0-21.5) × 15.5 (12.5-18.0) μm (n=44) with bilayered oocyst wall consisting of thin colourless inner layer (~0.5 μm) and thicker yellowish outer layer (~1.0 μm) with markedly rugged outer surface (Fig. 1.B). Oocyst residuum and micropyle are absent. One, rarely two polar granules, 2-6 μm in diameter, irregular, seemingly composed of a few fused granules. Sporocysts dizoic, elliptical, often asymmetrical – flattened at one side, with length/width ratio 2.3 (range 1.7-3.1), measuring 13.5 (11.5-15.5) × 6.0 (4.0-8.0) μm (n=21). Stieda body well-recognizable, 1.5-2.0 μm wide, 0.5-1.0 μm high (Fig. 1.A). Transparent, barely visible sub-Stieda body might be present. The sporocyst pole bearing the SB often slightly tapered (Fig. 1.A). Sporocysts usually lying parallelly, tightly appressed to each other, leaving almost no free space within oocyst. Each sporozoite possesses one large refractile body 3-5 μm long and another smaller one measuring 3.0 × 2.5 μm (Fig. 1.A). Dense granulation of sporozoite cytoplasm sometimes did not allow for exact recognition of internal sporocyst structures. The sporocyst residuum consists of a dense irregular cluster of fine granules, ~6.5 μm in diameter (Fig. 1.A). In incompletely sporulated oocysts, the sporocyst residuum consists of relatively larger granules of variable size scattered among sporozoites.

Molecular characterization of sequences of *Eimeria nkaka* n. sp.

Partial sequences of 3 genes were obtained for this eimerian species.
Nuclear 18S rDNA: total length 1376 bp, GC content of 47 %.
Plastid ORF 470: total length 449 bp, GC content of 25 %, 149 amino acids.
Mitochondrial COI: total length 768 bp, GC content of 35 %, 256 amino acids.

Molecular characterization of sequences of *Eimeria* cf. *tenggilingi*.

Partial sequences of 2 genes were obtained for this eimerian species.

Nuclear 18S rDNA: total length 1432 bp, GC content of 45 %.

Mitochondrial COI: total length 771 bp, GC content of 33 %, 257 amino acids.

Phylogenetic position of *Eimeria* spp. from pangolins.

Phylogenetic analyses based on the 18S rRNA, ORF 470 and COI genes yielded unstable positions of both *E. nkaka* and *E.* cf. *tenggilingi* sequences within the other eimerian species. Nevertheless, this unstability is not due to the overall lack of the phylogenetic signal in the matrix or poor tree resolution; other sequences included in the matrices formed stable and robust host-specific clusters (i.e. fowl-, rabbit- and rodent-specific) in all analyses performed (MP, ML and BI) (Figs. 2-4).

In most analyses, the African *E. nkaka* clusters with *Eimeria myoxi* from the garden dormouse, close to the *Cyclospora* and fowl-*Eimeria* clade, but always with low bootstrap support (Figs. 2, 3). Similarly, the unstable position was previously shown for *E. myoxi* (Kvičerová et al. 2011). Only in ORF 470 phylogenies, *E. nkaka* was placed inside the rodent-specific cluster (Fig. 4). However, ORF 470 data set contains only 16 *Eimeria* sequences since no other species are available in the GenBank. It is therefore obvious that the results can be distorted due to the lack of taxa.

In the tree based on 18S rDNA sequences, *E.* cf. *tenggilingi* clusters to *Eimeria pilarensis* from bat; in COI tree, however, it falls to the fowl-*Eimeria* group. The low bootstrap support for the *E.* cf. *tenggilingi* nodes is shown in analyses of both genes (Figs. 2, 3). Unfortunately, we were not successful in obtaining the ORF 470 sequence of *E.* cf. *tenggilingi*. Details on phylogenetic analyses are provided below (Table 1). Accession numbers of sequences used in the analyses are provided in Table 2.

Table 1. Information on phylogenetic analyses of molecular data and parameters used.

Matrix	MP (PAUP)	ML (Phyml)	BI (MrBayes)
18S rDNA 46 sequences, alignment length 1620 bp	hsearch + TBR 1000 replicates best tree = 1004, strict consensus of 42 trees CI = 0,6026	GTR + Γ +I 1000 replicates -ln: 7920.252959	GTR + Γ +I mcmc = 10,000,000 gens. burn-in = 1100 trees
ORF 470 16 sequences, alignment length 585 bp	hsearch + TBR 1000 replicates best tree = 463, strict consensus of 6 trees CI = 0,6847	GTR + Γ +I 1000 replicates -ln: 2989.351591	GTR + Γ +I mcmc = 10,000,000 gens. burn-in = 2000 trees
COI 26 sequences, alignment length 714 bp	hsearch + TBR 1000 replicates best tree = 484, strict consensus of 9 trees CI = 0,6054	GTR + Γ +I 1000 replicates -ln: 3338.626500	GTR + Γ +I mcmc = 10,000,000 gens. burn-in = 2000 trees

Discussion

Comparison among morphology, host specificity and phylogeny of the two pangolin *Eimeria* species reveals an interesting phenomenon. While these parasites are phylogenetically distant, they display a striking morphological similarity. It includes a combination of the following traits: a relatively thick oocyst wall composed of thin colourless inner layer and thicker yellowish to brownish outer layer with markedly rugged surface; delicate thin-walled, colourless, usually asymmetrical sporocysts; absence of oocyst residuum (for visual comparison of oocyst morphology, see Results and/or publication of Else and Colley 1976). The above mentioned traits present in both species are particularly conspicuous, because such a combination of features is only rarely found in eimerians. It is thus interesting to hypothesize that especially the relatively thick oocyst wall may be an independent adaptation facilitating high resistance of the oocysts to environmental conditions and their long-term

viability outside the host. Necessity for the long-term survival of oocysts outside host might reflect the relatively low population densities resulting from solitary life style, large home ranges and sedentarity of pangolins (Kingdon 1997), which logically result in infrequent encounters of oocysts with potential new hosts.

While it is clear from the resulting trees that the two species are not closely related, their exact phylogenetic position could not be resolved. It is generally known that inferring the evolutionary history of phylogenetically isolated, deep-branching groups of taxa may be difficult because their close relatives are not available for the analyses. This is the case of both eimerians from pangolins; their phylogenetic analysis is hampered by the lack of *Eimeria* species infecting closely related host taxa, such as nandinia, lisangs, mongooses, meerkats, coatis, skunks and other members of the order Carnivora (Meredith et al. 2011). *Eimeria nkaka* represents a second described (and the first sequenced) coccidium from the order Pholidota. Enrichment of the existing data set by *Eimeria* sequences from the closest relatives of Manidae, as well as carnivores, might help to fill in this missing link and resolve the topology of coccidia infecting this ancient group of placental mammals. Unfortunately, no coccidium has yet been described from some of these host groups (namely Eupleridae, Nandiniidae and Prionodontidae).

This study thus confirms the often stressed importance of a representative sampling. In Eimeriidae, the available taxon sampling is quite uneven. While several host groups (e.g. rodents, rabbits, poultry, livestock) are relatively well-studied from the phylogenetic point of view, other groups of hosts (both diverse and species-poor) are undersampled or even absent. Diversified homeothermic host taxa that are surprisingly poorly represented are for example wild-living birds (molecular studies are focused mostly on coccidia from domestic fowl) and wild-living ungulates. In these groups, however, extension of sampling might be quite easy. In contrast, numerous host groups will probably remain difficult to sample due to their restricted distribution ranges or rarity, which applies also to their parasites.

Another feature that deserves particular attention is a presence of SB. It is a plug-like structure located at one pole of the sporocyst wall that disintegrates after ingestion of infectious developmental stage (the sporulated oocyst) by a new host in its digestive tract. An opening appears at the place of former SB,

which allows motile sporozoites to leave the sporocyst, enter the gut lumen, and eventually find and infect receptive host cells. SB is therefore essential in the initial stage of infection and its uniqueness is of a great taxonomic significance, as it represents the only evident synapomorphy of Eimeriidae (Jirků et al. 2002, Barta et al. 2005, Jirků et al. 2009b).

In the original description of *E. tenggilingi*, it is explicitly stated that it does not possess SB. However, SB is present in all mammal-host *Eimeria* species, including the very similar and closely related *E. nkaka* described in this work (Fig. 1. A). Therefore, we believe that also *E. tenggilingi* possesses, though probably barely discernible, SB that was overlooked by Else and Colley (1976).

TAXONOMIC SUMMARY

***Eimeria nkaka* n. sp.**

Type host: African Tree Pangolin or African White-bellied Pangolin *Phataginus tricuspis* (Rafinesque, 1821) (Mammalia, Pholidota, Manidae)

Type locality: Kungutadi, Cabinda province, Angola, 4°42'31.76"S, 13° 0'52.14"E.

Prevalence: Only a single animal was examined.

Site of infection: Epithelial cells of the whole intestine – enterocytes of villar bases and glandular crypts of colon.

Type material/Hapantotype: Histological sections of infected intestine, oocysts in absolute ethanol, digital photomicrographs (photosyntypes) and liver tissue sample of the symbiotype *P. tricuspis* are deposited at the protistological collection of the Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, no. IPASCR ProtColl 17.

DNA sequences: Sequences of nuclear 18S rRNA, plastid ORF 470 and mitochondrial COI genes of *Eimeria nkaka* are available in the GenBank database (NCBI) under the Accession numbers xx-xx.

Etymology: The specific epithet is name for pangolin in local Ibinda language of Cabinda.

Acknowledgements

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Table 2. Sequences included in the phylogenetic analyses.

n.d.: our sequences, not deposited in the GenBank yet.
- : the sequence is not available.

Organism	**Acc. number 18S rDNA**	**Acc. number ORF 470**	**Acc. number COI**
Eimeria acervulina	U67115	-	FJ236419
E. ahsata	AF338350	-	-
E. alabamensis	AF291427	-	-
E. albigulae	AF307880	AF311630	-
E. alorani	-	-	n.d.
E. antrozoi	AF307876	-	-
E. apionodes	-	-	n.d.
E. arizonensis	AF307878	AF311631	-
E. arnyi	AY613853	-	-
E. bovis	U77084	-	-
E. catronensis	AF324213	-	-
E. cf. *mivati*	-	-	FJ236441
E. chobotari	AF324214	-	-
E. coecicola	EF694015	-	n.d.
E. crandallis	AF336339	-	-
E. dipodomysis	AF339490	-	-
E. exigua	-	n.d.	n.d.
E. falciformis	AF080614	AF311632	-
E. faurei	AF345998	-	-
E. flavescens	EF694011	JF304149	n.d.
E. gruis	AB205165	-	-
E. intestinalis	-	n.d.	n.d.
E. irresidua	-	-	n.d.
E. langebarteli	AF311640	AF311639	-
E. leucopi	AF339491	-	-
E. magna	EF694016	JF304150	n.d.
E. maxima	-	-	FJ236459
E. mivati	U76748	-	EF174185
E. myoxi	JF304148	JF304151	n.d.
E. necatrix	-	-	EU025108
E. nieschulzi	U40263	AF311633	-
E. nkaka	n.d.	n.d.	n.d.
E. onychomysis	AF307879	AF311634	-
E. peromysci	AF339492	-	-

E. pilarensis	AF324215	-	-
E. piriformis	-	-	n.d.
E. polita	AF279667	-	-
E. porci	AF279666	-	-
E. reedi	AF311642	AF311636	-
E. reichenowi	AB205175	-	-
E. rioarribaensis	AF307877	-	-
E. scabra	AF279668	-	-
E. scholtysecki	AF324216	-	-
E. separata	AF311643	AF311637	-
E. sevilletensis	AF311644	AF311638	-
E. telekii	AF246717	-	-
E. tenella	U67121	Y12333	FJ236458
E. tenggilingi	n.d.	-	n.d.
E. trichosuri	FJ829323	-	-
E. tropidura	AF324217	-	-
E. vejdovskyi	-	-	n.d.
E. sp. TKC-1-2005	DQ072716	-	-
E. sp. TKC-2-2005	DQ167480	-	-
Cyclospora cayetanensis	AF111183	-	-
Goussia neglecta	FJ009242	-	-
Isospora hypoleucae	-	-	FJ269363
Isospora sp. iSAT1	-	-	FJ269357
Isospora sp. iSAT2	-	-	FJ269358
Isospora sp. iSAT3	-	-	FJ269359
Isospora sp. iSAT4	-	-	FJ269360
Isospora sp. iSAT5	-	-	FJ269361
Isospora sp. iSAT6	-	-	FJ269362
Toxoplasma gondii	M97703	-	-

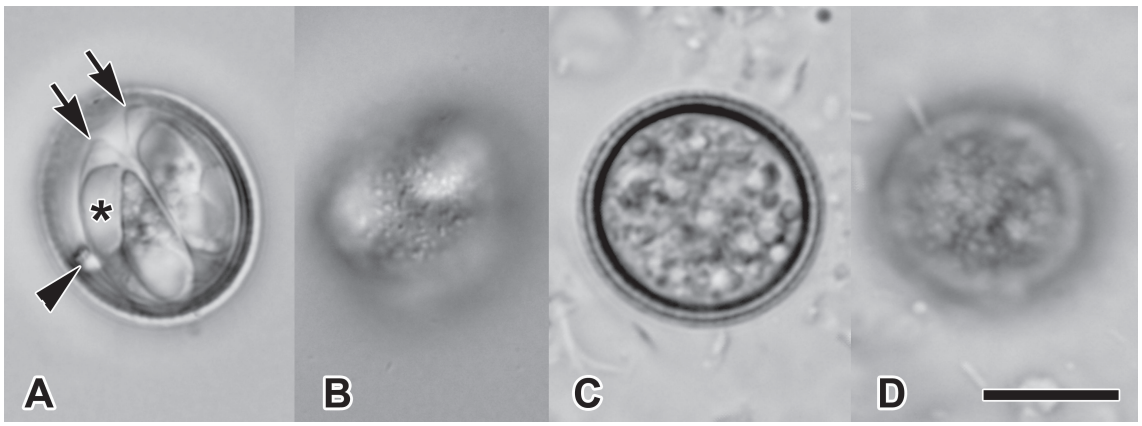

Fig. 1. A-D. Oocyst morphology of *Eimeria* species from pangolins.
A-B. *Eimeria nkaka* n. sp. from *Phataginus tricuspis*. C-D. *Eimeria* cf. *tenggilingi* from *Manis javanica*. Oocysts concentrated from faeces by flotation; Nomarski interference contrast. All in the same scale, scale bar = 10 µm.

Fig. 1.A. Morphology of sporulated oocyst of *E. nkaka* showed in optical section. Note a distinct refractile body of the sporozoite (*), irregular polar granule (arrowhead), assymetrical shape of the sporocysts and clearly discernible Stieda bodies (arrows).
Fig. 1.B. Oocyst wall of *E. nkaka* showing irregular granulation of its external surface.
Fig. 1.C. Unsporulated oocyst of *Eimeria* cf. *tenggilingi* showing typical character of oocyst wall.
Fig. 1.D. Oocyst wall of *E.* cf. *tenggilingi* showing granulation of its external surface.

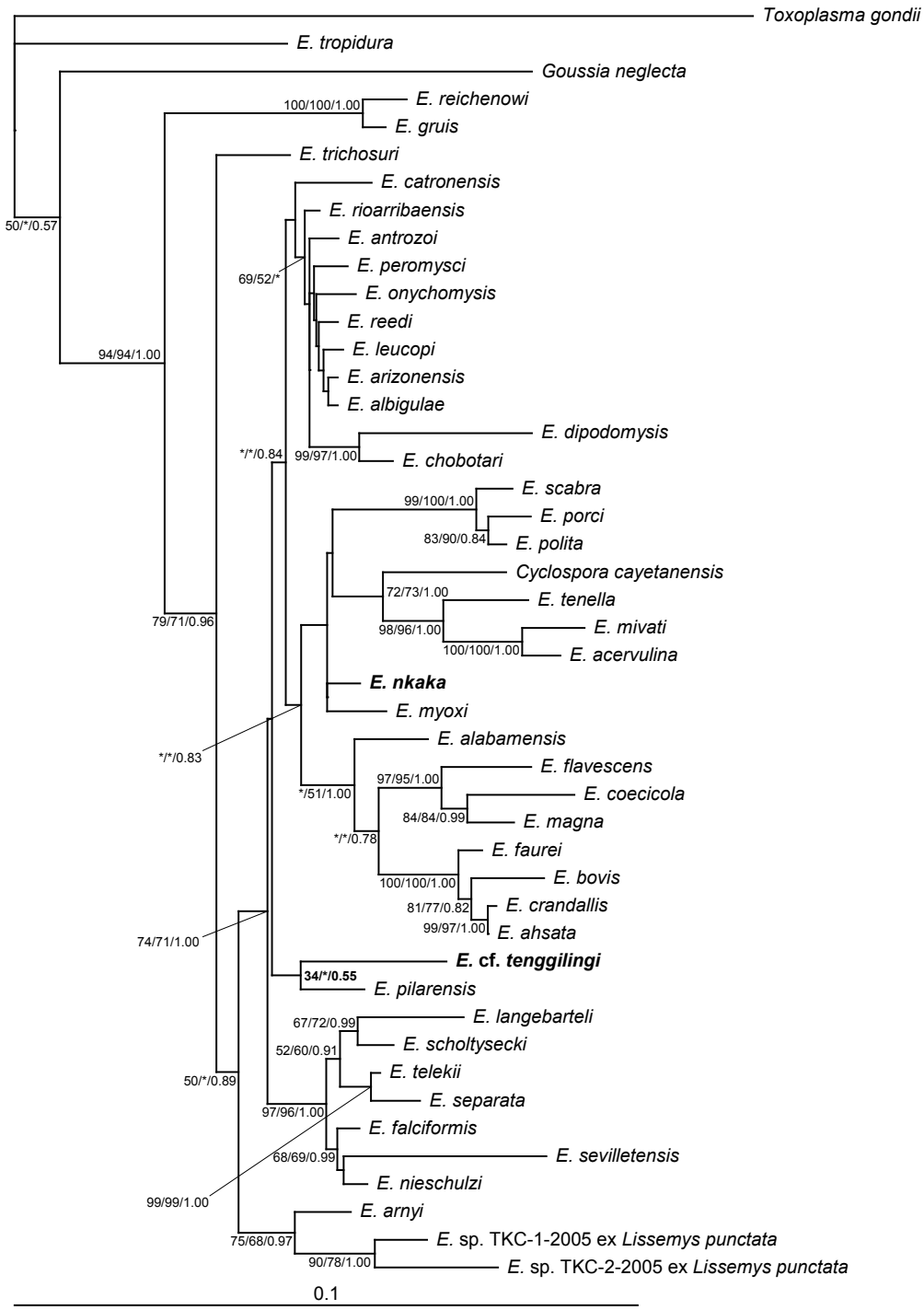

Fig. 2. Phylogenetic tree of the 18S rDNA obtained by BI. The tree is rooted with *Toxoplasma gondii*. Numbers at the nodes show bootstrap values for ML and MP, and posterior probability under BI (the values are provided only for the nodes also present in ML and MP trees). Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are marked with asterisk (*).

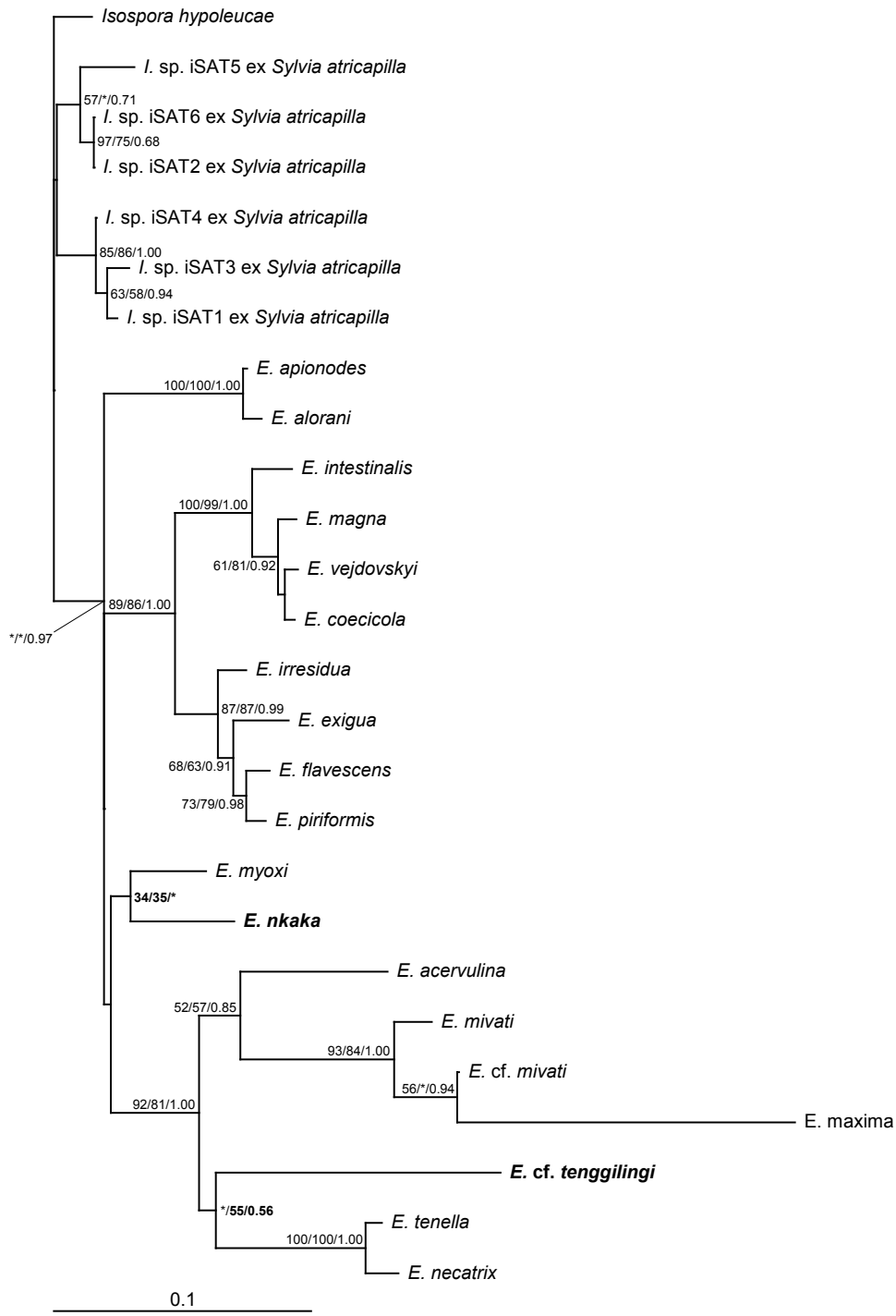

Fig. 3. Phylogenetic tree of the COI obtained by BI. The tree is rooted with *Isospora hypoleucae*. Numbers at the nodes show bootstrap values for ML and MP, and posterior probability under BI (the values are provided only for the nodes also present in ML and MP trees). Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are marked with asterisk (*).

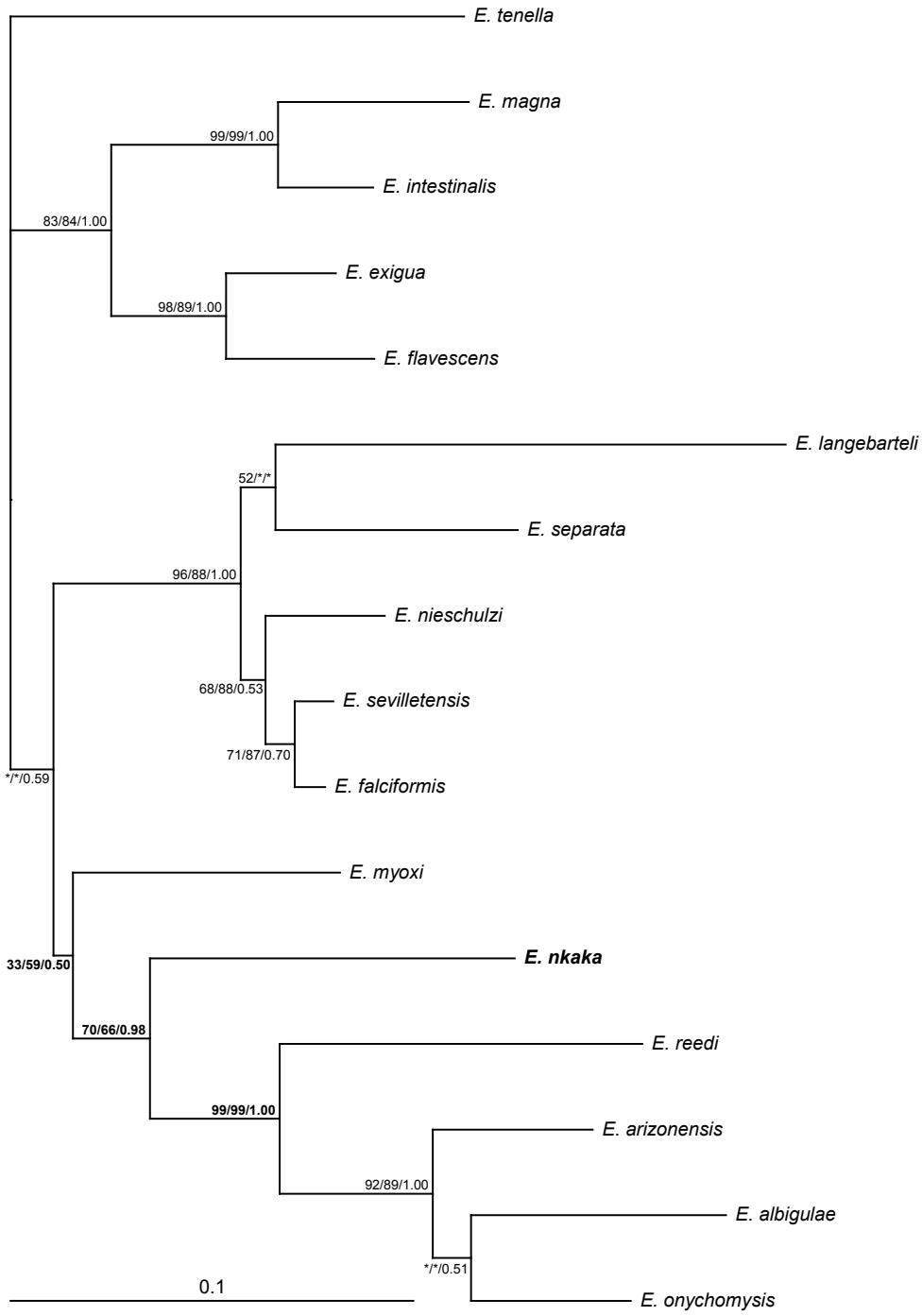

Fig. 4. Phylogenetic tree of the ORF 470 obtained by BI. The tree is rooted with *Eimeria tenella*. Numbers at the nodes show bootstrap values for ML and MP, and posterior probability under BI (the values are provided only for the nodes also present in ML and MP trees). Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are marked with asterisk (*).

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Extended set of *Eimeria* spp. indicates that eimerian host specificity is conserved due to adaptive rather than cophylogenetic processess

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Abstract

The degree of host specificity, its phylogenetic conservativeness and underlying processess are virtually unknown. This is largely due to inadequate sample of eimerians represented by molecular data that can be used for reliable phylogenetic analyses. In this study, we extend the data on *Eimeria* spp. with 86 new sequences of eimerians from 16 small mammals genera, mostly rodents. According to the feasibility of the genes amplification, the new samples are represented by one or more of the following genes: 18S rRNA, ORF 470 and COI. The results confirm the previous suggestion that *Eimeria*, in its current morphology-based delimitation, is not a monophyletic group. Several samples corresponding morphologically to other genera are scattered among the *Eimeria* lineages. More importantly, the distribution of eimerians from different hosts indicates that the clustering of eimerian species is influenced by their host specificity, but does not arise from a cophylogenetic/copseciation process; while several clusters are specific to particular host group, inner topologies of these clusters do not reflect the host phylogeny. This suggests that host specificity in *Eimeria* is caused by adaptive rather than cophylogenetic processess.

Key words: host specificity, phylogenetic relationships, cophylogeny, adaptive processess, coccidia, Eimeriidae, small mammals.

Introduction

Specificity to more or less restricted group of hosts is one of the fundamental characteristics of most parasitic taxa. In parasitological research, this trait has traditionally been considered highly conserved from the phylogenetic point of view. This led to establishment of a broad spectrum of concepts and methods dealing with coevolution/cospeciation between the host and parasite (Brooks 1988, Brooks and McLennan 1991, 1993, Page 1991, 1993, 1994, Thompson 1994, Huelsenbeck et al. 1997, Paterson and Gray 1997). More recently, analyses based on molecular data revealed a tendency to the conservativeness of host specificity and even strong cospeciation signal in many parasitic groups (Page 1996a, Hafner and Nadler 1990, Ricklefs et al. 2004). On the other hand, they also demonstrated that such a conservativeness is not a "rule", and found many surprising inconsistencies among the host and parasite phylogenies (Charleston 1998, Page et al. 1998, Huelsenbeck et al. 2000, Jousson et al. 2000, Ricklefs and Fallon 2002). Moreover, many other features, morphological or ecological, presumed to be reliable determinants of taxonomy and classification, proved to suffer the same phylogenetic inconsistencies (Relman et al. 1996, Pieniazek and Herwaldt 1997, Carreno et al. 1998, Fiala 2006, Štefka and Hypša 2008). Consequently, traditional classifications of many taxa remain artificial and many generic names do not designate monophyletic groups.

Currently, there is no consensus or general view on how might be the host specificity in various parasites phylogenetically conserved. Apart from many methodological problems (Page 1996a, Paterson and Banks 2001), one drawback is the traditional focus on several model groups (e.g. chewing lice, lice and nematodes; Hafner and Nadler 1988, 1990, Brant and Gardner 2000, Perlman et al. 2003, Weckstein 2004, Whiteman et al. 2004) and unsufficient data for many others. The situation may be particularly difficult and the analyses misleading in taxonomically rich groups for which only poor sampling is currently available; any pattern observed on the phylogenetic background

may be a random outcome of the inadequate arbitrary sampling rather than reflection of real tendencies within the group. Considering their importance, it is quite surprising that coccidia of the genus *Eimeria* provide such an example. Majority of the traditional studies on coccidia with taxonomic implications are based solely on morphology of sporulated oocysts (e.g. Pellérdy 1974, Lewis and Ball 1983, Levine and Ivens 1990, Higgs and Nowell 1991, Hůrková et al. 2005, Seville et al. 2005, Golemansky and Koshev 2007, Lynch et al. 2007). Several other publications deal with the host specificity (mostly laboratory cross-transmission studies) and pathogenicity of coccidia (de Vos 1970, Upton et al. 1992, Koudela and Vítovec 1994, Schito et al. 1996).

Only few comprehensive molecular studies have been performed so far (Barta et al. 1997, Franzen et al. 2000, Morrison et al. 2004, Matsubayashi et al. 2005, Kvičerová et al. 2008). They show that some morphological features of the oocyst (i.e. oocyst size, sporocyst size and shape index) are phylogenetically incosistent and can not be used as taxonomic determinats. In addition, several morphological studies indicate that these features even vary during the development/patency of the oocyst (Long and Joyner 1984, Parker and Duszynski 1986, Gardner and Duszynski 1990). Moreover, determination of the "oocyst shape" is a subjective criterion that depends on the microscopic experience of the individual observer (e.g. oval vs. ovoidal vs. ellipsoidal shape; the "spherical" or "subspherical" shape is often detected in dependence on the view angle).

In this study, we further explore phylogenetic significance of host specificity within *Eimeria* by adding 86 new eimerian sequences. Since the most frequently utilized phylogenetic marker, the 18S rDNA, proved to be unsufficient for this group, we also sequenced two additional DNA regions where possible, cytochrome c oxidase subunit I (COI) and ORF 470. To obtain a consistent picture allowing for evolutionary inference, we mainly focused on the rodent-derived *Eimeria*; the complete set thus contains 46 eimerian parasites from various rodent groups, covering/involving 8 rodent families. This representative set demonstrates that with increasing number of available taxa, the phylogenetic relationships become less host-dependent.

Materials and Methods

Samples collection and treatment

Rodents were trapped using the Sherman live-traps or classic wooden traps, with official permissions. Fresh faeces or the gut content of each individual animal were placed into 4% (w/v) potassium dichromate solution ($K_2Cr_2O_7$) and stored at 4 ^oC. Several samples (e.g. shrews, moles and mole-rats) were obtained from already deceased animals. Faecal samples were examined for the presence of coccidian oocysts by the standard flotation technique with Sheather´s sucrose solution (sp.gr. 1.30). An Olympus BX51 microscope equipped with the Olympus Camedia C-5060W camera and Quick Photo Pro v. 2.0 PC software was used for species-specific identification of oocysts found. Morphological and morphometrical features were evaluated according to Duszynski and Wilber (1997).

Molecular analyses

Genomic DNA of coccidia was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals). Three different genes (nuclear 18S rRNA, plastid ORF 470 and mitochondrial COI) were amplified using the HotStarTaq DNA polymerase (Qiagen) and PCR protocols according to Zhao and Duszynski (2001b), Kvičerová et al. (2008) and Schwarz et al. (2009). PCR products of expected sizes (18S rDNA ~1500 bp, ORF 470 ~700 bp and COI ~700 bp) were enzymatically purified and cloned into the pGEM-T Easy Vector (Promega). Five clones of each sample were used for the plasmid extraction by the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Plasmids were sequenced on an automatic 3730XL DNA analyzer in Macrogen, Inc. (Korea) with the PCR primers and inner primers (Zhao and Duszynski 2001b, Kvičerová et al. 2008, Schwarz et al. 2009). Sequences were identified by BLAST analysis, adjusted using the DNASTAR program package (DNASTAR Inc.) and deposited in the GenBank database (NCBI) under the Accession numbers xx-xx.

Phylogenetic analyses

To explore phylogenetic signal in the obtained sequences in a complex way, we built several different single-gene and multi-gene matrices. Three

single-gene matrices, *18S rDNA*, *COI* and *ORF 470*, were designed with different taxa samplings according to the availability of the given sequences for individual taxa (Table 1). The *Skeleton* matrix included taxa for which all three genes were available. The *Concatenate* matrix encompassed all taxa for which at least one gene was available. To achieve stable and reliable placement of the root, multiple taxa were used as outgroups (Table 1). All matrices were aligned and analyzed at nucleotide level. Alignments were constructed in MAFFT v. 6 program (Katoh et al. 2002, 2005) and corrected manually in BioEdit program (Hall 1999). Maximum likelihood (ML) and Bayesian inference (BI) were used for phylogenetic analyses. Most suitable models of sequence evolution were identified in jModelTest (Posada 2008, 2009) and MrModel (Nylander 2004) programs using Akaik's criterion. ML was performed in Phyml v. 2.4.3 (Guindon and Gascuel 2003) with GTR + Γ + I model and parameters estimated from the data. BI was done using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001) with GTR + Γ + I model for 50 million generations. Chain convergence and burn-in were estimated according to the indices implemented in the MrBayes program (deviation of split frequencies, potential scale reduction factor – PSRF) and using the program Tracer (Rambaut and Drummond 2007). The trees were summarized after removing 20% burn-in and visualized using TreeView v. 1.6.6. (Page 1996b).

Results

While the trees obtained by phylogenetic analyses with different data sets and methods vary in positions of individual branches, they are well-compatible in their overall structure and arrangement. Since the aim of this study was to analyse the monophyly and composition of the whole clusters characterized by various biological features (e.g. morphology, host specificity) rather than relationships among individual species, we focused on comparison among particular internal nodes in the trees. To allow for a transparent comparison among the trees inferred from different data sets, we established a specific reference method. We chose the *Concatenated* ML tree with bootstrap values to delimit clusters of two types. First, we labelled all monophyletic groups that were characterized by well-defined spectrum of the host taxa

(vertical lines in the Fig. 2); second, we "fixed" all nodes that were strongly supported by the bootstrap values and were also preserved in the BI tree (open squares at the branches; Fig. 2). We then identified whether each of these "fixed" groups is represented by at least one sample in the *Skeleton* tree (asterisks at the taxa names in Fig. 2).

The *Skeleton* tree divides the included taxa into 6 main arbitrary delimited clades (A-D, Fig. 1). When fixed according to the *Skeleton* taxa, these clades are also preserved and well-supported in all performed single-gene analyses and in the *Concatenated* tree. The single-gene trees as well as the *Concatenated* tree also demonstrate that whereas some genera (e.g. *Cyclospora*) are monophyletic, others (*Eimeria* and *Isospora*) are polyphyletic. In all analyses performed, the rodent *Eimeria* species are divided into several (6-8) paraphyletic lineages. Composition of these clades corresponds to the presence/absence of the oocyst residuum (OR). Other criteria (oocyst shape and size, presence/absence of micropyle and other inner oocyst structures, location of endogenous development, pre-patent and patent periods, sporulation time), if known for the studied taxa, do not correlate with the topology. Of our new rodent samples, three species from the newly added hosts fall within the OR+ rodent cluster (namely *E. cahirinensis*, *E. callospermophili* and *Eimeria* sp. from *Acomys* sp.). Another twelve samples (i.e. *E. caviae*, *E. chinchillae*, *Eimeria* spp. from *Apodemus*, *Cricetus*, *Heliophobius*, *Mastomys*) branched within the OR- rodent cluster. While most of *Eimeria* tend to cluster according to the host (i.e. distinct and stable fowl-, wild living bird-, porcine-, bovine-, rabbit- and rodent- lineages), the *Concatenated* tree also indicates that the sampling is still insufficient and several taxa lack the clear phylogenetic position (e.g. eimerians from the tree pangolin, garden dormouse, sheep, ferret and marsupials) (Fig. 2).

Discussion

This study provides the most up-to-date insight into the phylogeny of eimerian parasites. Altogether, 86 new sequences of *Eimeria* species belonging to 16 small mammals genera (8 rodent families, 2 insectivores and 1 manid) and 1 new rodent *Isospora* sequence were analyzed together with coccidian

sequences available from the GenBank. Two main conclusions arise from the presented results. Firstly, they confirm the previous suggestion that *Eimeria*, in its current morphology-based delimitation, is not a monophyletic group. Secondly, and more importantly, they show an interesting relationship between the host specificity and phylogeny: the distribution of eimerians from different hosts indicates that clustering of eimerian species is influenced by their host specificity but does not stem from a cophylogenetic process. Before attempting any serious evolutionary conlusion, however, it should be admitted that the current sample of molecularly characterized *Eimeria* spp. and spectrum of their available genes is extremely poor and incosistent. Nevertheless, despite this drawback, both conclusions stated above are well-supported by all data and analyses.

The non-monophyly issue of the genus *Eimeria* has been indicated by several previous studies (Morrison et al. 2004, Matsubayashi et al. 2005, Yabsley and Gibbs 2006). It introduced into the recognition of the inconsistency between various phenotypic traits (most typically the oocyst morphology) and phylogenetic relationships in coccidia (Relman et al. 1996, Pieniazek and Herwaldt 1997, Franzen et al. 2000, Kvičerová et al. 2008). However unpleasant this finding may had been for the coccidian taxonomists, it is hardly surprising that similar decoupling of the morphology of resistant stages and phylogenetic positions was also demonstrated in some other groups of parasites (e.g. Myxosporea; Fiala 2006).

This situation brings a serious problem with future reclassification of the family Eimeriidae. Several species corresponding morphologically to different genera (e.g. *Cyclospora* and *Isospora*) branch within the *Eimeria* cluster. For example, genus *Isospora* is undoubtedly polyphyletic, with several lineages scattered among *Eimeria* species (mammal-associated species on the base of coccidian topology/related to Sarcocystidae, bird-associated species split into 2 lineages, one scattered among rodent *Eimeria* species and one related to *Eimeria* from cattle and rabbits, mole isosporans and *Isospora* sp. from field mouse form separate clusters within Eimeriidae) (Fig. 2; Franzen et al. 2000, Jirků et al. 2002, Samarasinghe et al. 2008, Dolnik et al. 2009, Jirků et al. 2009). Sporulated oocysts of *Isospora* spp. are quite morphologically uniform (for examples, see Pellérdy 1974 and/or Duszynski and Upton 2000); nevertheless, the genus *Isospora* was divided into 2 individual genera according

to the phylogeny, host specificity and the presence/absence of a Stieda body (SB): bird-associated *Isospora* (former *Atoxoplasma*) with SB belonging to Eimeriidae and mammal-associated *Cystoisospora* lacking SB belonging to Sarcocystidae (Carreno et al. 1998, Franzen et al. 2000, Barta et al. 2005). However, it is pertinent to stress that only 10 *Isospora*/*Cystoisospora* species from mammals (mainly cats and dogs) out of >130 described species (Duszynski and Upton 2001) have been sequenced so far. Moreover, the comprehensive descriptions including photomicrographs show that several *Isospora* species infecting mammals (namely those parasitising moles and shrews) evidently possess conspicuous SB (Duszynski and Upton 2000). Sequences from these species could potentially bring new, surprising insight into isosporan phylogeny. Similarly, the genus *Cyclospora* keeps to cluster strikingly within *Eimeria* species, related to fowl-associated *Eimeria* (Relman et al. 1996, Pieniazek and Herwaldt 1997, Eberhard et al. 1999, Li et al. 2007). However, only data on *Cyclospora* spp. from man, primates and dairy cattle are currently available, while inclusion of additional *Cyclospora* species from other hosts (e.g. insectivores or reptiles) may bring more surprises.

The issue of the host specificity and its phylogenetic significance has been much less explored in the published studies. One of the main reasons is an inadequate representation of the host-specific groups. In fact, only the rodent-derived *Eimeria* are currently represented by a reasonable number and diversity of samples, whereas the other so-called host-specific lineages are mostly derived from very closely related hosts or even a single host species. Alternatively, they are defined by various artificial rather than taxonomic characteristics of their hosts (e.g. poultry parasites, livestock parasites, etc.).

Previous phylogenetic studies tended to group the rodent-specific *Eimeria* species in two distant but monophyletic clusters with unclear dependency on the taxonomic position of the hosts (Zhao and Duszynski 2001a, b, Zhao et al. 2001). Taking the number of eimerian samples from rodents and taxonomic diversity of their hosts into account, these two clusters could be potentially envisaged as two main evolutionary sources of rodent eimerians. Identification of a third lineage formed by *Eimeria myoxi* suggested that the situation may be more complex (Kvičerová et al. 2011). The 26 new rodent-derived samples added in this study further support this view. While many of these new samples from so far unexplored hosts (e.g. black-bellied hamster,

chinchilla, ground squirrel, guinea pig, mole-rats, spiny mice, and several field mice) clearly belong to the "1st" and "2nd" rodent clades, the position of others (garden dormouse, gerbil, multimammate rat, and some field mice) is more variable. It is also interesting to note that no rodent sample of *Eimeria*–like morphology falls into the A group, containing mainly parasites from poultry, livestock, rabbits, and the isosporan lineage: the only *Apodemus*–isolated sample branching in this group clearly corresponds to the *Isospora* morphology (Fig. 2).

The relationship between host specificity and phylogeny displays an interesting pattern. While host specificity provides useful characteristics for many clusters (e.g livestock, pigs, poultry, rabbits), species arrangements within the clusters do not show any correlation with host phylogenies. The host conservativeness of the clusters is thus likely to reflect ecological, physiological or other adaptations to particular host group rather than host-parasite cospeciation. Perhaps the most surprising outcome of this study is the phylogenetic diversity of *Eimeria* samples obtained from the genus *Apodemus*. While an exact taxonomic status of the 11 analyzed samples and their precise position may not be entirely clear from the available topologies, they demonstrably cluster at least at four different places in the tree and cover quite a large phylogenetic span (Fig. 2). This suggests that apart from the availability of representative taxonomic sample of the host, another serious problem rests in the knowledge of the eimerian diversity within a single host genus or species. Considering the composition of the available data set (with only rodents sufficiently sampled in respect to the taxonomic representativeness as well as parasite diversity within a single host species), the trends pointed out in this study have to be further examined using similar representative samples of other host groups.

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Table 1. Taxa and sequences included in the phylogenetic analyses.

*: sequences included in the *Skeleton* matrix.
•: taxa used as outgroups for the phylogenetic analyses.
Taxa for which new sequences were obtained in this study are printed in bold.
n.d.: our sequences, not deposited in the GenBank yet.
- : the sequence is not available.

Organism	**Acc. number 18S rDNA**	**Acc. number ORF 470**	**Acc. number COI**
Eimeria acervulina	U67115	-	FJ236419
E. adenoeides	AF324212	-	-
E. ahsata	AF338350	-	-
E. alabamensis	AF291427	-	-
E. albigulae	AF307880	AF311630	-
E. antrozoi	AF307876	-	-
E. arizonensis	AF307878	AF311631	-
E. arnyi	AY613853	-	-
E. attwateri	EU481858	-	-
E. auburnensis	AY876927	-	-
E. auritusi	DQ398107	-	-
E. banffensis	n.d.	-	-
E. bovis	U77084	-	-
E. brunetti	U67116	-	-
***E. cahirinensis* NFS**	n.d.	-	n.d.
***E. cahirinensis* SFS**	n.d	-	-
***E. cahirinensis* WR**	n.d.	-	n.d.
E. callospermophili	n.d.	-	n.d.
E. catronensis	AF324213	-	-
***E. caviae* ***	n.d.	n.d.	n.d.
E. cf. *mivati*	FJ236378	-	FJ236441
E. chaetodipi	AF339489	-	-
E. chinchillae	n.d.	-	-
E. chobotari	AF324214	-	-
E. coecicola	EF694015	n.d.	n.d.
E. crandallis	AF336339	-	-
E. cylindrica	AY876928	-	-
E. dipodomysis	AF339490	-	-
E. ellipsoidalis	AY876929	-	-
***E. exigua* ***	EF694007	n.d.	n.d.

E. falciformis	AF080614	AF311632	-
E. faurei	AF345998	-	-
E. flavescens *	EF694011	JF304149	n.d.
E. furonis	AB239130	-	-
E. gruis	AB205165	-	-
E. intestinalis *	EF694012	n.d.	n.d.
E. irresidua *	EF694009	n.d.	n.d.
E. langebarteli	AF311640	AF311639	-
E. leucopi	AF339491	-	-
E. magna *	EF694016	JF304150	n.d.
E. maxima	DQ538348	-	FJ236459
E. media	EF694013	-	-
E. meleagrimitis	AF041437	-	-
E. mitis	U40262	-	-
E. mivati	U76748	-	EF174185
E. myoxi *	JF304148	JF304151	n.d.
E. necatrix	DQ136185	-	EU025108
E. nieschulzi	U40263	AF311633	-
E. nkaka *	n.d.	n.d.	n.d.
E. onychomysis	AF307879	AF311634	-
E. ovinoidalis	AF345997	-	-
E. papillata	AF311641	AF311635	-
E. perforans	EF694017	n.d.	n.d.
E. peromysci	AF339492	-	-
E. phalacrocoraxae	DQ398106	-	-
E. pilarensis	AF324215	-	-
E. piriformis	EF694014	n.d.	n.d.
E. polita	AF279667	-	-
E. porci	AF279666	-	-
E. praecox	U67120	-	-
E. ranae	EU717219	-	-
E. reedi	AF311642	AF311636	-
E. reichenowi	AB205175	-	-
E. rioarribaensis	AF307877	-	-
E. scabra	AF279668	-	-
E. scholtysecki	AF324216	-	-
E. separata	AF311643	AF311637	-
E. sevilletensis	AF311644	AF311638	-
E. stiedai	EF694008	n.d.	n.d.
E. subspherica	AY876930	-	-
E. synaptomysis	n.d.	-	-
E. telekii	AF246717	-	-

E. tenella *	U67121	Y12333	FJ236458
E. trichosuri	FJ829323	-	-
E. tropidura	AF324217	-	-
E. vejdovskyi	EF694010	n.d.	n.d.
E. vilasi	n.d.	-	-
E. weybridgensis	AY028972	-	-
E. wyomingensis	AY876931	-	-
E. zuernii	AY876932	-	-
E. sp. DAM-2009	FN298443	-	-
E. sp. ESP-181	AB447983	-	-
E. sp. TKC-1-2005	DQ072716	-	-
E. sp. TKC-2-2005	DQ167480	-	-
E. sp. ex *Acomys* sp.	n.d.	-	-
E. sp. ex *A. agrarius* 21439	n.d.	-	-
E. sp. ex *A. agrarius* 21455	n.d.	-	-
E. sp. ex *A. agrarius* 21615	n.d.	-	-
E. sp. ex *A. agrarius* 21617 *	n.d.	n.d.	n.d.
E. sp. ex *A. agrarius* 21655 *	n.d.	n.d.	n.d.
E. sp. ex *A. agrarius* 21668	n.d.	-	n.d.
E. sp. ex *A. flavicollis* 1	-	-	n.d.
E. sp. ex *A. flavicollis* 4	-	-	n.d.
E. sp. ex *A. flavicollis* 12	-	-	n.d.
E. sp. ex *A. sylvaticus* 08/50	n.d.	-	n.d.
E. sp. ex *A. sylvaticus* 08/53 *	n.d.	n.d.	n.d.
E. sp. ex *C. cricetus* K7	n.d.	-	-
E. sp. ex *G. dasyurus*	n.d.	-	-
E. sp. ex *Heliophobius* E1	n.d.	-	n.d.
E. sp. ex *Heliophobius* K12 *	n.d.	n.d.	n.d.
E. sp. ex *M. natalensis*	n.d.	-	-
E. sp. ex *S. araneus*	-	n.d.	n.d.
Caryospora bigenetica	AF060975	-	-
Choleoeimeria sp.	AY043207	-	-
Cyclospora cayetanensis	AF111183	-	-
C. cercopitheci	AF111184	-	-
C. colobi	AF111186	-	-

C. papionis	AF111187	-	-
Cystoisospora belli •	AF106935	-	-
C. felis •	L76471	-	-
C. ohioensis •	AF029303	-	-
C. orlovi •	AY365026	-	-
C. rivolta •	AY618554	-	-
C. suis •	U97523	-	-
C. timoni •	AY279205	-	-
Goussia janae	AY043206	-	-
G. metchnikovi	FJ009244	-	-
G. neglecta	FJ009242	-	-
G. noelleri	FJ009241	-	-
G. ex *Bufo bufo*	FJ009243	-	-
Intranuclear coccidium JW-2004	AY728896	-	-
Isospora gryphoni	AF080613	-	-
I. robini	AF080612	-	-
Isospora sp. iSAT1	-	-	FJ269357
Isospora sp. iSAT2	-	-	FJ269358
Isospora sp. iSAT3	-	-	FJ269359
Isospora sp. iSAT4	-	-	FJ269360
Isospora sp. iSAT5	-	-	FJ269361
Isospora sp. iSAT6	-	-	FJ269362
***I.* sp. ex *A. flavicollis* B13**	-	-	n.d.
***I.* sp. ex *Talpa* 106**	n.d.	-	n.d.
***I.* sp. ex *Talpa* 151**	n.d.	-	n.d.
***I.* sp. ex *Talpa* 156**	n.d.	-	-
***I.* sp. ex *Talpa* 218**	-	n.d.	n.d.
Toxoplasma gondii •	M97703	U87145	DQ228959

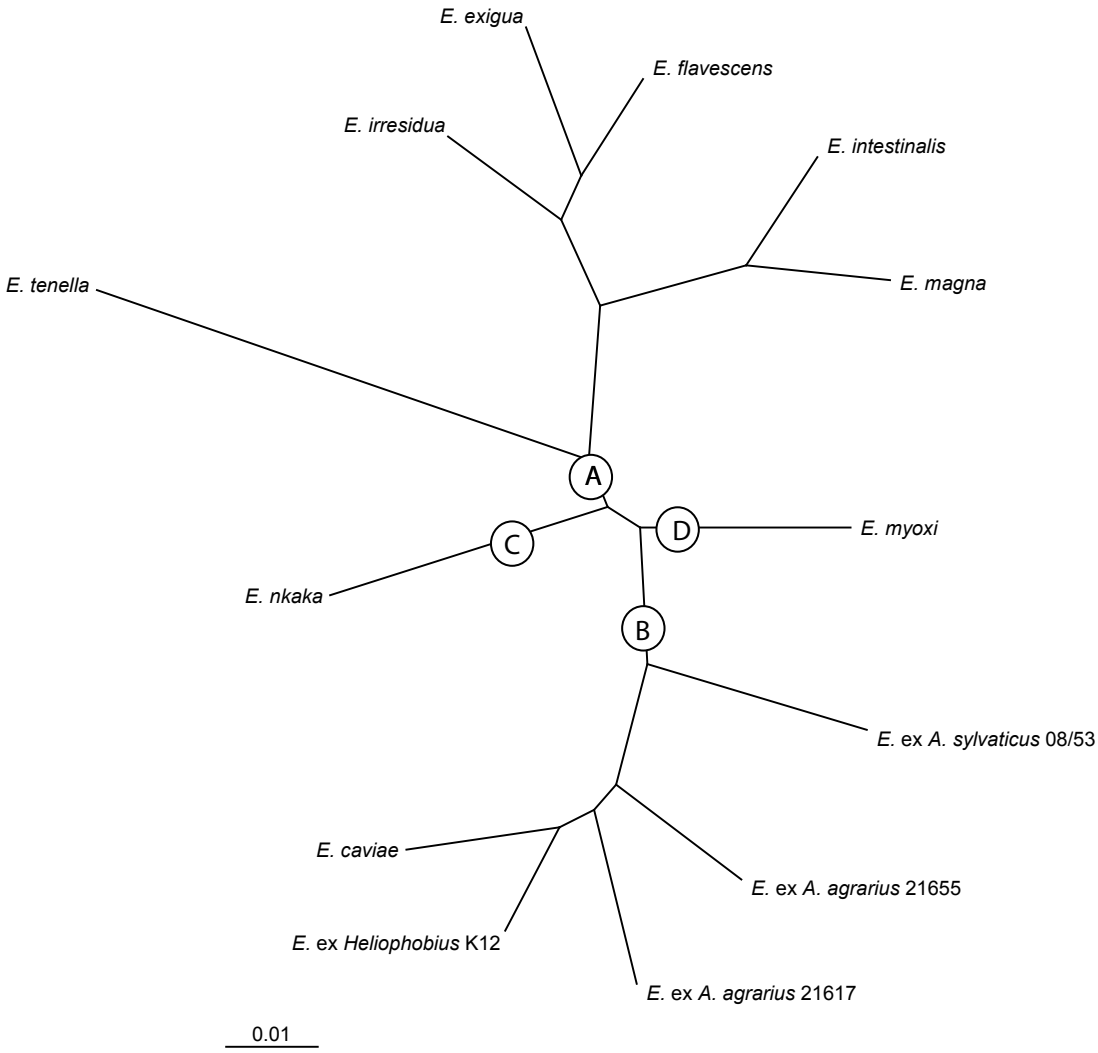

Fig. 1. A *Skeleton* tree (ML and BI) including taxa for which all three genes (18S rDNA, ORF 470 and COI) are available.

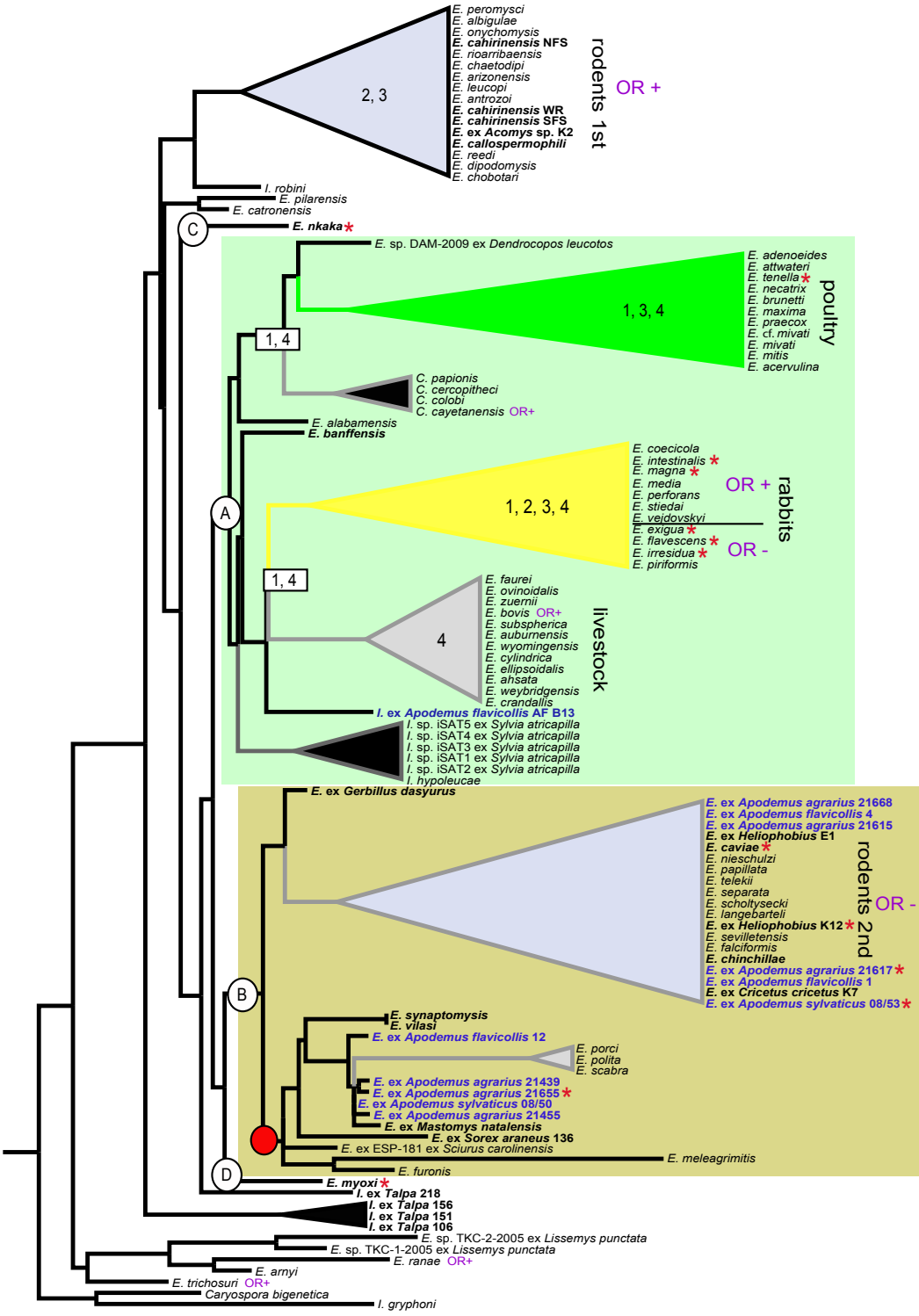

Fig. 2. *Concatenated* ML tree. Letters A-D show clusters delimited according to the *Skeleton* trees (taxa present in the *Skeleton* trees are marked with asterisk). The clades A and B are also supported by BI and ML analyses of *Concatenated* matrix as well as the *Skeletons*. The red node indicates a low-host-specific cluster, containing taxa from several different host groups. Numbers 1-4 indicate lineages also supported by the BI analyses of the following matrices: 1, *Concatenated*; 2, ORF 470; 3, COI; 4, 18S rDNA. The newly added taxa are printed in bold; coccidia from rodents are printed in blue. To decrease the size of the tree for the printed presentation, we removed several most basal outgroups.

Draft no. 1:

Preliminary results for the population structure, host specificity and biogeography in *Apodemus* and *Eimeria*.

Preliminary results for the population structure, host specificity and biogeography in *Apodemus* and *Eimeria*

Background

Apodemus Kaup, 1829 (Old World Field mice) is a Palearctic genus of murid rodents, distributed throughout the whole Europe and Asia. Twenty species have been described based on morphological features, geographic distribution and genetic structure (Wilson and Reeder 2005). However, the number of valid species changes regularly (for examples, see Wilson and Reeder 2005), and the genus systematics is still not settled (Aulagnier et al. 2009). In Europe, 8 *Apodemus* species have been recorded: *A. agrarius*, *A. alpicola*, *A. epimelas*, *A. flavicollis*, *A. mystacinus*, *A. sylvaticus*, *A. uralensis* (former *A. microps*) and *A. witherbyi* (Wilson and Reeder 2005). Only four of them have been reported from the Czech Republic: *A. agrarius*, *A. flavicollis*, *A. sylvaticus* and *A. uralensis*. Geographic distribution and habitats of these species overlap, so they often live in sympatry (Anděra and Beneš 2002, Anděra and Horáček 2005), competing for the food resources. They are omnivorous, the diet usually includes grains, seeds, nuts, roots, insects and other invertebrates. They have been recorded from a variety of habitats, often in connection with grassy fields, woodlands, forests, shrubs, water streams, but also from cultivated areas and human vicinities (Štefančíková et al. 1994, Nowak 1999, Anděra and Beneš 2002, Wilson and Reeder 2005).

It is generally known that based on morphological features, it may be difficult to distinguish among *A. flavicollis*, *A. sylvaticus* and *A. uralensis* in the field. This problem occurs especially in juveniles and subadults, in which the morphological features (body size and colour, hind foot length, collar spot) are overlapping among these species (Štusák 1987, Filippucci et al. 1989, Anděra and Beneš 2002, Anděra and Horáček 2005). Therefore, methods of molecular biology proved to be the most efficient tool for resolving the species identity (Filippucci et al. 1989, Martin et al. 2000, Michaux et al. 2001, 2002). Ecology, phylogeny, phylogeography, genetics and genealogy of the genus *Apodemus* have been studied extensively within last three decades (Tsuchiya and Yosida

1971, Tsuchiya 1974, Filippucci et al. 1989, Martin et al. 2000, Michaux et al. 2001, 2002, Sakka et al. 2010).

Coccidia of the genus *Eimeria* Schneider, 1875, members of the largest apicomplexan genus, are frequently found in faeces or gut contents of field mice (Lewis and Ball 1983, Higgs and Nowell 2000). To date, these parasites have been recorded from 6 *Apodemus* species (*A. agrarius*, *A. argenteus*, *A. flavicollis*, *A. mystacinus*, *A. speciosus* and *A. sylvaticus*) (Higgs and Nowell 1991, Wash et al. 1985, Hůrková et al. 2005). The first *Eimeria* species reported from the field mice was *Eimeria muris*, described from *Apodemus flavicollis* by Galli-Valerio (1932). Later, 22 more *Eimeria* species were described (Higgs and Nowell 1991, Hůrková et al. 2005); from today's perspective, however, many of these descriptions are inadequate and do not allow unequivocal species identification. Since they do not provide the photomicrographs/line drawings or enough details on inner structures of the oocysts (see Musaev and Veisov 1965 or Pellérdy 1974), the observations reported by other authors were difficult to relate to the original descriptions, and also to each other (Wash et al. 1985, Higgs and Nowell 1991, Hůrková et al. 2005).

From the phylogenetic point of view, the rodent-associated *Eimeria* species are among the most extensively studied coccidia; till now, 22 *Eimeria* species from 11 rodent genera have been sequenced and analyzed using the methods of molecular phylogeny (www.ncbi.nlm.nih.gov). However, these samples still represent only a small portion of the known diversity of the rodent eimerians (more than 350 *Eimeria* species have been described from rodents; Levine and Ivens 1990, Duszynski and Upton 2001). Phylogenetic analyses indicate that the rodent-associated *Eimeria* species cluster in several (at least 3) different and phylogenetically unrelated lineages (Zhao and Duszynski 2001a, b, Power et al. 2009, Kvičerová et al. 2011). In general, phylogenetic studies also show that most of the biological and morphological characteristics are phylogenetically inconsistent (Eberhard et al. 1999, Kvičerová et al. 2008, Samarasinghe et al. 2008). A future taxonomic revision in eimerians is thus inevitable.

No molecular data are yet available for any of *Eimeria* exploiting *Apodemus* hosts. The situation is further complicated by the potential degree of host specificity of these eimerians. It is evident that some *Eimeria* species can infect several species of *Apodemus* (e.g. *E. alorani*, *E. apionodes*, *E. apodemi*,

E. argenteus, *E. hungaryensis*, *E. inuyamensis*, *E. montgomeryae*, *E. uptoni*) (Lewis and Ball 1983, Wash et al. 1985, Higgs and Nowell 1991, Hůrková et al. 2005, Kvičerová, this study), while others have so far been described only from a single host species. Moreover, molecular analyses may be further complicated by the multi-species *Eimeria* infections that often occur in *Apodemus* individuals.

In *Apodemus* hosts, genealogy and genetic diversity were previously analyzed for populations of a nematode *Heligmosomoides polygurus* (Nieberding et al. 2004, 2005) and lice *Polyplax serrata* (Štefka and Hypša 2008). The aim of this study is twofold: to place *Apodemus* – specific eimerians into the phylogenetic tree of *Eimeria* and to study for the first time genealogy and population structure of these rodent parasites.

Materials and Methods

Collections of host & parasite samples

Rodents of the genus *Apodemus* (*A. agrarius*, *A. flavicollis* and *A. sylvaticus*) were trapped in the field using Sherman live-traps or classic wooden traps. All animals were trapped with official permissions (Nos. PP 42/2006 and KUJCK 11134/2010 OZZL/2/Ou). Host tissues (a piece of ear, finger or tail) were collected for molecular identification of *Apodemus* species. Oocysts of *Eimeria* species were recovered from fresh faeces or the gut content of the hosts.

Sample treatment, oocyst morphology and determination

Faecal material was examined by standard flotation technique with Sheather´s sucrose solution (sp.gr. 1.30) (Sheather 1923). Coccidia-positive samples were allowed to sporulate on air for several days, and then stored individually in 4% (w/v) potassium dichromate solution ($K_2Cr_2O_7$) at 4 ^oC. Sporulated oocysts were measured and evaluated according to Duszynski and Wilber (1997) using an Olympus BX51 light microscope equipped with the Olympus Camedia C-5060W camera and Quick Photo Pro v. 2.0 PC software. Morphology of sporulated oocysts was then compared with published descriptions of coccidia species infecting *Apodemus* (Musaev and Veisov 1965,

Pellérdy 1974, Arnastauskiene et al. 1978, Wash et al. 1985, Hůrková et al. 2005).

DNA extraction, PCR and sequencing

Genomic DNA of coccidia was isolated by commercial kit (FastDNA SPIN Kit for Soil, MP Biomedicals). Mitochondrial gene for cytochrome c oxidase subunit I (COI, ~700 bp) was selected as the most suitable genetic marker: this gene has previously been successfully applied to resolve intraspecific variability within fowl *Eimeria* species (Schwarz et al. 2009). PCR reactions were performed at a 25 µl volume with HotStarTaq DNA polymerase (Qiagen). Primers and PCR protocols were adopted from a publication by Schwarz et al. (2009). PCR products were enzymatically purified and sent to Macrogen, Inc. (Amsterdam, the Netherlands) for sequencing on an automatic 3730XL DNA analyzer. For the correct identification of *Apodemus* species, the host DNA was extracted by commercial kit (NucleoSpin Tissue, Macherey-Nagel) and mitochondrial cytochrome b gene together with mitochondrial DNA control region (D-loop) were amplified by PCR (Martin et al. 2000, Bellinvia 2004) and sequenced.

Sequence alignment, phylogenetic analyses and population structure

Obtained sequences were identified by BLAST analysis (www.ncbi.nlm.nih.gov), manually adjusted using the SequenceScanner (Applied Biosystems), EditSeq and SeqMan (DNASTAR Inc.) programs, and deposited in the GenBank database (NCBI) under the Accession Nos. xx-xx. Alignments were created and adjusted in BioEdit program (Hall 1999) in the aminoacid mode. The alignments were then switched to nucleotide mode and used for the analyses. Evolutionary relationships and population structure in *Eimeria* spp. were analyzed using 3 phylogenetic approaches (maximum parsimony - MP, maximum likelihood - ML, and Bayesian inference - BI) and methods of population genetics (haplotype networks generated by TCS program). Four different computer programs were employed for phylogenetic and genealogical analyses - PAUP v. 4.0b10 (Swofford 2001), Phyml v. 2.4.3 (Guindon and Gascuel 2003), MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001) and TCS v. 1.21 (Clement et al. 2000). Most suitable evolutionary models were selected with jModeltest program (Posada 2008, 2009). The trees

were visualized using TreeView v. 1.6.6 (Page 1996) and adjusted in the Adobe Illustrator CS5 v. 15.0 (Adobe Systems Inc.).

Results and discussion

Altogether, 44 coccidia specimens (43 *Eimeria* and 1 *Isospora*) of *Apodemus* spp. were gathered for the population structure. The parasites were retrieved from 3 host species (11 individuals of *Apodemus agrarius*, 29 *A. flavicollis* and 4 *A. sylvaticus*) with overlapping areas of distribution, by sampling across Europe (Czech Republic, England, France, Germany, Italy, Macedonia and Slovak Republic) (Fig. 1; Table 1). Morphological traits of sporulated oocysts of collected *Eimeria* samples corresponded to the descriptions of four species, *E. alorani*, *E. apionodes*, *E. jerfinica* and *E. kaunensis* (Musaev and Veisov 1965, Pellérdy 1974, Arnastauskiene et al. 1978, Hůrková et al. 2005) (Table 2). Sporulated oocysts obtained from *A. flavicollis* sample B13 and identified as *Isospora* sp. did not correspond to a so far described species from the genus *Apodemus*, *Isospora uralicae* Svanbaiev, 1956. Compared to isosporan species reported from murid rodents and also from animals that may occur in sympatry with field mice, the oocyst morphology of this coccidium is most similar to *I. araneae* Golemansky, 1978 described from shrews (Pellérdy 1974).

The length of 43 COI sequences of obtained *Eimeria* samples ranged between 500 and 779 bp, with the GC content of ~36 %. The COI sequence of a single specimen, morphologically corresponding to the genus *Isospora* and found in *A. flavicollis*, was 771 bp long, with the GC content of 35 %. When analyzed by MP, ML and BI, the COI sequences of *Eimeria* spp. split into 6 distinct and well-supported clades. While the relationships among the clades varied with the method (Fig. 2), the composition of the clades was identical in all analyses. The deep distinction among the clades obtained by phylogenetic analyses also reflected the results of haplotype network analysis performed in TCS program. When collapsed into haplotypes, the 43 eimerian sequences of COI generated 3 major clades (A, B and C) with 11 unique haplotypes (Fig. 3; Tables 1, 2). The haplotype distribution was relatively uneven; while a majority of the haplotypes was represented by 1-5 sequences, the two most abundant

haplotypes, H1 and H6, were represented by 13 and 8 samples, respectively (Tables 1, 2). As expected, the single sequence of the sample morphologically determined as *Isospora* sp. formed a distant, separate branch in both phylogenetic tree and TCS haplotype network.

The current state of knowledge on coccidia provides only limited information on intraspecific structure and the significance of both host-preference and geography. For example, Hnida and Duszynski (1999) did not find any intraspecific variability for the 18S rRNA gene within multiple isolates of 4 rodent *Eimeria* species of different geographic origin. On the contrary, a notable genetic variation between strains of chicken *Eimeria* species was described by Barta et al. (1998) and Lew et al. (2003), based on analyses of the ITS regions.

Indication of a possible intraspecific pattern in coccidia infecting wild-living rodents was already noted in MS no. 5. Against expectation, analyses in a broader phylogenetic context revealed great phylogenetic diversity of 11 *Eimeria* samples obtained from the genus *Apodemus*. While an exact taxonomic status of analysed samples and their precise position could not be entirely clear from the available topologies, they evidently clustered at least at 4 different places in the tree and covered quite a large phylogenetic span (MS no. 5, Fig. 2).

This study brings several interesting findings regarding the origin and genealogy of *Apodemus*–specific *Eimeria*. Phylogenetic position of different samples from single *Apodemus* sp. in several distant eimerian lineages shows that these parasites switched multiple times independently to the same host. Their branches are intertangled not only with the eimerians from other *Apodemus* spp. but even with samples obtained from different host genera (Fig. 2). This is in contrast to for example rabbit-specific eimerians, where 11 previously described species proved to form a monophyletic clade, indicating that they diversified on the host (Kvičerová et al. 2008).

Another interesting phenomenon is that despite their distribution among several clusters in the tree, all *Apodemus*–associated *Eimeria* fall only in some particular subtrees. It is remarkable that eimerians infecting *A. agrarius* always form separate lineages even inside the clades of eimerians from *A. flavicollis*. However, only samples from Eastern Slovakia are yet available for *A. agrarius*. Therefore, before attempting any serious conlusion, it would be particularly

interesting to enlarge the present data set also with samples from the Czech Republic (and/or from other countries). Similarly, *Eimeria* species from *A. flavicollis* tend to cluster together; however, eimerians from *A. sylvaticus*, represented only by 4 samples, are scattered among the *A. flavicollis* lineages. Our results indicate that *Apodemus*-associated *Eimeria* tend to cluster according to the host species rather than to the geographic origin (Figs. 1, 3).

Regarding the *Eimeria* species/morphotypes revealed by microscopy of sporulated oocysts, it is evident that at least one species, namely *E. apionodes*, is not monophyletic and its 5 haplotypes cluster at three different places in the trees (Fig. 2; Table 2).

This study also reveals new aspects regarding the host specificity of *Apodemus*–associated *Eimeria*; it is evident that these species are not as strictly host-specific as was previously believed. At least ten of them (namely *E. alorani*, *E. apionodes*, *E. apodemi*, *E. argenteus*, *E. hungaryensis*, *E. inuyamensis*, *E. jerfinica*, *E. kaunensis*, *E. montgomeryae* and *E. uptoni*) – that is almost a half of so far described species, are able to infect more than a single *Apodemus* species (Lewis and Ball 1983, Wash et al. 1985, Higgs and Nowell 1991, Hůrková et al. 2005, Kvičerová, this study).

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Table 1. Origin of the obtained haplotypes.

(CZ – Czech Republic, DE – Germany, FR – France, IT – Italy, MK – Macedonia, SK – Slovak Republic, UK – England)

Apodemus agrarius (11 COI sequences of *Eimeria* spp.)

Sample name	Locality	District, Region/Province	Country of origin	Haplotype
AGR 21455	Rozhanovce	Košice-Okolie, Košický	SK	**H8**
AGR 21617	Šebastovce	Košice, Košický	SK	**H7**
AGR 21649	Rozhanovce	Košice-Okolie, Košický	SK	**H7**
AGR 21650	Rozhanovce	Košice-Okolie, Košický	SK	**H3**
AGR 21655	Rozhanovce	Košice-Okolie, Košický	SK	**H3**
AGR 21657	Rozhanovce	Košice-Okolie, Košický	SK	**H7**
AGR 21668	Rozhanovce	Košice-Okolie, Košický	SK	**H3**
AGR 21831	Botanic Garden of Košice	Košice, Košický	SK	**H11**
AGR 21882	Botanic Garden of Košice	Košice, Košický	SK	**H3**
AGR 21906	Botanic Garden of Košice	Košice, Košický	SK	**H10**
AGR 21914	Botanic Garden of Košice	Košice, Košický	SK	**H8**

Apodemus flavicollis (28 COI sequences of *Eimeria* spp. and 1 COI sequence of *Isospora* sp.)

Sample name	Locality	District, Region/Province	Country of origin	Haplotype
AF 1	Solany	Litoměřice, Ústecký	CZ	**H6**
AF 2	Solany	Litoměřice, Ústecký	CZ	**H9**

AF 2 VK	Velký Kosíř	Prostějov, Olomoucký	CZ	**H2**
AF 4	Boršov nad Vltavou	České Budějovice, Jihočeský	CZ	**H1**
AF 4 VM	Pastýřské kameny	Děčín, Ústecký	CZ	**H1**
AF 8	Stružná	Karlovy Vary, Karlovarský	CZ	**H6**
AF 10	Stružná	Karlovy Vary, Karlovarský	CZ	**H5**
AF 11	Chotěborky	Trutnov, Královéhradecký	CZ	**H6**
AF 12	Stružná	Karlovy Vary, Karlovarský	CZ	**H9**
AF 15	Stružná	Karlovy Vary, Karlovarský	CZ	**H1**
29 AF	Stružná	Karlovy Vary, Karlovarský	CZ	**H1**
SB 3	Litvínov	Most, Ústecký	CZ	**H1**
SB 5	Litvínov	Most, Ústecký	CZ	**H1**
SB 11	Litvínov	Most, Ústecký	CZ	**H5**
RR 196	Litvínov	Most, Ústecký	CZ	**H6**
OB I 173	Litvínov	Most, Ústecký	CZ	**H6**
AF 21423	Rozhanovce	Košice-Okolie, Košický	SK	**H1**
AF 21833	Botanic Garden of Košice	Košice, Košický	SK	**H1**
AF 21898	Botanic Garden of Košice	Košice, Košický	SK	**H1**
AF 22027	Hýľov, Hlboká dolina	Košice-Okolie, Košický	SK	**H5**
ITAF 10	Brinzio	Varese	IT	**H1**
ITAF 20	Civitanova del Sannio	Isernia, Molise	IT	**H6**
AF 2 D	Pinkowitz	Meissen	DE	**H5**
AF 10 D	Pinkowitz	Meissen	DE	**H1**
AF 95 D	Torgau	Torgau-Oschatz	DE	**H1**
MAC 1/3	Popova Šapka	Tetovo, Tetovo	MK	**H6**
MAC 9/8	Nižepole (Pelister)	Bitola	MK	**H4**
MAC 10/8	Kruševo	Krusevo	MK	**H9**

AF B 13 (*Isospora*)	Litvínov	Most, Ústecký	CZ	-

Apodemus sylvaticus (4 COI sequences of *Eimeria* spp.)

Sample name	**Locality**	**District, Region/Province**	**Country of origin**	**Haplotype**
AS 08/50	Ashford	South East	UK	**H10**
AS 08/53	Ashford	South East	UK	**H5**
AS 07/104	Toulouse	Haute-Garonne	FR	**H1**
ItBA 7	Bubbiano	Milano	IT	**H6**

Table 2. *Eimeria* species/morphospecies determined based on oocyst morphology and the details of sampled specimens.

***Eimeria* species (morphospecies)**	**Sample name**	**Host species**	**Haplotype**
E. alorani	AGR 21650	*A. agrarius*	H3
	AGR 21655	*A. agrarius*	H3
	AGR 21668	*A. agrarius*	H3
	AGR 21882	*A. agrarius*	H3
E. apionodes	AF 1	*A. flavicollis*	H6
	AF 8	*A. flavicollis*	H6
	AF 11	*A. flavicollis*	H6
	RR 196	*A. flavicollis*	H6
	OB I 173	*A. flavicollis*	H6
	ITAF 20	*A. flavicollis*	H6
	MAC 1/3	*A. flavicollis*	H6
	ItBA 7	*A. sylvaticus*	H6
	AGR 21617	*A. agrarius*	H7
	AGR 21649	*A. agrarius*	H7
	AGR 21657	*A. agrarius*	H7
	AGR 21831	*A. agrarius*	H11
	AF 2 VK	*A. flavicollis*	H2
	AF 4	*A. flavicollis*	H1
	AF 4 VM	*A. flavicollis*	H1
	AF 15	*A. flavicollis*	H1
	AF 29	*A. flavicollis*	H1
	SB 3	*A. flavicollis*	H1
	SB 5	*A. flavicollis*	H1
	AF 10 D	*A. flavicollis*	H1
	AF 95 D	*A. flavicollis*	H1
	ITAF 10	*A. flavicollis*	H1
	AF 21423	*A. flavicollis*	H1
	AF 21833	*A. flavicollis*	H1
	AF 21898	*A. flavicollis*	H1
	AS 07/104	*A. sylvaticus*	H1
E. jerfinica	AF 2	*A. flavicollis*	H9
	AF 12	*A. flavicollis*	H9
	MAC 10/8	*A. flavicollis*	H9

	AS 08/50	*A. sylvatius*	H10
	AGR 21455	*A. agrarius*	H8
	AGR 21906	*A. agrarius*	H10
	AGR 21914	*A. agrarius*	H8
E. kaunensis	AF 10	*A. flavicollis*	H5
	SB 11	*A. flavicollis*	H5
	AF 2 D	*A. flavicollis*	H5
	AF 22027	*A. flavicollis*	H5
	AS 08/53	*A. sylvaticus*	H5
	MAC 9/8	*A. flavicollis*	H4

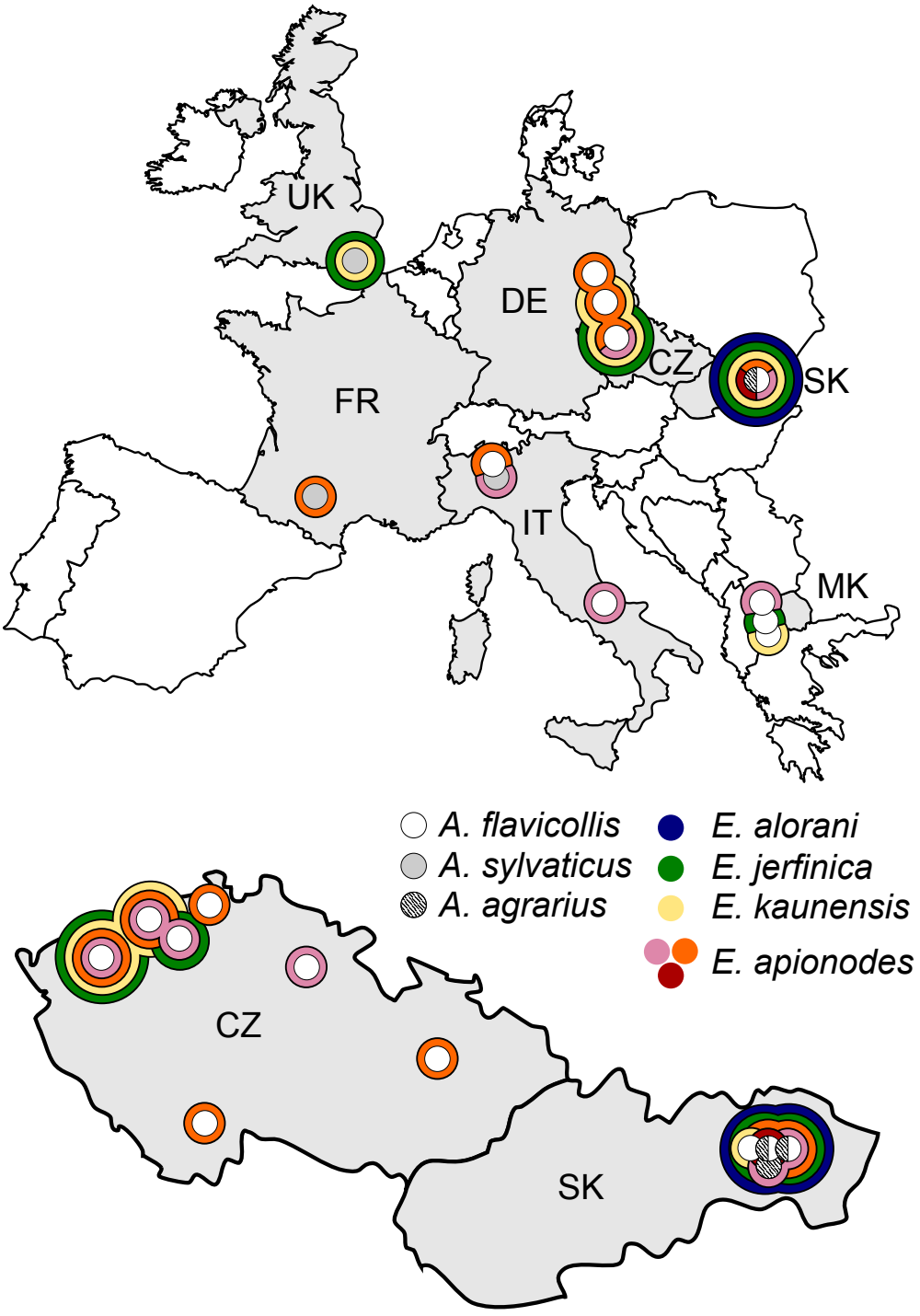

Fig. 1. The origin of the samples and individual genetic lineages.

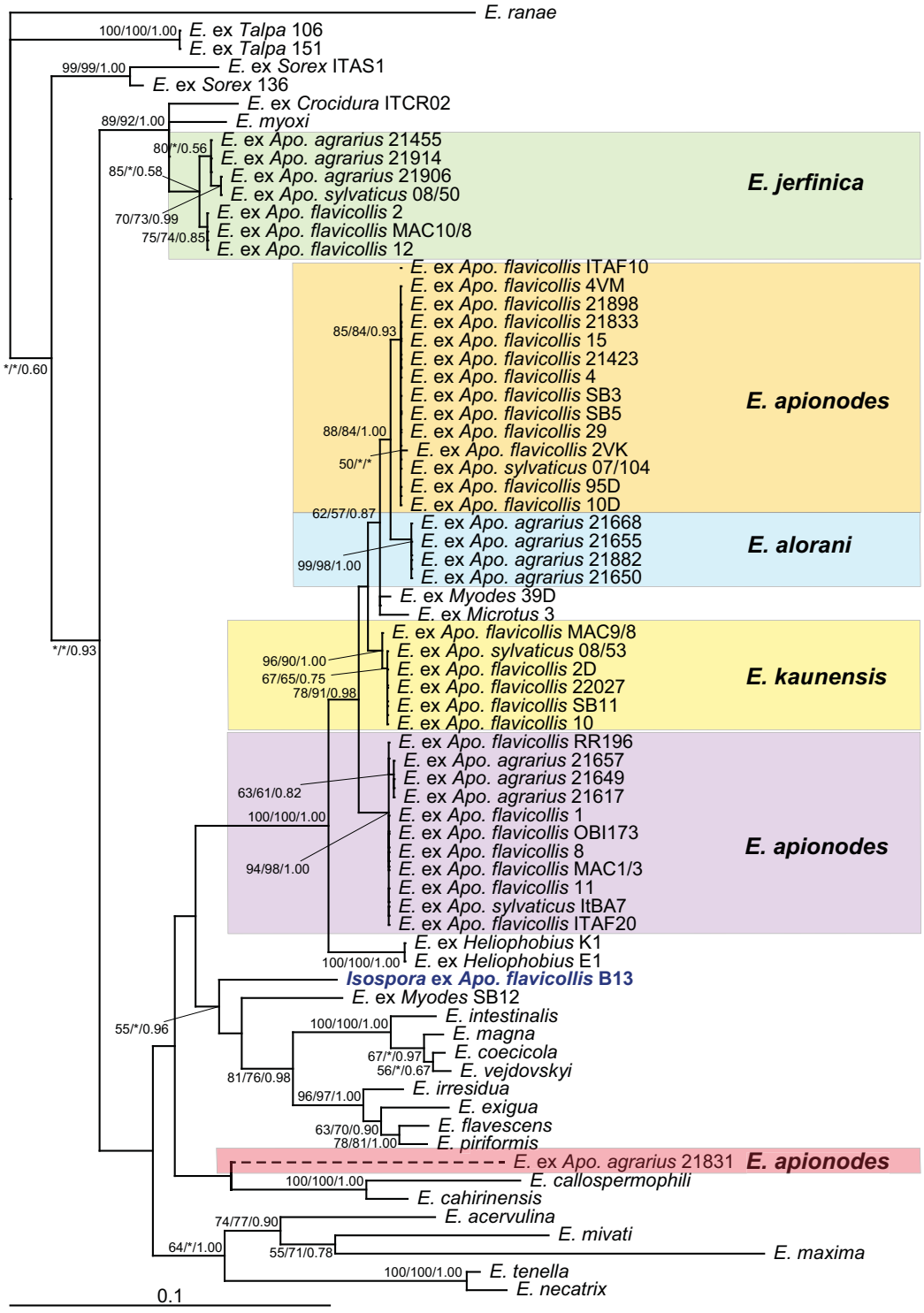

Fig. 2. Phylogenetic tree of the COI obtained by ML. The tree is rooted with *Eimeria ranae*. Numbers at the nodes show bootstrap values for ML and MP, and posterior probability under BI (the values are provided only for the nodes also present in ML and MP trees). Bootstrap supports and posterior probabilities lower than 50% or 0.50, respectively, are marked with asterisk (*). The interrupted line indicates branching not corresponding to the BI analysis.

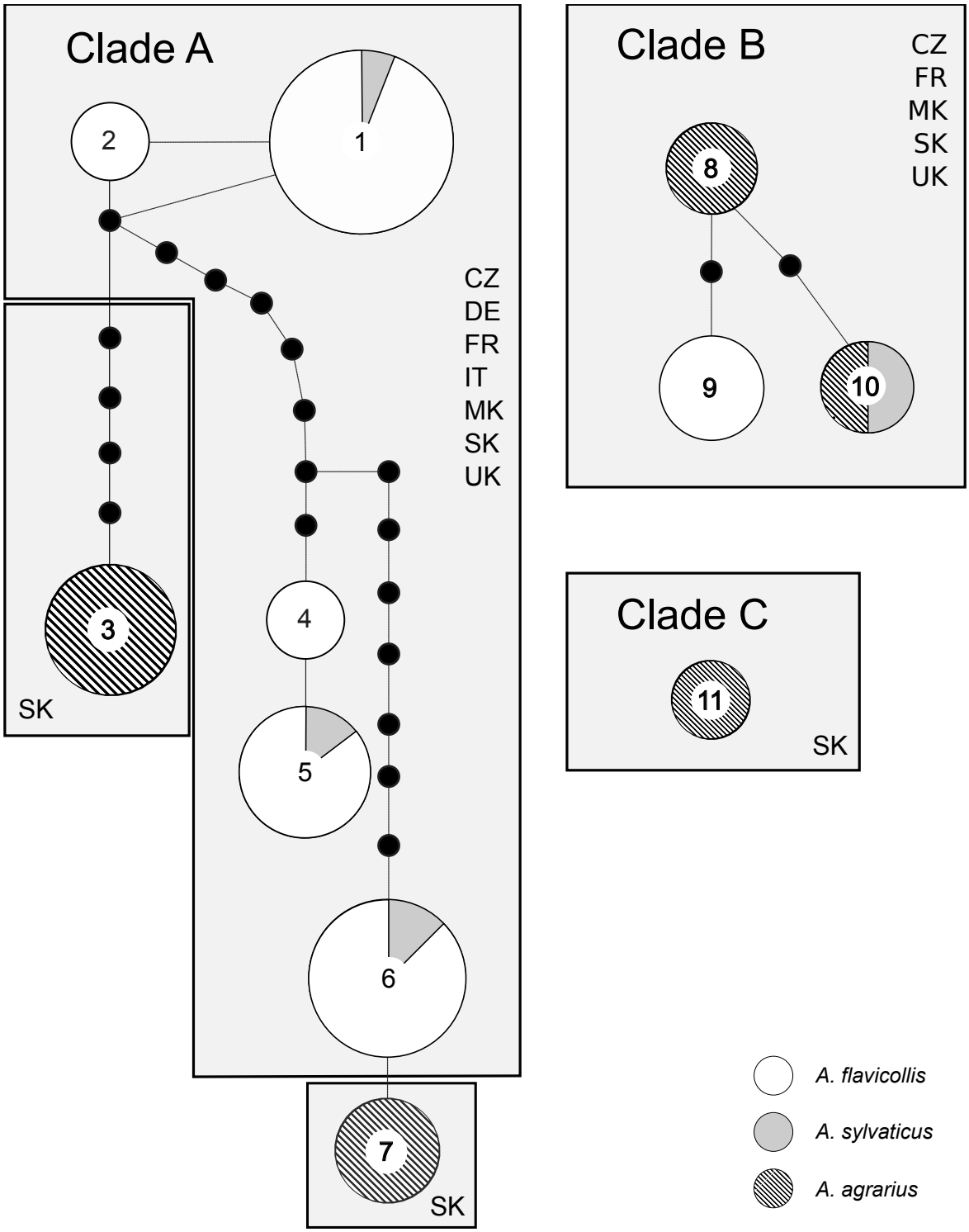

Fig. 3.
Haplotype networks of 43 *Eimeria* specimens from *Apodemus* spp. obtained by TCS analysis. Geographic origin and host species are listed in Table 1.

CURRICULUM VITAE

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1998 - 2005: Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Brno.

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Thesis: Coccidia of the genus *Eimeria* from the spiny mice (*Acomys* spp.) – experimental study on biology, pathogenicity and life cycle. [121 pp., in English].
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2005 - present: internal Ph.D. study in Parasitology, Faculty of Science, University of South Bohemia, České Budějovice.
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2005 - present: Research Scientist, Biology Centre, Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic.

2005 - present: Research Scientist, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Abroad stays and fellowships:

2008 (April - May):
University of Wyoming in Casper, Casper, Wyoming, USA:
Faecal samples of small mammals from Alaska and Siberia, primates from Ghana, and passerine birds from China: microscopy and molecular analyses.

2008 - 2011 (several short-term stays):
Institute of Parasitology (former Zoology), Slovak Academy of Sciences, Košice, Slovakia. KONTAKT Programme.
Field samplings with the focus on ectoparasites and endoparasites of wild-living rodents.

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2008 – 2010:
Supervisor of Bachelor degree student Anna Mácová (project: Coccidia in rodents of the genus *Apodemus*), Department of Parasitology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

2010 – present:
Supervisor of Master degree student Anna Mácová (project: Population structure and phylogenetic relationships in coccidia infecting rodents of families Muridae and Arvicolidae), Department of Parasitology, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

2007 – 2009:
Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Biology of Parasitic Arthropods*. (all seminars). Spring 2007 and 2009.

2008 – 2010:
Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Mammal keeping*. (1 lecture and 1 seminar). Spring 2008 and 2010.

2006 – 2011:
Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Field practice "Vomáčka"– rodent trappings & dissections, demonstration of parasites*. Autumn 2006, 2007, 2010 and 2011.

Successful grant applications or participations:

GA JU 46/2006/P-BF (2006) (J. Kvičerová):
Eimeria of rodents and lagomorphs – a model for studying cophylogenetic associations between protozoan parasites and their hosts. Applicant.

International Scientific and Technical Cooperation (KONTAKT) MEB 080897 (2008 -2009) (J. Štefka):
Ekologie hlodavců rodu *Apodemus* a *Microtus* a genetika jejich parazitů ve vybraných oblastech Slovenska a České republiky. (Ecology of rodents of the genus *Apodemus* and *Microtus* and genetics of their parasites in selected localities in Slovakia and Czech Republic). Co-applicant.

VaV SP/2d4/61/08 (2008 - 2010) (V. Vohralík):
Investigation on the biology, ecology and distribution of the ground squirrel (*Spermophilus citellus*) on the context of its Action plan in the Czech Republic. Member of the research team.

GA ČR 206/08/1019 (2008 - 2011) (V. Hypša):
Genealogy and population structure of parasites in relation to host specificity and biogeography. Member of the research team.

International Scientific and Technical Cooperation (KONTAKT) MEB 0810106 (2010 -2011) (J. Kvičerová):
Epidemiologický význam drobných savců a jejich parazitů v modelových oblastech České a Slovenské republiky. (Epidemiological significance of small mammals and their parasites in model localities in the Czech Republic and Slovak Republic). Applicant.

GA ČR P506/11/1738 (2011 - 2014) (V. Hypša):
Population structure and evolutionary relationships of the intracellular parasite *Hemolivia mauritanica* (Sergent and Sergent, 1904). Member of the research team.

GA ČR P505/12/1620 (2012 - 2015) (V. Hypša):
Population genetics, demography and molecular evolution in interspecific associations: comparative study of two complex parasitic/symbiotic systems. Member of the research team.

Publications:

Kvičerová J, Ptáčková P, Modrý D (2007) Endogenous development, pathogenicity and host specificity of *Eimeria cahirinensis* Couch, Blaustein, Duszynski, Shenbrot, and Nevo, 1997 (Apicomplexa: Eimeriidae) from *Acomys dimidiatus* (Cretzschmar, 1826) (Rodentia: Muridae) from the Near East. *Parasitology Research* 100 (2): 219-226. doi: 10.1007/s00436-006-0251-7.

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Gustavsen CR, **Kvičerová J**, Dickinson H, Heller RS (2009) *Acomys*, the closest relatives to Gerbils, do express Pdx-1 protein and have similar islet morphology to Gerbils. *Islets* 1 (3): 191-197. http://dx.doi.org/10.4161/isl.1.3.9557.

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Fričová J, Stanko M, Mošanský L, **Kvičerová J** (2011) Small mammals - reservoir hosts of blood pathogens in urban surroundings. *Folia Veterinaria* 55, Supplementum 1: 36-38.

Conference presentations:

Kvičerová J (2005) Coccidia of the genus *Eimeria* from the spiny mice (*Acomys* spp.) – experimental study on biology, pathogenicity and life cycle. Tomáškovy mikrobiologické dny, June 8-10, Brno, CZ. Lecture.

Kvičerová J, Mikeš V (2007) Parazitace plcha zahradního (*Eliomys quercinus*) v ČR. Jírovcovy protozoologické dny, April 30-May 4, Vranov nad Dyjí, CZ. Lecture.

Kvičerová J, Mikeš V, Hulová Š (2007) Parazitofauna drobných savců. Tomáškovy mikrobiologické dny, June 7-8, Brno, CZ. Lecture.

Kvičerová J (2007) Parazitace sysla obecného (*Spermophilus citellus*) v ČR. Výskum a ochrana cicavcov na Slovensku (VOCS), October 12-13, Zvolen, SK. Lecture.

Kvičerová J, Hypša V (2008) Phylogenetic relationships and evolutionary patterns of *Eimeria* (Apicomplexa: Eimeriidae). ASP, June 27-30, Arligton, Texas, USA. Lecture.

Kvičerová J, Matějů J, Hulová Š, Nová P, Uhlíková J (2008) Endoparasites of ground squirrels (*Spermophilus citellus*) from the Czech Republic and Slovakia. II. European ground squirrel meeting (EGSM), October 1-5, Svatý Jan pod Skalou, CZ. Poster.

Stanko M, Fričová J, Várfalvyová D, Čisláková L, **Kvičerová J**, Jareková J, Karbowiak G (2008) Parasite - host relationships and epidemiological role of *Mus spicilegus* (Rodentia) in Slovakia. EMOP, August 24-28, Paris, FR. Poster.

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Stanko M, Fričová J, Mošanský L, **Kvičerová J** (2011) Akú úlohu zohráva myš kopčiarka (*Mus spicilegus*) v prírodných ohniskách ochorení? Zoologické dny, February 17-18, Brno, CZ. Lecture.

Kvičerová J, Mácová A, Hypša V (2011) *Apodemus* and *Eimeria*: Population structure, host specificity and biogeography. ASP, June 1-4, Anchorage, Alaska, USA. Lecture.

Fričová J, Stanko M, Mošanský L, **Kvičerová J** (2011) Drobné cicavce – rezervoároví hostitelia krvných patogénov v urbánnom prostredí. Ekológia a veterinárna medicína VIII., September 22-23, Košice, SK. Lecture.

Široký P, **Kvičerová J**, Hypša V (2011) The past and the future of research on host-parasite complex *Testudo-Hyalomma-Hemolivia*. Mediterranean Congress "Animal Biodiversity and Ecology of Health", October 15-18, Annaba, Algeria. Lecture.

Invited lecture:

Kvičerová J (2009) Coccidia from Mammals: Molecular Insight on Phylogenetic Relationships, Host Specificity and Morphology. ASP, August 14-17, Knoxville, Tennessee, USA.

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