

Doctoral Thesis Evaluation for Jana Kvičerová

Thesis title: Phylogeny of coccidia and coevolution with their hosts.

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Prior to evaluating the specific manuscripts that comprise this doctoral thesis, it is important to put into context, the prodigious amount of work that remains to be done, both in terms of taxa sampled and genes sequenced, before confidence can emerge in phylogenies derived from these taxa and their genes. This context is needed to understand the difficulties faced by Ms. Kvičerová in her work and to appreciate her immense effort to collect and make sense of the numerous coccidians she has studied in a wide variety of small mammals.

The Apicomplexa is a diverse phylum of parasitic protists with ~6,000 species that traditionally are placed into 1 of 4 groups: coccidia, gregarines, haemosporidia, and piroplasms. It's estimated that only 0.1% of apicomplexan species have been discovered (Adl et al., 2007). The coccidia, the most species-rich of the 4 groups, has been studied in the most breadth, but, unfortunately, not in as much depth as those with medical and veterinary importance (e.g., haemosporidia - human malarial; piroplasms - domestic animal blood pathogens). Using best approximations of extant vertebrates and the coccidia known from them, Duszynski (2011) estimated there may be, minimally, 124,300 coccidia species that use vertebrates as hosts. The 1,800 named species at present are only 1.4% of the likely number of coccidia that parasitize all vertebrates, and molecular sequence data from these and the other apicomplexan groups is a critically small and biased sample. For example, GenBank contains nucleotide sequence data for only a small number of apicomplexans, with about 98% of these sequences in only 5 genera: *Babesia*, *Cryptosporidium*, *Plasmodium*, *Theileria* and *Toxoplasma* (Morrison, 2008). As pointed out repeatedly in the papers that comprise Kvičerová's thesis, there are relatively few sequences for the largest coccidian genera, *Eimeria* and *Isospora*.

Clearly, many more taxa must be sampled and their genes sequenced before any real concordance can emerge among phylogenies derived. Adequate taxon sampling must be in hand to provide a convincing case for the particular taxonomic boundaries studied. Unfortunately, knowledge of the morphology and biology of eimeriid coccidia of virtually all host groups/clades is clearly and woefully inadequate and sequence data unknown for most coccidia species. Another problem is a lack of sequence data for potential basal groups (e.g., *Blastocystis*) within the Apicomplexa. Basal taxa (those most similar to the ancestor of the ingroup) are vital for rooting the phylogenetic tree which defines the clade and determines the relationships among its taxa. Finally, molecular phylogenies in the Apicomplexa are usually based on nucleotide sequence of the 18S rRNA gene. But a cladogram of a single molecular sequence represents only the phylogeny of that 1 gene, which may not be the phylogeny of all the species in that group; thus, there is a need to have concordance between phylogenies derived from several different molecular sequences. These points are made to emphasize what I believe are the real strengths of the papers comprising this thesis: numerous taxa sampled and multiple genes sequenced.

I. Kvičerová, Pakandl & Hypša, 2008. They were the first to examine the phylogenetic relationships among 11 of the 15 (73%) valid eimerians known in the domestic rabbit, *Oryctolagus cuniculus*. The genus is monotypic, and because of its importance to humans as pets and in both biomedical research and food production, *O. cuniculus* has been better studied for its intestinal parasites than any other lagomorph species. Thus, by sampling the majority of species in this clade, real confidence can be given to the species boundaries they considered, including the morphometrics of their sporulated oocysts, location and number of endogenous developmental stages, sporulation time, pathogenicity, and more. Using partial sequences of 18S rRNA from the 11 species, and the 50+ *Eimeria* sequences available in GenBank (at the time), they found that the rabbit eimerians produced a well-formed, monophyletic cluster. Interestingly, despite all of the morphological and biological characters used in their analysis, only the presence or absence (+/-) of an oocyst residuum (OR) strictly followed the phylogenetic division into 2 monophyletic sister lineages. This was of interest because it mirrored previous work with eimerians in 3 rodent families that also showed distinct phylogenetic relationships based on the (+/-) of the OR, rather than on morphologic and/or biologic characters, which might be expected to be more phylogenetically informative. The only shortcoming of this paper is that it would have been interesting to learn if any congruence could be found between phylogenies had multiple molecular sequences been examined. It also would have been interesting to include oocysts of *E. neoleporis* in their analysis because this species, although described originally from a cottontail rabbit, *Sylvilagus floridanus*, has been successfully transmitted several times to *O. cuniculus* and has been reported a number of times in the wild form.

II. Kvičerová, Mikeš & Hypša, 2011. As the authors point out, our knowledge of the coccidia that infect rodents in the family Gliridae is almost non-existent, with only about 10 poorly-described species known from 3 host species. The reason for this is endemic to the discipline and beyond anyone's control. That is, taxon sampling of both hosts and their parasites, by its nature, usually is opportunistic, which can't create statistically representative taxon samples. In this paper, however, the authors direct their energy to collecting a relevant sample size of a single endangered rodent from several localities. Serendipity played a key role in that a high percentage (85%) of the 54 *Eliomys quercinus* they collected were infected with only a single morphotype of oocyst, which they were able to characterize as one previously, but poorly, described species, *Eimeria myoxi*. This gave them large numbers of oocysts to study, measure and describe morphologically and sufficient DNA to be able to sequence nuclear, plastid and mitochondrial genes. Thus, they were able to accomplish their goal, which was to unequivocally characterize *E. myoxi* so that it could be used in future phylogenetic and evolutionary studies. This is something that should be done with every species re-description and every new species description; that is, provide a thorough morphological characterization using at least 100 sporulated oocysts, provide photomicrographs and a line drawing of the oocyst that are archived, and add 1 or more molecular sequences that are deposited in GenBank. It should also be a goal to contribute life cycle, cross-transmission and other biological information to known species, but since they were dealing with an endangered host species, which likely does not grow or reproduce well under lab conditions, they were understandably unable to gather these kinds of data.

III. Kvičerová, Ptáčková & Modrý, 2007 + Supplement. Couch et al. (1997) named *E. cahirinensis* and did a basic species description based on oocysts sent to them in the feces of *Acomys cahirinus* that had been collected in Evolution Canyon near Mt. Carmel, Israel. A decade later, Kvičerová et al. (2007) did an outstanding, comprehensive and detailed study of *E. cahirinensis* using oocysts from wild-caught *A. dimidiatus* to infect lab strains of various *Acomys* and other rodent species that were reared and maintained in their laboratory facilities. Their work supplied extensive biological information on prepatent and patent periods, histological evidence of the location and details of asexual and sexual endogenous development and stages, sporulation time, immunogenicity of infective doses, pathogenicity of the infection, cross-transmission information and host-specificity. Their Supplement gave a phylogenetic analysis based on nuclear (18S rDNA) and mitochondrial (COI) gene sequences that placed *E. cahirinensis* (OR+) into the rodent-derived *Eimeria* lineage of those species that all possess an OR. Overall, this is really an outstanding piece of work and if we had these kinds of biological, structural and molecular knowledge for every known coccidian species, we would have a much better understanding about coccidial speciation, evolution, phylogeny, and host-specificity. Wouldn't it be wonderful to have these data for all eimerians known from *Acomys* species and those from all murid rodent genera and species?

IV. Jirků, Kvičerová, Modrý & Hypša, 2012. This manuscript examines the only 2 eimerians known from scaly anteaters, an ancient order of mammals with only 1 family and 7 species. Only 1 previous *Eimeria* species had been described from 1 animal in the family, so the authors are immediately confronted with the issue of inadequate taxon sampling noted in the introduction to this review. They are clearly aware that working with this lack of information will not allow them to provide a convincing case for the taxonomic boundaries of this group and they stress the importance of representative sampling. Nonetheless, they are able to partially sequence 3 genes for both species, 2 of which were phylogenetically informative enough to allow them to conclude that the 2 eimerians are unrelated to each other. This is exactly the kind of detailed information needed from all future descriptions of coccidia. If everyone who published species descriptions supplied such detail, we could begin to fill in the many gaps that exist in our knowledge of the coccidia and any concordance that may emerge among the phylogenies derived. Just a few comments on the manuscript: First, Ms. Kvičerová has a sterling record of getting her work into publications. Nonetheless, since this manuscript is still unpublished, and part of a university thesis, it would be best for the authors not to assign a name to their new species; if the work doesn't get published, the name is preoccupied and cannot be used again by another author. Second, there seem to be several items missing from this pre-publication, namely a composite line drawing of the sporulated oocyst of the new species and photomicrographs of endogenous stages from the tissue stages they found in the large and small intestines that they attribute to the life cycle of the new species. I question their statement that sporocysts of their new species are asymmetrical, which is not seen in their photomicrograph (Fig. 1-A). Perhaps it was the power of suggestion from Else and Colley (1976), who stated that sporocysts of *E. tenggilingi*, which they described, had asymmetrical sporocysts? This is not evident in their only photomicrograph of *tenggilingi*, which is of rather poor quality, unfortunately. Finally, I agree with the authors that Else and Colley (1976) overlooked a "barely discernible" SB in *E. tenggilingi*.

V. Kvičarová & Hypša, 2012. This manuscript will add a large number of partial new sequences, many from 3 different genes for both *Eimeria* and *Isospora* species, to the NCBI database when they are deposited. This is a very important and Herculean contribution to the molecular sequences known from all coccidians, more than doubling the current number of sequences. However, what is the exact number? Table I lists the sequences included in their phylogenetic analyses. I interpret their "n.d." designation ("not yet deposited in GenBank") to represent these new sequences. If true, then the authors need to readjust the numbers they cite throughout the manuscript. For example, I count 198 sequences (all 3 genes) used in their analysis (Table I); of these, 74 are new (n.d. designation) with 66 from various *Eimeria* species and 8 from "*I. sp. ex Talpa* (7)" and "*I. sp. ex A. flavicolis* B13 (1)." Yet their manuscript (abstract, introduction, discussion) states that "86 new sequences of *Eimeria* species" were added from rodents and insectivores. Similarly, under their *Phylogenetic analysis* they state, "The Skeleton matrix included taxa for which all three genes were available." The Skeleton tree has 13 species listed that divide into 4 arbitrary clades. However, Table I lists 19 species (18 *Eimeria* species, *Toxoplasma gondii*) for which all 3 gene sequences were available. Why weren't the other 6 species (*E. coecicola*, *E. perforans*, *E. piriformis*, *E. stiedai*, *E. vejdvoskyi*, *T. gondii*) included with this analysis. Or am I overlooking/missing something in my interpretation?

Using these 198 sequences, the authors produced a concatenated ML tree that also was supported internally by BI and ML analyses. The aim of this study was to, "analyze the monophyly and composition of the whole clusters characterized by various biological features (morphology, host specificity) rather than relationships among individual species." I would define morphology as a structural feature (not biological) and an explanation of how the authors knew which species were host specific and which were not needs a cleaner, clearer explanation. If we don't strictly define host specificity and list the species that meet those criteria, how can we see "an interesting relationship between the host specificity and phylogeny?"

VI. Kvičarová, Mácová & Hypša, 2012 (preliminary draft). *Apodemus* species, common Old World field mice, are distributed throughout Europe, their biology, ecology, genetics and geographic distributions are reasonably well-studied, and they've had >20 *Eimeria* species described from them (although most descriptions are inadequate by any standards); thus, the authors have proposed using this natural host-parasite system as a model in which to study population structure, host specificity and biogeography. This is a commendable undertaking and if the authors can study this model as thoroughly as they have *Oryctolagus* eimerians (Kvičarová et al., 2008) and *E. cahirinensis* in *Acomys* species (Kvičarová et al., 2007) they will have made an important and lasting contribution that can be used in the future as a template and example for all other workers in the field. The results, however, as presented in this preliminary manuscript, are both incomplete and confusing (to me). First, how many total *Apodemus* were collected? The authors stated they used 44 samples (43 infected with *Eimeria*, 1 infected with *Isospora*) from 3 *Apodemus* species for the analysis of population structure. Were there no multi-species *Eimeria* infections found, as often occurs in this host genus? I find it extremely unusual that they could collect only 44 animals from 7 European countries and find every animal infected with only and exactly 1 coccidian species each. Figure 1 is confusing as presented because the legend is not sufficiently explanatory. For example, in the (lower) enlarged line drawing of the CZ, the far left

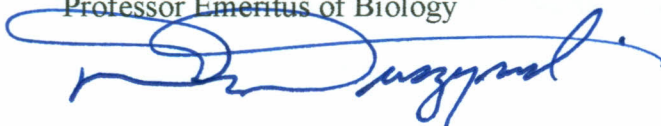
white dot is presumed to represent *A. flavicollis*. It has 4 colored circles around it (pink, orange, yellow, green), representing 3 of the 4 eimerians identified. Is this intended to tell the reader that 4 *A. flavicollis* were collected in this location, 2 infected with *E. apionodes* and the other 2 with either *E. kaunensis* or *E. jerfinica*? If this is what is intended, it should be spelled out clearly in the legend to avoid confusion. And although field mouse B-13 is listed in Table 1, it is omitted from Table 2 and from Figure 1, but having had some of its genes sequenced, it appears again in Fig. 2. This seems inconsistent. The authors state that the descriptions of many of the eimerians known from *Apodemus* spp. are inadequate and do not allow proper species identifications. How then did they arrive at identifying the 4 eimerian species present in their samples? I would expect to see at least the thoroughness and quality of species description, including measurements, photomicrographs, line drawing and gene sequences, as were presented in their earlier work (cited above). The authors state that "No molecular data are yet available for any *Eimeria* exploiting *Apodemus* hosts," yet here they isolate DNA from 34 samples and sequenced mitochondrial COI genes for the 5 coccidians they found (which presumably will be deposited in GenBank). Finally, although the authors used a program (TCS) to identify 11 unique haplotypes, the haplotype distribution network represented by the 3 clades in Fig. 3 is unclear, at least to me. This needs to be explained much better in the Figure legend and the Results/Discussion. Would there be any interest or relevance to note, for example, that *E. alorani* only possessed Haplotype-3 (H-3) and infected 4 of the 11 (36%) *A. agrarius*, but not the other 2 host species? Similarly, of 26 mice infected with *E. apionodes*, 13 had H-1, 8 had H-6, and these 2 haplotypes infected *A. flavicollis* and *A. sylvaticus*, but not *A. agrarius*?

Overall Summary

I must re-emphasize the status of taxonomy in biology, because understanding its importance solidifies the value of the numerous contributions made by Ms. Kvičerová and her colleagues. Repeating what I wrote for a previous contribution (Tenter et al., 2001): "Taxonomy is the most basic activity in biology because it involves the discovery, analysis of variation (quantitatively or qualitatively), naming (nomenclature), ordering (classification/systematics), and communication (publication) of the patterns of all life forms. It makes these life-forms (species) historical, temporal, and spatial entities that are the essential elements of biodiversity, i.e., the genealogical packages that store and transmit the information that leads to the interactions within complex ecosystems. Species are the atoms of biology, they comprise our periodic table." And right now coccidiologists are attempting to understand the biology of their organisms knowing only 1 or 2 elements in the periodic table of coccidians.

The work done by Ms. Kvičerová and her colleagues over the last 5 years is Herculean in volume, carefully executed, substantive in quality, and continually pushes the boundaries of our thinking in its conceptual nature. The body of work in this thesis certainly would be accepted to satisfy the dissertation requirement for a Ph.D. in the United States and I STRONGLY RECOMMEND TO PASS for satisfying that requirement here in the Czech Republic.

Respectfully submitted,
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Opponent's evaluation of the Ph.D. thesis entitled "Phylogeny of coccidia and coevolution with their hosts" presented by Jana Kvičarová

1. Subject of the Ph.D. study and its significance for parasitology. Coccidia, parasitic protists of the phylum Apicomplexa, represent the most numerous and diverse class of the phylum. For their both theoretical and practical importance, they are a "hot" research subject in modern biology and parasitology. The present research on coccidia is generally oriented to a limited number of organisms which are either causing animal or human disease, or are convenient laboratory models for biochemical and molecular studies (e.g. poultry coccidia, *Toxoplasma*, *Neospora*). The merit of Jana Kvičarová's Ph.D. study is that it opens to the reader a less known world of some "unconventional" coccidian species of the genus *Eimeria* of small mammals. Her Ph.D. study brings interesting data and observations on (1) several aspects of the evolutionary relationships between hosts and *Eimeria* species parasitizing on different rodent families and other small mammal (insectivores, rabbits, tree pangolin); (2) evaluation of the intraspecific variability and population structure of *Eimeria* species from field mice of the genus *Apodemus* (*Eimeria* - *Apodemus* model); and (3) comparison of morphological traits of sporulated oocysts of *Eimeria* species with results of molecular phylogeny. The individual results are discussed in more detail below.

2. Has the Ph.D. study reached its goal? The core of the Ph.D. consists of 3 papers published in international parasitological journals of good standing and three papers which are being prepared. As Jana Kvičarová is the first author in all published papers and two of papers in preparation, one can assume that her contribution to these papers was essential. The three published papers in peer-reviewed journals fulfil thus the general requirement for Ph.D. study as far as the number and quality of publications is concerned. I also found the background section of the Ph.D. thesis comprehensive and concise, providing the introductory information needed for understanding the papers that follow in an organised and well thought manner. This forty-five page long text then complements the publications and merges author's observations with a general discussion on the molecular insight into phylogenetic relationships, host specificity and morphology; summarizes the knowledge of the host-cophylogeny, presents the general picture of the molecular phylogeny of coccidian parasites and in more depth deals with the representatives of the genus *Eimeria* in different species of small mammals. The text complementing the publications documents the excellent knowledge and orientation of Jana Kvičarová in literary data concerning current research of coccidian parasites in small mammals and current state of their phylogeny.

3. Methods used in the research are modern, up to date and their spectrum corresponds to that currently used in both molecular/phylogenetic area and "classical" coccidian research.

4. Important results of the Ph.D. study. Besides the description of the endogenous development, pathogenicity and host specificity of *Eimeria cahirinensis* from *Acomys dimidiatus* from the Near East, this Ph.D. study presents results of the first study published on rabbit coccidia in the field of molecular phylogeny and evolution. Based on analyses of nuclear 18S rDNA sequences, Jana Kvičerová found 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. One of the most important results in the view of this reference is the conclusion on population-genetics, genomics and proteomics approaches (microsatellites/STRs, minisatellites/VNTRs, AFLPs, SNPs, ESTs) in parasitology. Jana Kvičerová concluded that some of these methods (e.g. AFLP) cannot be applied for coccidian parasites, 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. Jana Kvičerová finally deduced that 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.

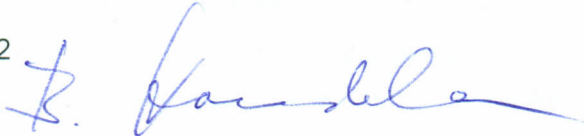
5. The importance of the thesis for the field of parasitology. This Ph.D. study brings numerous new data both for parasitology, as well as for the biological disciplines of systematic biology and evolutionary biology. The molecular data which enabled the author (and her co-workers) to produce trees tracing and precisising evolutionary history of coccidian parasites of small mammals and to try to graft this history to the coevolutionary history of the hosts have special value.

6. Comments and one question. Out of the published papers and the prepared manuscripts presenting the factual basis of the thesis, three papers were published in peer-reviewed journals and it would be superfluous to discuss them here. The paper No. V, presented as "in preparation" looks as the "crown" paper in which Jana Kvičerová with co-authors attempted the analysis of the data on *Eimeria* spp. with 86 new sequences of eimerians from 16 small mammals genera, mostly rodents. The results of this analysis 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 authors concluded that the distribution of eimerian parasites from different hosts indicates that the clustering of *Eimeria* species is influenced by their host specificity, but does not arise from a cophylogenetic/cospeciation 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 processes.

What would be the solution/result for applying of the criterion based on the host specificity for the taxonomy of monoxenous coccidian species of the family Eimeriidae which are traditionally known as host specific parasites?

7. Conclusion. I believe that Jana Kvičarová's thesis proves beyond any doubt her qualification for obtaining the doctoral degree and I warmly recommend the acceptance of the Ph.D. thesis. I also recommend that after satisfactory defence is Jana Kvičarová awarded the title „ Philosophiae doctor – Ph.D.“.

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