Jílové u Prahy, November 24, 2014

Reviewer's report for the Ph.D. thesis of Alena Kodádková, School of Doctoral Studies in Biological Sciences, University of South Bohemia in České Budějovice.

The thesis presented by RNDr. Alena Kodádková is focused on the diversity and molecular as well as morphological evolution of Myxozoa, an enigmatic taxon of structurally extremely reduced metazoans. The thesis is a document of scientific quality and scholarship. The introductory chapter and summary of the results show clear understanding by the candidate of the deeper issues in the field. The text is written in a clear way and contains only negligible, usually punctuation errors, which do not interfere with understanding; they will not be dealt with here, except for one typo that I found quite funny. The candidate states (on page 20) that "These findings contribute to ... an important topic that emphasizes the general necessity of species elimination." Personally, I do not sympathize with human-driven extinctions, but maybe the myxozoans are so peculiar that an exception can be made.

The thesis consists of seven papers. Five of them have already been accepted or published in peer-reviewed journals. Three papers have been published/accepted in journals with impact factor between 2.5 and 3.5; two papers have been published in International Journal for Parasitology: Parasites and Wildlife, which, as a new journal, does not have an impact factor. In addition, two unpublished manuscripts are included in the thesis that have been sent to press and presumably are now undergoing the review process. While I might have reached slightly different conclusions from some of the data or have performed some different tests on some of the issues, the analyses and conclusions are reasonable, and I believe both manuscripts will be accepted in good journals, though paper VI still needs a little more work. From the seven papers/manuscripts included in the thesis, Alena Kodádková is the first author of two of them and co-authored the others. She is also the corresponding author of one of the submitted manuscripts. I think all papers are relevant in the field of Myxozoa, and I like especially paper IV dealing with Malacosporea.

Questions and comments:

1. The candidate mentions on page 10 that "It was found to be extremely problematic to amplify myxosporean protein-coding genes". Can she explain it more?

2. There were several new myxosporean species described in the papers included in the thesis. The type material is often represented only by a DNA sample. Despite this approach does not violate the International Code of Zoological Nomenclature, it is not very common. Do the DNA samples represent a syntype or holotype (or hapantotype, as a special variant of the latter)? More importantly, it seems from the descriptions that the samples contained only DNA of Myxosporidia and were not contaminated by host's DNA. How was this achieved?

3. *Zschokkella siegfriedi* was distinguished from the closely related and morphologically identical *Z. hildae* on the basis of differences in SSU rDNA, slightly different host and localization of sporogenic stages. However, the authors admit that both host and tissue specificity may not be significant in this case. This leaves us with genetic distance being the only important feature justifying the existence of *Z. siegfriedi* as a separate species. Species delimitation on the basis of molecular data is always difficult task; this is true particularly for taxa where no obvious "barcoding gap" exists. SSU rDNA of the two *Zschokkella* species

Actinopterygii is assessed. By the way, is anything known about the presence of Myxozoa in coelacanths or Dipnoi?

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8. "*Ceratomyxa* spore morphology represents an evolutionary old morphotype which is supported by the firm position of the *Ceratomyxa* clade as the basal taxon of the marine myxosporean lineage (Page 142)." Is there a rule which says that if two closely related clades considerably differ in the number of species, the smaller one is comparatively more plesiomorphic?

9. Members of Malacosporea have been found only in freshwater bryozoans. The authors of paper IV think that this is the result of an insufficient sampling of marine bryozoans, and "the existence of malacosporeans in the marine environment is highly probable due to the fact that the vast majority of bryozoans are marine species." Coincidentally (or maybe not coincidentally), the class Phylactolaemata, which comprises all fresh-water bryozoans, is a sister lineage of the rest of Bryozoa. Is there any estimate when the basal split within crown Bryozoa occurred? In case the split occurred before the emergence of Malacosporea, is it possible that Malacosporea have, in fact, evolved in freshwater environments? Also, in what part of the colony of marine bryozoans would malacosporeans parasitize? It is known (or at least I think) that the zooids of marine Gymnolaemata are much smaller than that of freshwater Phylactolaemata.

Conclusion

Despite my comments, I am convinced that the papers contribute significantly to the understanding of the studied aspects of Myxozoa. This thesis was a pleasure to read, and it is certainly worthy of a PhD.

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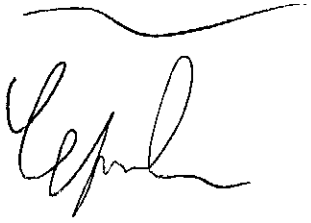

Opponent's evaluation of the PhD. Thesis of Alena Kodádková **„Myxosporean phylogeny and evolution of myxospore morphotypes"**

The PhD. dissertation of Alena Kodádková reviews the phylogeny of parasitic cnidarians of the unranked subphylum Myxozoa and analyses the evolutionary trends of morphotypes of their class Myxosporea, representing the majority of the presently known myxozoa.

The subject of dissertation is of research importance. With more than two thousands nominal species in about 60 genera, Myxozoa represent one of the most important group of parasites of animals and provide a rich material for studying the origin of parasitic cnidaria, their diversification, evolution and host-parasite relationships. And, as the dissertation under defense shows, the known myxozoa and their known life cycles represent evidently only a fragment of parasitic cnidaria in existence. Thus the understanding of the rich repertoire of myxozoan species and morphotypes is of crucial importance not only in taxonomy, faunistics and understanding of myxozoan biology but is relevant for the understanding of biological evolution in general.

The dissertation consists of two parts: a review of the present biology of myxozoa, stressing their phylogeny, and a supporting part consisting of 7 papers. Combination of classical microscopy methods (LM, EM) with usage of molecular markers and methods of phylogeny analysis is the methodology used throughout the dissertation. It allowed the candidate (and her co-authors) to tackle the old persisting problem of myxozoa research, how to weight different morphological and parasitized tissue characters in myxozoan classification and how to deal with many myxozoan polyphyletic genera. At the same time the usage of molecular markers allowed for the search of myxozoa occurrence across a range of hosts.

Let me comment briefly the supporting papers which represent the factual basis of the thesis and represent the foundations on which the narrative part of the thesis is based. These papers have to be evaluated in the context of more than 40 papers published in the last 12 years by the laboratory of fish protistology of the Institute of Parasitology, Academy of Science and of the Department of Parasitology of the Faculty of Science of the University of South Bohemia. These laboratories systematically pursue the phylogeny and evolution of myxozoa andare the followers of an older tradition of fish parasitology research represented by the work of late Jiři Lom and prof. Iva Dyková.

Out of the seven papers forming part of the dissertation, four were already published in peer reviewed journals, one paper is in press, and two others were submitted. In these papers Alena Kodádková participated in different degree, indicated for each paper. Her participation involved sampling of material, structural characterization of some myxozoa using light and electron microscopy, sequencing of a part of the genome, performing phylogenetic and molecular clock analyses, and drafting and writing the manuscript. Laboratory milieu in which Alena Kodádková worked was the guarantee that the PhD student got an ample opportunity to master a broad degree of techniques necessary for quality scientific research. As already mentioned, all papers forming part of the dissertation deal in some way with the phylogeny of myxozoa and their evolution and strive to contribute to a better understanding of their diversity. Their taxonomic "by product" is the description of several new species and the definition of some evolutionary clades. Of special importance in this respect are two papers of which Alena Kodádková is the first author. One of those papers deals with microsporidia in fish of high Arctic (paper III). It describes three new myxosporean species and more importantly tries to follow the evolutionary trends within marine myxosporeans infecting

urinary tracts of their hosts. I am of the opinion, however, that the second paper of this "submitted series" is the one which should be especially mentioned.
The paper No.VI. describes a new species of a phylogenetically ancient myxosporean. Its phylogeny then allowed the authors to trace the early phase of myxozoa evolution and to propose a hypothesis that the two myxozoan evolutionary clades (the Myxosporea and Malacosporea, respectively) split from a common ancestor in the Cambrian era and that cartilagenous fish represented an ancestral host for myxosporeans. The repeated entrance of myxozoa into bony fishes then led to their rapid diversification. It seems that the paper is the first attempt to trace the deep evolution of myxozoa using molecular methods and it brings a number of evolutionary hypotheses of how many times myxozoa entered bony fish as their hosts, when amphibians became hosts of myxozoa. Of interest is also the speculation of how and why different myxozoan spore ornamentation came into existence. The paper also indirectly implies that malacosporean myxozoa originated in marine environment. This opens the question: where are the marine malacosporea today? Only freshwater malacosporea, the second evolutionary clade of myxozoa, are known and are surprisingly diverse as shows another paper in the series and one of which the candidate is a co-author (paper IV).
This paper shows that the occurrence and diversity of malacosporea in freshwater fish is far greater than expected, implies besides other findings (5 new species of two respective malacosporean genera were found) the evidence of existence of a new malacosporean lineage. Surprisingly however, no molecular signature of malacosporea was found in marine fishes, despite the fact that malacosporea-like organisms were reported in literature to exist in marine bryozoa. This opens an interresting possibility that marine malacosporea are parasites of bryozoa only and fish are they accidental hosts only in freshwater environment. Or is it imaginable that a very diverse branch of malacospora in marine bryozoa and fish exists, while the malacosporean primers used for freshwater species are not able to amplify their DNA? Can the author of the dissertation comment that?

The second and most important part of the dissertation is its textual part. On 32 pages Alena Kodádková presents a general overview of Myxozoa, as it emerges from her work, work of her laboratory colleagues and the recent scientific literature.

General characteristics of Myxozoa are presented, followed by chapters on phylogeny, ecology, diversity and evolution. Of special interest is the chapter 5 summarizing author's research. Here are treated new myxosporean lineages, Myxozoan diversity and cryptic species phenomenon, phylogenetic position of newly found morphotype and the evolution of myxospore characters and the history of myxozoan evolution.
According my oppinion, the text part of the dissertation is a successful attempt to summarize the actual knowledge of myxozoa, stressing their phylogeny and evolution as based on molecular markers.

My criticism concerning the text part of the dissertation is minor.
While it is true that myxozoa infect aquatic-animals worldwide (p.1), it is certainly of interest that some myxozoa succeeded in infecting terrestrial mammals and semiterestric amphibians and birds. This information has to be reconciliated with more details given on page 4, 12 and 14. In the history of myxozoa research (section 1.1.) the whole period between the discovery of myxozoa (1841) and the year 1970, when myxozoa had been considered to belong to Protozoa, is covered only marginally. Thus, two important papers escaped author's attention. One is the classical monograph summarizing the early knowledge of myxozoa, written by the French author Thelohan (Thelohan P. "Recherches sur les myxosporidies", Bull. Scient. France Belgique, Vol. 26, 1895), the other is the important paper by P.P. Grassé from the year

1970 (Grassé, P.P. "Les myxosporidies sont les organismes pluricellulaires". C.R. Acad. Sci. Paris, 25, 1970). Grasse's paper was the first one bringing electron microscopy evidence that myxosporidia are not Protozoa. Thus was confirmed the pioneering observation of Antonín Štolc on Actinomyxidia published in 1899, (mentioned briefly in chapter one) that actinomyxidia are related to myxosporidia and that these organisms are animals. These historical milestones would certainly be appropriate to insert, if Alena Kodádková eventually decides to enlarge and publish the text part of her thesis in the form of a review article.
On page 4, the malacosporean life cycle is presented as occurring in the excretory system of a fish and the body cavity of a bryozoan. As only one such cycle has been described, is there any reason to believe that other malacosporea have the same life cycle?

Conclusions: the doctoral thesis of Alena Kodádková has brought important new data and views allowing better comprehension of the variability, phylogeny and evolution of myxozoa. The thesis corresponds to the requirements required for doctoral dissertation as it witnesses the ability of its author to work using classical and modern scientific methods and to present the results of her work in proper form required for publication. I recommend that the thesis is defended.

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November 13, 2014

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Eilat, 21 November, 2014–11–21

Myxosporean phylogeny and evolution of myxospore morphotypes

Evaluation of a thesis submitted for the degree of Doctor of Philosophy at the University of South Bohemia in Ceske Budejovice

This thesis deals with evolutionary aspects of the Myxozoa, a group of commercially important microscopic metazoan fish parasites. Myxosorean vegetative stages are typically amorphic, and for years only the spores which have some, although minimal, morphological features) were relied upon as a basis for species descriptions. The taxonomy of the group, using molecular methods, has become a target of intensive research in the last two decades, and the currently accepted approach is that a blend of classic, descriptive taxonomic coupled with molecular analyses can lead to incremental improvement of classification of the group. Nevertheless, revisions driven by phylogeny must be carefully weighed up against biological characteristics and bionomical traits (and preferably, supported by experimention). In this context, to cite a very appropriate paper entitled "The study of species in the era of biodiversity: a tale of stupidity" Diversity 2: 115-126 (2010, open access), F. Boero colorfully explains why a combination of traditional and novel molecular techniques is absolutely critical for any modern biodiversity study: "...before putting a specimen in a grinder, one has to guess what it is by inspecting a phenotype, and give it a name. Molecular taxonomists know phenotypes in a rather primordial way. So we might end up having very precise sequences, deposited in international data bases that are referred to nominal species that might have been identified in an incorrect way...". Indeed, it is crucial that careful morphometrics be an integral component in the analytical study. Such an approach bonds together pieces, as in a puzzle, and often results in a much improved resolution of "the big picture". The current study is in line with this general concept, and Alena has reconfirmed in her results the many advantages of this holistic approach.

The present study focuses on morphological and molecular characterization of selected myxosporeans with little or no previous molecular data was available. The objectives of the study (as outlined in Chapter 4) included investigation of cryptic myxosporean species from closely related fish hosts; and exploring myxosporean evolutionary trend and an attempt to trace myxosporean character evolution for the estimation of a timeline for Myxozoan divergence. In addition, Alena participated in a multifaceted project that investigated the diversity of the Malacosporea – a second class within the myxozoa.

I would characterize the core component chapters of the thesis as the following two studies:

- A study of the Myxozoa of benthic and pelagic fish in the Norwegian Arctic region showed that water depth is a more important factor than geographic distribution for myxosporean diversity. Three species belonging to the genera *Zschokkella, Parvicapsula* and *Sinuolinea* were described as new species, and included the study of a *Latyspora*-like organism. The study generated ample new data for the genera *Ceratomyxa, Myxidium* and *Schulmania*, and widened the rather limited knowledge on myxozoans infections in Arctic fish, in particular those inhabiting the urinary bladder of their fish hosts.
- Exploring the phylogenetic ambiguities of the myxosporea and attempting to elucidate some of them by comparing ancient vs. extant lineages. *Bipteria vetusta* n. sp. is a species that infects the gall bladder of *Chimaera monstrosa,* and the study by Alena is the first molecular characterization of such a species, essentially a living fossil, and using it to delve into myxozoans evolution. The species was distinguished as representing a separate basal sub-lineage within the marine myxosporean lineage. She went on to hypothesize that myxosporeans evolved at the time of the origin of cartilaginous fish, which then became their first vertebrate hosts; and later, species radiation facilitated the spread of myxozoa to freshwater and marine host species.

The additional chapters, to which Alena contributed considerably, but wereled by other researchers, are as follows:

- A study of *Chloromyxum careni*, the first of the genus from amphibians to be sequenced. Probably it represents a distinct lineage, which in the future will possibly warrant a new species.
- A study of *Sinuolinea* infection in *Cynoscion nebulosus* and *C. regalis* which focused on cryptic species within the genus and highlighted the hypothesis that genetic divergence may be strongly linked with ecological niche variability; and that in myxosporeans which have a biphasic, heteroxenous development (involving invertebrate and vertebrate hosts), environmental pressure can be a key determinant in species delimitation.
- A study on the hidden diversity of malacosporeans, which yielded many new records and significantly extended the knowledge on the phylogenetic character of the class, including enrichment of Genbank with sequence submissions, and, last but not least, an addition of a new, separate malacosporean lineage from Ohio, USA. The fact that no marine fish yielded any malacostreans is intriguing, since the primary hosts of these parasites, the Bryozoa, are much more diverse and common in the oceans as compared

to freshwater habitats. These results offer new challenges for future research on the evolution of this enigmatic group.

- A morphological and phylogenetic analysis of two *Sphaeromyxa* species from shallow water marine fish from the South African coast.
- A study of the evolutionary origin of *Ceratonova shasta.*

All of the objectives listed in Chapter 4 have been well addressed by the candidate in the series of 7 publications put forward in the thesis, four of which have been published (2011, 2013 and 2014), and one accepted for publication. Two are 'in review'. Such a large number of publications in a single thesis is quite extraordinary. All papers were published in highly regarded parasitology-related journals: the Journal of Eukaryotic Microbiology (IF=2.911) (1), the International Journal of Parasitology: Parasites and Wildlife (no IF, new Journal) (2), the Int. J. of Parasitology (IF=3.404) (1), and Parasitology (IF=2.350) (1). The two 'in review' manuscripts are in the International Journal of Parasitology and Molecular Phylogeny And Evolution. Alena is first author in two of the publications, second author in two, third author in two and 5th (of 8 authors) in one. Overall, the extensive new data generated by the collective publications, coupled with their very high scientific quality, are a clear endorsement for Alena: she in obviously a very capable and talented young researcher.

In the introductory part of the thesis, which precedes the published manuscripts, Chapter 1 presents a brief account of historical myxozoan research, their morphology and classification principles, developmental cycles and their significance as pathogens. Chapter 2 deals with myxozoan affinities to their taxa, the phylogeny within the group, and the issues arising from discrepancies between taxonomy and phylogeny. Chapter 3 discusses myxozoan ecology, their host specificity and tissue tropism, their host range, and the evolution of myxosporean spore types. Finally, Chapter 5 highlights the core findings of the thesis. I have a few comments (typos mainly) on these introductory chapters.

Page 1: infections are 'intramuscular' (not 'intermuscular')

Page 6: Enteromyxum does not develop intercellularly. All of its known stages are in the mucosal inter-cellular spaces.

Page 6: Line 13 from bottom. Sphaerospora dykova is not a marine species.

Page 7: Is it 'Phorocyte' or 'Pericyte'?

Page 8: should be 'plumatellae'

Page 13: Line 5 from bottom: 'begun' instead of 'become'.

Page 15: Line 5 from top: the term "highly polyxenous' seems awkward, as it would seem to me that a parasite species can be defined as either monoxenous or polyxenous, it is not really a spectrum. Suggest changing to "....cyprinid fishes), others (e.g. *E. leei* and *K. thyrsites*) infect many taxonomically different host species..."

Page 15 Line 18. Should be '*Tubifex tubifex*'

Page 16, top paragraph. There is a problem with an incomplete sentence: "Intracellular parasitism is also Sphaerospora (Kristmundsson & Freeman 2013)".

Page 18 Line 8 from bottom: should be 'amphibians'.

Page 19, section 5.2, second line: Should read: 'Our PCR screening of myxozoa from different fish hosts and habitats

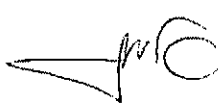
Page 21 Section 5.4, 3rd line: "an evolutionary ancient" seems redundant. Should read: '...data from such a host group, which is reflected by the fact...

Page 21, Line 6: should be 'adaptations'

Page 22 top line – "at that time" meaning 1966 or 100mA? ☺. Change of wording recommended.

In summary: This is an excellent thesis. It contains an important contribution to the knowledge of the myxozoa and provides insight into mechanisms that govern the evolution of this important parasitic group. The research methods employed are state of the art; the written English and presentation are crystal clear; and the figures are well constructed and of high graphic quality. The few errors found in the text indicate great attention and careful copy editing by the candidate. All these lead me to conclude that Alena's research is worthy of defense and I absolutely recommend that she be awarded the degree of Doctor of Philosophy.

Sincerely,



Ariel Diamant, PhD

As requested, here is a question for Alena's defense:

The differences in function of the "polar filament" in Myxosporeans and the "polar tube" in Microsporidia (as pointed out on page 1) is suggested that in the former, it

serves as an "anchor", while in the latter, as a "conveyer of infection". Is this commonly accepted view necessarily correct? What do you think?

(Comment: This long held "dogma" may soon turn out to be erroneous. In fact, that the myxosporean polar "filament" is actually a tube" has been known for many years, as it is formed by invagination into the primordial polar capsule (see the classic papers of Lom and co-workers of the early 1960s). The question is - what does the myxosporean tube "transport" through its lumen? What do you think?)