# Phylogenetic relationships among *Eimeria* spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features

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SUMMARY

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.

Key words: coccidia, *Eimeria*, oocysts, *Oryctolagus*, host specificity, 18S rDNA, phylogenetic analysis.

## INTRODUCTION

Investigation of the relationship between parasite phylogeny and various biological traits can provide two important types of information. It serves as a background for understanding a parasite's evolutionary history, including evolution of the host-parasite interaction, and it indicates markers suitable for diagnostic and/or taxonomic purposes.

An immense amount of data and analyses were produced during the last few years for various groups of parasites (Page, 1996*a*; Barta *et al*. 1997; Hoberg *et al*. 1997; Proctor, 1999; Jousson *et al*. 2000; Paterson *et al*. 2000; Johnson and Clayton, 2001; Nieberding *et al*. 2004, 2005; Banks *et al*. 2006) including apicomplexan taxa, such as *Adelina*, *Atoxoplasma*, *Cyclospora*, *Goussia*, gregarines, etc. (Carreno *et al*. 1999, Eberhard *et al*. 1999, Barta *et al*. 2001, Jirků *et al*. 2002, Leander *et al*. 2006, Schrenzel *et al*. 2005, Kopečná *et al*. 2006). Within coccidia, attention has been mainly given to the evolution of Sarcocystidae, the tissue cyst-forming coccidia with a typically heteroxenous life-cycle. A number of studies investigated the phylogenetic significance of specificity to intermediate and definitive hosts, or affinity to various tissues (Votýpka *et al*. 1998; Doležel *et al*. 1999; Šlapeta *et al*. 2003). For example, the species of the genus *Sarcocystis* were suggested to co-evolve with their final, rather than intermediate, hosts (Doležel *et al*. 1999; Holmdahl *et al*. 1999; Šlapeta *et al*. 2003). Based on this finding, the final host is supposed to represent an ancestral type of the host within Sarcocystidae (Barta, 1989; Tenter *et al*. 1992).

Paradoxically, only few studies have been devoted to the largest group of coccidia, the monoxenous parasites of the genus *Eimeria* Schneider, 1875. Thus, evolution of various traits within this genus, such as morphology, host-specificity or pathogenicity, remains very unclear. Since *Eimeria* are typically parasites with extremely narrow host spectra, analysis of the dependence between phylogeny and host-specificity is relatively straightforward. The molecular data currently available show significant correlation between phylogenetic relationships and host-specificity; the genus splits into well-formed lineages, such as livestock-specific or fowl-specific species. This pattern, however, is not universal. For example, with an increasing number of available sequences, it became clear that rodent-associated fauna is composed of species from several lineages. While each of these lineages itself is monophyletic, their relationships are paraphyletic or even polyphyletic. Even single rodent species can harbour a set of parasites from different lineages. For example, certain species of the genera *Peromyscus* Gloger, 1841, *Onychomys* Baird, 1858 or *Dipodomys* Gray, 1841 were shown to host the parasite from at least

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2 lineages (Zhao and Duszynski, 2001*a*, *b*; Morrison *et al*. 2004; Matsubayashi *et al*. 2005).

An effort to put the morphological and biological features of *Eimeria* into a phylogenetic framework can be found in several studies; all of them focused on rodent species. First, Reduker *et al*. (1987) recognized 2 different *Eimeria* lineages by cladistic and phenetic analyses of isozyme banding patterns, sporulated oocysts morphology and life-history traits. Similar results, based on phylogenetic analyses of molecular sequence and riboprinting data, were reported by Hnida and Duszynski (1999*a*, *b*). Another study by Zhao and Duszynski (2001*a*, *b*) determined the phylogenetic relationships among *Eimeria* species parasitizing 3 rodent families (Muridae, Heteromyidae and Cricetidae) based on plastid 23S rDNA, ORF470 and nuclear 18S rDNA sequences. Their results split the rodent *Eimeria* into 2 lineages according to the oocyst size/shape and the presence/absence of the oocyst residuum (OR).

The establishment of any concise evolutionary scenario across *Eimeria* is, however, limited by relatively scarce sampling of molecular data. Here, we focus on a so far neglected group of *Eimeria*, a group of rabbit-associated species. Of 65 *Eimeria* spp. described from 10 genera of lagomorphs (Duszynski and Upton, 2001), 11 parasitize the domestic rabbit, *Oryctolagus cuniculus* f. *domesticus* and also the wild Old World rabbit, *Oryctolagus cuniculus* (Coudert *et al*. 1979; Pakandl, 1988; Coudert *et al*. 1995). The whole rabbit-specific fauna is relatively heterogeneous in respect to oocyst morphology, location within host or pathogenicity (Pellérdy, 1965; Coudert *et al*. 1995; Eckert *et al*. 1995). While 10 of the rabbit *Eimeria* species develop in the host intestine, *E. stiedai* parasitizes the epithelium of biliary ducts (Metzner, 1903; Minning, 1936; Rutherford, 1943). Some species are highly pathogenic, causing apathy, inapetency, severe growth depression, tympany, diarrhoea or even mortality (*E. stiedai*, *E. flavescens*, *E. intestinalis*); others express mild (*E. magna*, *E. piriformis*, *E. irresidua*, *E. media*) or low (*E. coecicola*, *E. perforans*, *E. exigua*, *E. vejdovskyi*) pathogenicity for their hosts (Pellérdy, 1965; Coudert *et al*. 1979; Licois and Mongin, 1980; Licois and Coudert, 1982; Vítovec and Pakandl, 1989; Coudert *et al*. 1995; Pakandl and Coudert, 1999).

A number of studies have been published on rabbit coccidia. The majority of them were based on morphological and ultrastructural characters and focused on the descriptions of the life-cycle and selection of precocious lines of these parasites. The 3 molecular studies published thus far focused on diagnostics and inter- and intraspecific variation using RAPD techniques (Ceré *et al*. 1995, 1996, 1997). However, many questions concerning the rabbit-specific species can only be addressed with sufficient molecular data providing a clear phylogenetic signal. It has never been tested whether the rabbit-specific *Eimeria* form a monophyletic branch of species that has diversified on the rabbit hosts, or represent an assemblage of unrelated species colonizing the same host.

In this study, we present the first molecular data on rabbit-associated parasites to address the two following questions. Is the morphologically diverse group of rabbit-specific *Eimeria* a monophyletic branch or a polyphyletic assemblage of distant species? Is there a correlation between the phylogeny and any of the morphological/biological features of these species?

## MATERIALS AND METHODS

### *Parasites*

Eimerian oocysts were obtained from faeces of naturally infected domestic rabbits (*Oryctolagus cuniculus* f. *domesticus*) from various localities in the Czech Republic and France. Pure strains were prepared by a single-oocyst infection, or by a method previously described by Pakandl *et al*. (2003). Briefly, about 20 individually collected oocysts examined by microscopy were administered into the oral cavity of a rabbit. The SPF (specific pathogen-free) rabbits originating from the Charles River Laboratories (Germany) and supplied by AnLab (Prague, Czech Republic) were used in the experiments.

Pure strains were obtained after 1 cycle of multiplication, and maintained in laboratory-reared rabbits. The purity of the strains was confirmed by microscopical examination. Oocysts were recovered and purified as described by Coudert *et al*. (1995), and were sporulated in 2·5% potassium dichromate ($K_2Cr_2O_7$) at room temperature on a shaker to ensure good aeration. Obtained oocysts were stored in potassium dichromate solution at ~4 °C until used. All experiments comply with the current laws of the Czech Republic and were officially permitted.

### *DNA extraction, PCR and sequencing*

Oocysts (approximately $3 \times 10^5$) were washed repeatedly by resuspension in sterile PBS, disrupted using a combination of glass-sand grinding and repetitive freeze-thaw cycles (liquid nitrogen – hot water) and incubated overnight on ice with lysis buffer (180 $\mu$l of NET 50, 24 $\mu$l of 30% sarcosin, 4 $\mu$l of pronase E). After digestion, total coccidian DNA was extracted using a standard phenol–chlorophorm protocol followed by ethanol precipitation.

Specific primers amplifying aproximately 1500 bp long region of 18S rDNA were designed according to published sequences of related *Eimeria* species. Their postion corresponded to the sites 62–86

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(primer EF: 5′-GAAACTGCGAATGGCTCA-TT-3′) and 1568–1586 (primer ER: 5′-CTTGCG-CCTACTAGGCATTC-3′) according to *E. tenella* sequence U67121. The PCR reaction was carried out in a 25 $\mu$l volume under standard conditions. The HotStarTaq DNA Polymerase (Qiagen) was used in all PCR reactions. The thermocycling protocol consisted of an initial inactivation-denaturation step at 95 °C for 4 min, followed by 30 cycles of 92 °C denaturation for 45 s, primer annealing at 53 °C for 45 s, and elongation at 72 °C for 90 s. A final primer extension of 10 min at 72 °C completed the amplification process. Amplicons were directly cloned into the pGEM – T Easy Vector (Promega). Plasmids with DNA inserts of the expected size were isolated using a Plasmid Miniprep Spin Kit (Genomed). The sequencing of 5 clones from each sample was performed with the PCR primers and 4 internal primers using the Big Dye terminator cycle sequencing ready reaction kit (ABI Prism, Perkin Elmer) on an automatic sequencer ABI 3130xl. Obtained sequences were identified by BLAST analysis. The sequences were deposited in the GenBank database (NCBI) under the Accession numbers EF694007-EF694017.

### *Sequence alignment and phylogenetic analysis*

Sequences were assembled and edited using the DNASTAR program package (DNASTAR, Inc). The phylogenetic position and arrangement of the new sequences was investigated in 2 steps. First, we designed an *Eimeria* matrix to examine whether the rabbit-specific *Eimeria* formed a monophyletic branch. For this purpose, we downloaded *Eimeria*, *Isospora*, *Caryospora* and *Cyclospora* sequences of 18S rDNA currently available in GenBank (NCBI) (Table 1). The sequences were aligned in the MegAlign program (DNASTAR, Inc) using the ClustalW algorithm, and the matrix was analysed by fast neighbour-joining method (NJ) implemented in PAUP* version 4.0b10 (Swofford, 2001). Since several studies demonstrated that 18S rDNA is insufficient to establish deeper phylogenetic relationships among coccidian lineages (Olsen and Woese 1993; Mugridge *et al*. 2000; Tenter *et al*. 2002), this step was only taken to verify that the rabbit-specific *Eimeria* form a monophyletic branch and can be further treated within taxonomically restricted context. After revealing the rabbit-specific *Eimeria* as a monophyletic cluster reliably characterized by long common branch, a *Rabbit* matrix was designed. It contained all sequences of rabbit-specific *Eimeria*, 6 eimerians from other hosts (*E. alabamensis*, *E. bovis*, *E. faurei*, *E. gruis*, *E. ovinoidalis*, *E. reichenowi*) and 2 outgroups, *Isospora belli* and *I. felis* (Table 1). This set of sequences was aligned in MegAlign program, manually adjusted in BioEdit (Hall, 1999) and analysed by more precise methods than the first matrix. It resulted in alignment of 1518 positions, containing 164 parsimony informative sites. Maximum parsimony (MP) was carried out using an exhaustive branch-and-bound search mode. Analysis was repeated with transition/transversion ratios set to 1 : 1, 1 : 2 and 1 : 3. A consensus cladogram was calculated from all trees obtained under the different parameter settings. Bootstrap values were calculated by 1000 replicates of heuristic search with ts/tv ratio set to 1 : 1. A maximum likelihood (ML) tree was calculated in PHYML version 2.4.3 (Guindon and Gascuel, 2003) under the GTR + Γ + I model, with parameters of gamma distribution and proportion of invariable sites estimated from the data. The trees were visualized and exported using TreeView version 1.6.6. (Page, 1996*b*).

## RESULTS

Partial sequences of 18S rDNA from 11 species of rabbit-infecting *Eimeria* were obtained. The sequences displayed a broad range of pairwise similarities, from 92% (e.g. *E. stiedai vs E. flavescens*) to more than 99% (*E. piriformis vs E. flavescens*) (Table 2). With 2 exceptions, the sequences were approximately of the same length (~1400 bp). The 2 exceptions were represented by *E. coecicola*, with a 20 bp long insertion, and particularly by *E. stiedai*, with an approximately 100 bp long deletion. When analysed in context of all *Eimeria* sequences available in GenBank, the rabbit-specific species formed a monophyletic cluster (result not shown; see the Materials and Methods section). This group was well-formed, possessing a relatively long common branch. The more restricted *Rabbit* matrix yielded several equally parsimonious trees; the number of the trees varied in relation to their ts/tv ratio (10, 26, 9). The trees exhibited high consistency indices (CI = 0·79–0·81, RC = 0·65–0·67) and their strict consensus produced a topology with several robust nodes supported by high bootstrap values (Fig. 1). The tree obtained by ML was entirely compatible with this topology. Of the morphological and biological characters (oocyst shape and size, presence/absence of oocyst inner structures, pathogenicity, site of infection, pre-patent period, patent period, sporulation time, number of asexual generations), mapped onto the topology, only the presence/absence of oocyst residuum strictly followed the phylogenetic division into 2 main branches (Fig. 1).

## DISCUSSION

Evolution of host specificity is one of the most intriguing questions of parasitological research. Amongst the coccidia, an important and diversified group of parasitic protists, *Eimeria* species are considered to exhibit high host specificity (Marquardt, 1973; Joyner, 1982; Long and Joyner, 1984; Hnida

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Table 1. Sequences included in the large *Eimeria* matrix

(Taxa used as outgroups for the *Rabbit* matrix are printed in bold.)

| Organism | Acc. number | Organism | Acc. number | Organism | Acc. number |
|---|---|---|---|---|---|
| *Caryospora bigenetica* clone 1 | AF060975 | *Eimeria gruis* clone G2-1/2 | AB205167 | *Eimeria reedi* | AF311642 |
| *Caryospora bigenetica* clone 2 | AF060976 | *Eimeria gruis* clone G2-3 | AB205168 | *Eimeria reichenowi* clone R1-1 | AB205170 |
| *Cyclospora cayetanensis* | AF111183 | *Eimeria gruis* clone G2-4 | AB205169 | *Eimeria reichenowi* clone R1-2 | AB205171 |
| *Cyclospora cercopitheci* strain Ethiopia 1 | AF111184 | *Eimeria gruis* type isolate GA | **AB243081** | *Eimeria reichenowi* clone R1-3 | AB205172 |
| *Cyclospora cercopitheci* strain Ethiopia 2 | AF111185 | *Eimeria gruis* type isolate GC | AB243082 | *Eimeria reichenowi* clone R1-4 | AB205173 |
| *Cyclospora colobi* | AF111186 | *Eimeria chaetodipi* | AF339489 | *Eimeria reichenowi* clone R1-5 | AB205174 |
| *Cyclospora papionis* | AF111187 | *Eimeria chobotari* | AF324214 | *Eimeria reichenowi* clone R2-1 | **AB205175** |
| *Cyclospora* sp. | U40261 | *Eimeria langebarteli* | AF311640 | *Eimeria reichenowi* clone R2-2 | AB205176 |
| *Cyclospora* sp. strain Gombe 22 | AF061566 | *Eimeria leucopi* | AF339491 | *Eimeria reichenowi* clone R2-3 | AB205177 |
| *Cyclospora* sp. strain Gombe 34 | AF061567 | *Eimeria maxima* strain Banksadde | DQ538348 | *Eimeria reichenowi* clone R2-4 | AB205178 |
| *Cyclospora* sp. strain Gombe 40 | AF061568 | *Eimeria maxima* strain Lianyungang | DQ538350 | *Eimeria reichenowi* clone R2-5 | AB205179 |
| *Eimeria acervulina* | U67115 | *Eimeria maxima* strain Shanghai | DQ136186 | *Eimeria reichenowi* type isolate RB | AB243083 |
| *Eimeria acervulina* strain Shanghai | DQ136187 | *Eimeria maxima* strain Shanghai | EF122251 | *Eimeria reichenowi* type isolate RD | AB243084 |
| *Eimeria acervulina* strain Yangda | DQ538351 | *Eimeria maxima* strain Townsend | DQ538349 | *Eimeria rioarribaensis* | AF307877 |
| *Eimeria adeneodei* | AF324212 | *Eimeria maxima* strain Tysons | DQ640012 | *Eimeria scabra* | AF279668 |
| *Eimeria ahsata* | AF338350 | *Eimeria meleagrimitis* | AF041437 | *Eimeria separata* | AF311643 |
| *Eimeria alabamensis* | **AF291427** | *Eimeria mitis* | U40262 | *Eimeria sevilletensis* | AF311644 |
| *Eimeria albigulae* | AF307880 | *Eimeria mitis* | U67118 | *Eimeria scholtysecki* | AF324216 |
| *Eimeria antrozoi* | AF307876 | *Eimeria mivati* | U76748 | *Eimeria telekii* | AF246717 |
| *Eimeria arizonensis* | AF307878 | *Eimeria necatrix* | DQ136185 | *Eimeria tenella* | U67121 |
| *Eimeria arnyi* | AY613853 | *Eimeria necatrix* | U67119 | *Eimeria tropidura* | AF324217 |
| *Eimeria bovis* | **U77084** | *Eimeria nieschulzi* | U40263 | *Eimeria weybridgensis* | AY028972 |
| *Eimeria brunetti* | U67116 | *Eimeria onychomysis* | AF307879 | *Isospora belli* | **U94787** |
| *Eimeria catronensis* | AF324213 | *Eimeria ovinoidalis* | **AF345997** | *Isospora felis* | **L76471** |
| *Eimeria crandallis* | AF336339 | *Eimeria papillata* | AF311641 | *Isospora gryphoni* | AF080613 |
| *Eimeria dipodomysis* | AF339490 | *Eimeria peromysci* | AF339492 | *Isospora ohioensis* | AF029303 |
| *Eimeria falciformis* | AF080614 | *Eimeria pilarensis* | AF324215 | *Isospora orlovi* | AY365026 |
| *Eimeria faurei* | **AF345998** | *Eimeria polita* | AF279667 | *Isospora robini* | AF080612 |
| *Eimeria gruis* clone G1-1 | AB205165 | *Eimeria porci* | AF279666 | *Isospora suis* | U97523 |
| *Eimeria gruis* clone G1-2/3/4 | AB205166 | *Eimeria praecox* | U67120 | *Eimeria reedi* | AF311642 |

Table 2. Percentage similarity calculated for rabbit-associated species of *Eimeria*

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *E. exigua* | | 97·7 | 98·3 | 97·8 | 98·1 | 98·2 | 91·7 | 97·3 | 98·6 | 98·5 | 97·2 |
| 2 | *E. intestinalis* | | | 97·5 | 98·7 | 98·9 | 99·0 | 92·3 | 98·1 | 97·6 | 97·5 | 97·7 |
| 3 | *E. irresidua* | | | | 97·5 | 97·9 | 98·0 | 92·9 | 97·1 | 98·1 | 98·1 | 97·3 |
| 4 | *E. magna* | | | | | 99·3 | 99·4 | 92·2 | 98·2 | 98·1 | 98·2 | 98·1 |
| 5 | *E. media* | | | | | | 99·8 | 92·6 | 98·6 | 98·4 | 98·4 | 98·5 |
| 6 | *E. perforans* | | | | | | | 92·6 | 98·7 | 98·4 | 98·5 | 98·6 |
| 7 | *E. stiedai* | | | | | | | | 91·8 | 91·9 | 92·0 | 92·2 |
| 8 | *E. vejdovskyi* | | | | | | | | | 97·3 | 97·4 | 97·8 |
| 9 | *E. flavescens* | | | | | | | | | | 99·7 | 97·2 |
| 10 | *E. piriformis* | | | | | | | | | | | 97·2 |
| 11 | *E. coecicola* | | | | | | | | | | | |

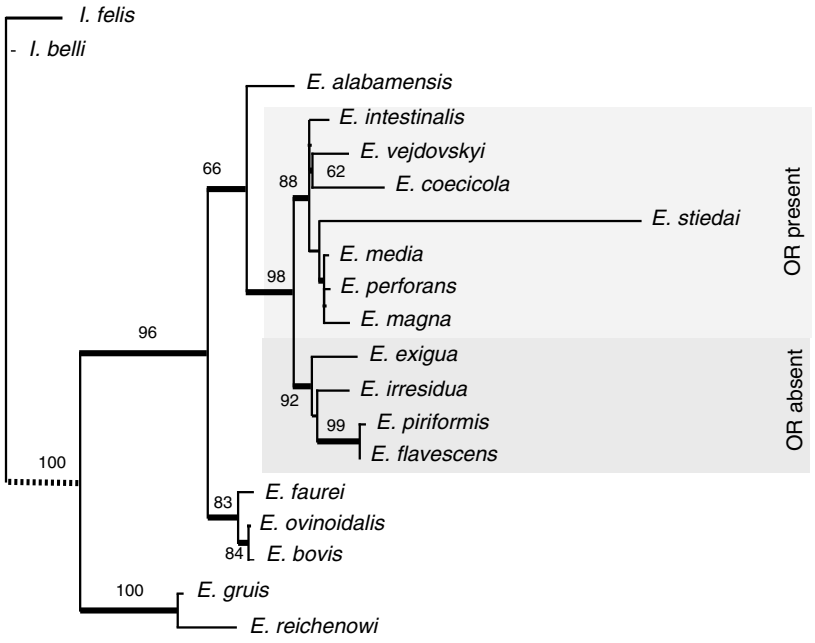


Fig. 1. A tree obtained by maximum parsimony analysis of the *Rabbit* matrix, with bootstrap values at the nodes. The branches preserved in the strict consensus of all MP trees and compatible with ML analysis are designated by bold lines. OR, oocyst residuum.

and Duszynski, 1999*a*). Also, cyst-forming heteroxenous species (e.g. *Sarcocystis*) show significant host-specificity in respect to the intermediate and definitive host, as well as a strong site-specificity, e.g. affinity to the brain, muscles etc. (Votýpka *et al*. 1998; Doležel *et al*. 1999; Šlapeta *et al*. 2003).

The results of our phylogenetic analysis indicate that rabbit-specific *Eimeria* species form a monophyletic branch. This finding is in line with the ultimate phylogenetic significance of host-specificity within *Eimeria* in other studies (Morrison *et al*. 2004; Matsubayashi *et al*. 2005; Li *et al*. 2007). It also implies that the 11 species with highly variable morphological characters diversified within a single host (or several closely related species at maximum). On the other hand, it should be recognized that in such a species-rich group as *Eimeria*, the study of its evolutionary history is usually highly compromised by the available samples. Thus, while more than 50 *Eimeria* sequences have so far been deposited into GenBank, this number represents only a small fraction of known species. The consequences of this problem can be easily demonstrated: for example, the fowl-infecting group of *Eimeria* is apparently monophyletic until the addition of *Cyclospora* species (Morrison *et al*. 2004; Matsubayashi *et al*. 2005).

An interesting aspect of the present study is a lack of congruence between phylogeny and most of the morphological and biological traits of the species. For example, the 2 most pathogenic species, *E. intestinalis* and *E. flavescens*, branch as very distant taxa; the latter particularly as one of the most derived offshots. At the same time, we have observed remarkable morphological and biological differences between genetically highly related species, supporting the 'recent' changes of some characteristics. Thus, while the 2 species with almost identical

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Table 3. Morphological and biological traits of the rabbit-associated species shown on Fig. 1

(The characteristics are adopted from the following descriptions: Eckert *et al*. 1995; Pakandl *et al*. 1996*a*,*b*,*c*, 2003; Pakandl and Coudert, 1999; Pakandl and Jelínková, 2006. Oocyst size is provided in $\mu$m (average size in parentheses). MP, micropyle; OR, oocyst residuum; GALT, gut-associated lymphoid tissue (i.e. Peyer's patches, sacculus rotundus, vermiform appendix); ND, not determined; Asexual, number of asexual generations; Prep., pre-patent period (days); Pat., patent period (days); Sporulation time is provided as hours at 18 °C/22 °C/26 °C.)

| Species | Oocyst shape | Oocyst size | MP | OR | Location | Pathogenicity | Asexual | Prep. | Pat. | Sporulation |
|---|---|---|---|---|---|---|---|---|---|---|
| *E. intestinalis* | piriform | 22–30 × 16–21 (26·7 × 18·9) | + | + + | jejunum-ileum | high | 4 | 8,5 | max. 10 | 105/70/60 |
| *E. vejdovskyi* | elongate-ovoid | 25–38 × 16–22 (31·5 × 19·1) | + | + | ileum | slight | 5 | 10 | ND | ND/50/35 |
| *E. coecicola* | elongate-ovoid | 27–40 × 15–22 (34·5 × 19·7) | + | + | GALT | none | 4 | 9 | 8–9 | 120/85/60 |
| *E. stiedai* | ellipsoid | 30–41 × 15–24 (36·9 × 19·9) | + | + | biliary ducts | mild-high | up to 6 | 14 | ND | 110/63/57 |
| *E. media* | ellipsoid-ovoid | 25–35 × 15–20 (31·1 × 17·0) | + + | + | jejunum-ileum | mild | 3 | 4,5 | 6–8 | 60/41/30 |
| *E. perforans* | ellipsoid-subrectangular | 15–27 × 11–17 (22·2 × 13·9) | – | + | duodenum-prox. jejunum | slight | 2 | 5 | 5–6 | 50/30/22 |
| *E. magna* | ovoid | 31–42 × 20–28 (36·3 × 24·1) | + + | + + | jejunum-ileum | mild | 4 | 6,5 | 15–19 | 115/80/46 |
| *E. exigua* | spherical-subspherical | 10–18 × 11–16 (15·1 × 14·0) | – | – | ileum | slight | ND | 7 | ND | ND/23/17 |
| *E. irresidua* | ovoid-barrel shaped | 31–44 × 20–27 (39·2 × 23·1) | + + | – | jejunum-ileum | mild | 4 | 9 | 10–13 | 105/85/50 |
| *E. piriformis* | piriform | 25–33 × 16–21 (29·5 × 18·1) | + + | – | colon | mild | 4 | 9 | 10 | 150/90/70 |
| *E. flavescens* | ovoid | 25–35 × 18–24 (30·0 × 21·0) | + + | – | cecum-prox. colon | high | 5 | 9 | ND | 120/80/48 |

sequence of 18S rDNA, *E. piriformis* and *E. flavescens*, still share a unique location within the large intestine, they differ significantly in oocyst shape. Similarly, all of the 3 closely related species, *E. media*, *E. magna* and *E. perforans*, develop within the walls and tips of the small intestine villi, but only the first 2 possess a micropyle on their oocysts. Such a mosaic of phylogenetically conservative and versatile characteristics is likely to be a common phenomenon. An attempt to map biological and morphological traits on phylogeny within a monophyletic group of closely related eimerian species has previously been published for the fowl-specific group (Barta *et al*. 1997). While authors of the study stress a generally high degree of phylogenetic congruence for various features, they also show that some characteristics may change rapidly. For instance, they report a shift of location for 3 related species, *E. brunetti*, *E. maxima* and *E. praecox*, and attribute it to a niche specialization process within the host. Within our set, the most striking example of uncoupled biology and phylogeny is the position of *E. stiedai*. Unlike all the other species inhabiting the host intestine, *E. stiedai* infects bile ducts (Pellérdy, 1965; Pellérdy and Dürr, 1970; Eckert *et al*. 1995), and has also been reported in hares (*Lepus europaeus* Pallas, 1778) (Varga, 1976; Entzeroth and Scholtyseck, 1977; Sugár *et al*. 1978; Scholtyseck *et al*. 1979). Moreover, the structure of its OR is highly unusual, incompact, consisting of 1–7 or more granules situated centrally in the oocyst (Norton *et al*. 1977; Eckert *et al*. 1995). This fact even lead Pellérdy (1965) to claim that this species does not possess an OR at all. In our matrix, *E. stiedai* formed a relatively long branch due to a 100 bp deletion and many autapomorphic substitutions along the sequence. Despite the unique biological and molecular features, this species branched firmly within the OR+ cluster of rabbit-associated eimeria in all analyses, regardless of the method, program and parameters.

Interestingly, we identified a convincing correlation between the phylogenetic position of the species and presence/absence of an OR. This feature has previously been reported as the only phylogenetically consistent character differing in 2 lineages of rodent *Eimeria* (Zhao and Duszynski, 2001*a*, *b*). In our analysis, the OR+ and OR- groups form 2 monophyletic sister lineages. It is therefore impossible to decide which state is plesiomorphic for rabbit *Eimeria*. Also, using light microscopy, we did not observe any further correlation between the inner phylogeny of the OR+ group and OR characteristics, such as size, shape or structure. Other morphological and biological characters (oocyst shape, size and presence/absence of oocyst inner structures, pathogenicity, pre-patent period, patent period, sporulation time, number of asexual generations) showed no influence on the phylogeny (Fig. 1; Table 3).

Since the presence/absence of OR seems to be an evolutionarily conserved feature, it would be interesting to determine the phylogenetic relationships of the rabbit-specific cluster to other OR+ groups. However, this was beyond the scope of the present study. In addition, resolving the OR issue at a higher phylogenetic level is problematic for 2 reasons. First, as demonstrated by conflicting results of several phylogenetic studies (Morrison *et al*. 2004; Matsubayashi *et al*. 2005; Li *et al*. 2007), the 18S rDNA data currently available for analysis may be riddled with inconsistencies, or at least difficulties when extracting a phylogenetic signal. An overall sensitivity of the 18S rDNA dataset to selection of a particular type of analysis and evolutionary model has been demonstrated in a thorough study of Morrison *et al*. (2004). Second, and more importantly, as mentioned above, poor sampling across all known *Eimeria* species makes such an attempt naive. We are convinced that a much more complete data set, in terms of host spectrum and sequenced genes, has to be available before the evolution of various traits within *Eimeria* can be seriously addressed.

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## REFERENCES

**Banks, J. C., Palma, R. L. and Paterson, A. M.** (2006). Cophylogenetic relationships between penguins and their chewing lice. *Journal of Evolutionary Biology* **19**, 156–166.

**Barta, J. R.** (1989). Phylogenetic analysis of the class Sporozoea (phylum Apicomlexa Levine 1970): evidence for the independent evolution of heteroxenous life cycles. *Journal of Parasitology* **75**, 195–206.

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

**Barta, J. R., Martin, D. S., Carreno, R. A., Siddall, M. E., Profous-Juchelka, H., Hozza, M., Powles, M. A. and Sundermann, C.** (2001). Molecular phylogeny of the other tissue coccidia: *Lankesterella* and *Caryospora*. *Journal of Parasitology* **87**, 121–127.

**Carreno, R. A., Martin, D. S. and Barta, J. R.** (1999). *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85**, 899–904.

**Ceré, N., Licois, D. and Humbert, J. F.** (1995). Study of the inter- and intraspecific variation of *Eimeria* spp. from

the rabbit using random amplified polymorphic DNA. *Parasitology Research* **91**, 324–328.

**Ceré, N., Humbert, J. F., Licois, D., Corvione, M., Afanassieff, M. and Chanteloup, N.** (1996). A new approach for the identification and the diagnosis of *Eimeria media* parasite of the rabbit. *Experimental Parasitology* **82**, 132–138.

**Ceré, N., Licois, D. and Humbert, J. F.** (1997). Comparison of the genomic fingerprints generated by the random amplification of polymorphic DNA between precocious lines and parental strains of *Eimeria* spp. from the rabbit. *Parasitology Research* **83**, 300–302.

**Coudert, P., Licois, D. and Streun, A.** (1979). Characterization of *Eimeria* species. I. Isolation and study of pathogenicity of a pure strain of *Eimeria perforans* (Leuckart, 1879; Sluiter and Swellengrebel, 1912). *Zeitschrift für Parasitenkunde* **59**, 227–234.

**Coudert, P., Licois, D. and Drouet-Viard, F.** (1995). *Eimeria* species and strains of the rabbits. In *Biotechnology. Guidelines on Techniques in Coccidiosis Research* (ed. Eckert, J., Braun, R., Shirley, M. W. and Coudert, P.), pp. 52–71. COST 89/820, Luxembourg.

**Doležel, D., Koudela, B., Jirků, M., Hypša, V., Oborník, M., Votýpka, J., Modrý, D., Šlapeta, J. R. and Lukeš, J.** (1999). Phylogenetic analysis of *Sarcocystis* spp. of mammals and reptiles supports the coevolution of *Sarcocystis* spp. with their final hosts. *International Journal for Parasitology* **29**, 795–798.

**Duszynski, D. W. and Upton, S. J.** (2001). The common coccidia of wild mammals. *Cyclospora*, *Eimeria* (Eimeriidae) and *Cryptosporidium* (Cryptosporidiidae) spp. In *Parasitic Diseases of Wild Mammals* (ed. Samuel, W. M., Pybus, M. J. and Kocan, A. A.), pp. 416–433. Iowa State University Press, Ames, IA, USA.

**Eberhard, M. L., Da Silva, A. J., Lilley, B. G. and Pieniazek, N. J.** (1999). Morphologic and molecular characterization of new *Cyclospora* species from Ethiopian monkeys: *C. cercopitheci* sp. n., *C. colobi* sp. n., and *C. papionis*, sp. n. *Emerging Infectious Diseases* **5**, 651–657.

**Eckert, J., Braun, R., Shirley, M. W. and Coudert, P.** (1995). *Biotechnology. Guidelines on Techniques in Coccidiosis Research*. COST 89/820, Luxembourg.

**Entzeroth, R. and Scholtyseck, E.** (1977). The life cycle of *E. stiedai* from rabbits in hares. *Fifth International Congress on Protozoology, New York City 26.6.-2.7.*

**Guindon, S. and Gascuel, O.** (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696–704.

**Hall, T. A.** (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

**Hnida, J. A. and Duszynski, D. W.** (1999*a*). Taxonomy and systematics of some *Eimeria* species of murid rodents as determined by the ITS1 region of the ribosomal gene complex. *Parasitology* **199**, 349–357.

**Hnida, J. A. and Duszynski, D. W.** (1999*b*). Taxonomy and phylogeny of some *Eimeria* (Apicomplexa: Eimeriidae) species of rodents as determined by polymerase chain reaction/restriction-fragment-length polymorphism analysis of 18s rDNA. *Parasitology Research* **85**, 887–894.

**Hoberg, E., Brooks, D. R. and Siegel-Causey, D.** (1997). Host-parasite cospeciation: history, principles, and prospects. In *General Principles and Avian Models Host-Parasite Evolution* (ed. Clayton, D. H. and Moore, J.), pp. 212–235. Oxford University Press, Oxford.

**Holmdahl, O. J., Morrison, D. A., Ellis, J. T. and Huong, L. T.** (1999). Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNA sequences. *Molecular Phylogenetics and Evolution* **11**, 27–37.

**Jirků, M., Modrý, D., Šlapeta, J. R., Koudela, B. and Lukeš, J.** (2002). The phylogeny of *Goussia* and *Choleoeimeria* (Apicomplexa; Eimeriorina) and the evolution of excystation structures in coccidia. *Protist* **153**, 379–390.

**Johnson, K. P. and Clayton, D. H.** (2001). Coevolutionary history of ecological replicates: comparing phylogenies of wing and body lice to Columbiform hosts. In *Tangled Trees: Phylogeny, Cospeciation, and Coevolution* (ed. Page, R. D. M.), pp. 262–286. Chicago University Press, Chicago, IL.

**Jousson, O., Bartoli, P. and Pawlowski, J.** (2000). Cryptic speciation among intestinal parasites (Trematoda: Digenea) infecting sympatric host fishes (Sparidae). *Journal of Evolutionary Biology* **13**, 778–785.

**Joyner, L. P.** (1982). Host and site specificity. In *The Biology of the Coccidia* (ed. Long, P. L.), pp. 35–62. University Park Press, Baltimore, Maryland, USA.

**Kopečná, J., Jirků, M., Oborník, M., Tokarev, Y. S., Lukeš, J. and Modrý, D.** (2006). Phylogenetic analysis of coccidian parasites from invertebrates: search for missing links. *Protist* **157**, 173–183. doi: 10.1016/j.protis.2006.02.005.

**Leander, B. S., Lloyd, S. A. J., Marshall, W. and Landers, S. C.** (2006). Phylogeny of marine gregarines (Apicomplexa) – *Pterospora, Lithocystis* and *Lankesteria* – and the origin(s) of coelomic parasitism. *Protist* **157**, 45–60. doi:10.1016/j.protis.2005.10.002.

**Li, G., Xiao, S., Zhou, R., Li, W. and Wadeh, H.** (2007). Molecular characterization of *Cyclospora*-like organism from dairy cattle. *Parasitology Research* **100**, 955–961.

**Licois, D. and Coudert, P.** (1982). Coccidioses et diarrhées du lapin à l'engraissement. *Bull GTV* **5**, 109–122.

**Licois, D. and Mongin, P.** (1980). An hypothesis of the pathogenesis of diarrhoea in the rabbit based on a study of intestinal contents. *Reproduction Nutrition Development* **20**, 1209–1216.

**Long, P. L. and Joyner, L. P.** (1984). Problems in the identification of species of *Eimeria*. *Journal of Protozoology* **31**, 535–541.

**Marquardt, W. C.** (1973). Host and site specificity. In *The Coccidia* (ed. Hammond, D. M. and Long, P. L.), pp. 23–43. University Park Press, Baltimore, Maryland, USA.

**Matsubayashi, M., Takami, K., Niichiro, A., Kimata, I., Tani, H., Sasai, K. and Baba, E.** (2005). Molecular characterization of crane coccidia, *Eimeria gruis* and *E. reichenowi*, found in feces of migratory cranes. *Parasitology Research* **97**, 80–83.

**Metzner, R.** (1903). Untersuchungen an *Coccidium cuniculi*. *Archiv für Protistenkunde* **2**, 13–72.

**Minning, W.** (1936). Zur Entstehung der Coccidienknoten in der Leber von Kaninchen. *Zeitschrift für Parasitenkunde* **9**, 61–72.

**Morrison, D. A., Bornstein, S., Thebo, P., Wernery, U., Kinne, J. and Mattsson, J. G.** (2004). The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). *International Journal for Parasitology* **34**, 501–514.

**Mugridge, M. B., Morrison, D. A., Jakel, T., Heckeroth, A. R., Tenter A. M. and Johnson, A. M.** (2000). Effects of sequence alignment and structural domains of ribosomal DNA on phylogeny reconstruction for the protozoan family Sarcocystidae. *Molecular Biology and Evolution* **17**, 1842–1853.

**Nieberding, C., Morand, S., Libois, R. and Michaux, J. R.** (2004). A parasite reveals cryptic phylogeographic history of its host. *Proceedings of the Royal Society of London, B* **271**, 2559–2568.

**Nieberding, C., Libois, R., Douady, C. J., Morand, S. and Michaux, J. R.** (2005). Phylogeography of a nematode (*Heligmosomoides polygyrus*) in the western Palearctic region: persistence of northern cryptic populations during ice ages? *Molecular Ecology* **14**, 765–779.

**Norton, C. C., Catchpole, N. and Rose, M. E.** (1977). *Eimeria stiedai* in rabbits: the presence of an oocyst residuum. *Parasitology* **75**, 1–7.

**Olsen, G. J. and Woese, C. R.** (1993). Ribosomal RNA: a key to phylogeny. *FASEB Journal* **7**, 113–123.

**Page, R. D. M.** (1996*a*). Temporal congruence revisited: comparison of mitochondrial DNA sequence divergence in cospeciating pocket gophers and their chewing lice. *Systematic Biology* **45**, 151–167.

**Page, R. D. M.** (1996*b*). TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357–358.

**Pakandl, M.** (1988). Description of *Eimeria vejdovskyi* sp. n. and redescription of *Eimeria media* Kessel, 1929 from the rabbit. *Folia Parasitologica* **35**, 1–9.

**Pakandl, M. and Coudert, P.** (1999). Life cycle of *Eimeria vejdovskyi* Pakandl, 1988: electron microscopy study. *Parasitology Research* **85**, 850–854.

**Pakandl, M., Černík, F. and Coudert, P.** (2003). The rabbit coccidium *Eimeria flavescens* Marotel and Guilhon, 1941: an electron microscopic study of its life cycle. *Parasitology Research* **91**, 304–311.

**Pakandl, M., Ahmed, N. E., Licois, D. and Coudert, P.** (1996*c*). *Eimeria magna* Pérard 1925: study of the endogenous development of parental and precocious strains. *Veterinary Parasitology* **65**, 213–222.

**Pakandl, M., Gaca, K., Drouet-Viard, F. and Coudert, P.** (1996*a*). *Eimeria coecicola* Cheissin 1947: endogenous development in gut-associated lymphoid tissue. *Veterinary Research* **82**, 347–351.

**Pakandl, M., Gaca, K., Licois, D. and Coudert, P.** (1996*b*). *Eimeria media* Kessel 1929: comparative study of endogenous development between precocious and parental strains. *Veterinary Research* **27**, 465–472.

**Pakandl, M. and Jelínková, A.** (2006). The rabbit coccidium *Eimeria piriformis*: Selection of a precocious line and life-cycle study. *Veterinary Parasitology* **137**, 351–354.

**Paterson, A. M., Wallis, G. P., Wallis, L. J. and Gray, L. D.** (2000). Seabird and louse coevolution: complex histories revealed by 12S rRNA sequences and reconciliation analyses. *Systematic Biology* **49**, 383–399.

**Pellérdy, L. P.** (1965). Oryctolagus. In *Coccidia and Coccidiosis* (ed. North, H. F. and Farkas, I.), pp. 323–330. Akademiai Kiado, Budapest.

**Pellérdy, L. P. and Dürr, U.** (1970). Zum endogenen Entwicklungszyklus von *Eimeria stiedai* (Lindemann 1865) Kisskalt & Hartmann, 1907. *Acta Veterinaria of the Academy of Sciences of Hungary* **20**, 227–244.

**Proctor, H. C.** (1999). *Gallilichus jonesi* sp.n. (Acari: Ascouracaridae): a new species of feather mite from the quills of the Australian brush-turkey (Aves: Megapodiidae). *Australian Journal of Entomology* **38**, 77–84.

**Reduker, D. W., Duszynski, D. W. and Yates, T. L.** (1987). Evolutionary relationships among *Eimeria* spp. (Apicomplexa) infecting cricetid rodents. *Canadian Journal of Zoology* **65**, 722–735.

**Rutherford, R. L.** (1943). The life-cycle of four intestinal coccidia of the domestic rabbit. *Journal of Parasitology* **29**, 10–32.

**Scholtyseck, E., Entzeroth, R. and Pellérdy, L.** (1979). Transmission of *Eimeria stiedai* from the rabbit (*Oryctolagus cuniculus*) to the hare (*Lepus europaeus*). *Acta Veterinaria of the Academy of Sciences of Hungary* **27**, 365–373.

**Schrenzel, M. D., Maalouf, G. A., Gaffney, P. M., Tokarz, D., Keener, L. L., McClure, D., Griffey, S., McAloose, D. and Rideout, B. A.** (2005). Molecular characterization of isosporoid coccidian (*Isospora* and *Atoxoplasma* spp.) in passerine birds. *The Journal of Parasitology* **91**, 635–647.

**Šlapeta, J. R., Modrý, D., Votýpka, J., Jirků, M., Lukeš, J. and Koudela, B.** (2003). Evolutionary relationships among cyst-forming coccidia *Sarcocystis* spp. (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle. *Molecular Phylogenetics and Evolution* **27**, 464–475.

**Sugár, L., Murai, E. and Mészáros, P.** (1978). Über die Endoparasiten der wildleben Leporidae Ungarns. *Parasitologica Hungarica* **11**, 63–85.

**Swofford, D. L.** (2001). *Phylogenetic Analysis Using Parsimony* (**and Other Methods*), *Version 4*. Sinauer Associates, Sunderland, Massachusetts, USA.

**Tenter, A. M., Baverstock, P. R. and Johnson, A. M.** (1992). Phylogenetic relationships of *Sarcocystis* species from sheep, goats, cattle and mice based on ribosomal RNA sequences. *International Journal for Parasitology* **22**, 503–510.

**Tenter, A. M., Barta, J. R., Beveridge, I., Duszynski, D. W., Mehlhorn, H., Morrison, D. A., Thompson, R. C. A. and Conrad, P. A.** (2002). The conceptual basis for a new classification of the coccidia. *International Journal for Parasitology* **32**, 595–616.

**Varga, I.** (1976). Experimental transmission of *E. stiedai* to the hare. *Acta Veterinaria of the Academy of Sciences of Hungary* **26**, 105–112.

**Vítovec, J. and Pakandl, M.** (1989). The pathogenicity of rabbit coccidium *Eimeria coecicola* Cheissin, 1947. *Folia Parasitologica* (Praha) **36**, 289–293.

**Votýpka, J., Hypša, V., Jirků, M., Flegr, J., Vávra, J. and Lukeš, J.** (1998). Molecular phylogenetic relatedness of *Frenkelia* spp. (Protozoa, Apicomplexa) to *Sarcocystis falcatula* Stiles 1893: is the genus *Sarcocystis* paraphyletic? *Journal of Eukaryotic Microbiology* **45**, 137–141. www.ncbi.nlm.nih.gov

**Zhao, X. and Duszynski, D. W.** (2001*a*). Phylogenetic relationships among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. *International Journal for Parasitology* **31**, 715–719.

**Zhao, X. and Duszynski, D. W.** (2001*b*). Molecular phylogenies suggest the oocyst residuum can be used to distinguish two independent lineages of *Eimeria* spp in rodents. *Parasitology Research* **87**, 638–643.

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