PhD thesis review

Title: Phylogenetic analyses of myxosporeans based on molecular data
PhD candidate: Pavla Bartosova
Institution where work was conducted: Institute of Parasitology, Biological Center, Academy of Sciences of the Czech Republic, Department of Protistology.
Reviewer: Astrid S Holzer, PhD (Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia)

Brief summary of content:

The current thesis focuses on Myxozoan relationships on three different levels using ribosomal (SSU and LSU) and protein-coding (EF2 and HSP70) gene sequences, most of which were produced during the present study. Analyses concentrate on the position of the myxozoans within the metazoans (general part, no paper published), the relationships and evolutionary trends within the phylum Myxozoa (papers I, II) as well as within different subclades of the phylum (papers III, IV and V). Morphological and bionomic characteristics are also observed and mapped to phylogenetic trees. The author of the thesis has contributed to three articles as first author and to two further as second author.

General impression of thesis contents and appearance:

I believe that the work conducted for this thesis is substantial and represents an important contribution to our knowledge of the phylogenetic relationships of the Myxozoa. The amount of new data produced and the generally sound approaches to critically investigate the obtained data is exemplary and shows that the candidate has matured to a scientist who develops and critically analyses ideas independently, and who definitively deserves the title PhD.

Despite the by far predominant positive aspects of this thesis, I would like to contribute to a further improvement of the manuscript by improving the structure of some parts (introduction), pointing out a few mistakes and by encouraging further analysis and discussion on certain points that, according to my opinion, did not receive sufficient attention by the author. I have been quite critical in several points which should not offend the candidate but help to improve the thesis and stimulate further discussion.

Formatting and language: Page numbering should be conducted throughout the manuscript and the text should be formatted justified. The English is good but could be improved in some parts of the manuscript; however, I don't think it leads to misunderstandings in any case.

Review including specific comments on different parts:

List of **OBJECTIVES OF RESEARCH**:

This list should be completed more comprehensively. It lacks several important aspects, e.g. the mapping of morphological characters of the spores to the phylogenetic trees of molecular data, or the development of multiplex PCR assay to differentiate between closely related species, etc.

GENERAL INTRODUCTION:

Classification & taxonomy (1.2) and life cycles (1.3):

- As the author has given it a special section and subheading in the introduction, the issues of intermediate / definitive host and their spore stages should be described in detail for both, myxosporeans and malacosporeans:
- In 1.2. the author develops on the morphological details of the malacosporean spore stage produced in the bryozoan hosts but only mentions details on the equivalent spore stage of myxosporeans (=actinosporeans), and their hosts (oligochaetes and polychaetes) later (in 1.3). Taxonomy (subheading 1.2) in both classes is based on the spore stage from the fish host and thus I believe the detailed description of the bryozoan malacosporean spore should be moved to 1.3. Furthermore, the author should provide the same amount of detail on the cellular composition and symmetry of the actinosporean spores as given for the malacasporean spore from the invertebrate host.
- In 1.3.2 the author mentions that "gametogony" takes place in the actinosporean stage. However, no statement is made on the occurrence of meiotic divisions in malacasporeans although it is known that these take place during spore formation in the bryozoan host [Canning EU, Curry A, Feist SW, Longshaw M, Okamura B (2000) J Eukaryot Microbiol 47:456–468].
- Care should be taken with the use of the terms "actinospore stage" and "actinosporean stage" as one refers to the mature spore and the other one may refer to stages previous to spore development, as the binucleate stages that show meiotic divisions in the oligochaete.
- In 1.3.2. add moles as potential vertebrate hosts [Friedrich C, Ingolic E, Freitag B, Kastberger G, Hohmann V, Skofitsch G, Neumeister U, Kepka O (2000) Parasitology 121, 483-492].

Importance of the Myxozoa (1.4): In this section the author provides a description of some species and the site of their infection but the pathology caused, mortality rates in infected fish (often 100%!) and the economical importance for the aquaculture industry is documented only very scarcely. According to the title of this section, it would be desirable to provide a bit more detail with this regard.

Relationships Myxozoa-Metazoa (2.1): Well documented with detailed information.

Phylogenetic relationships within the Myxozoa (2.2): Generally well written and cited, however, the author missed out on mentioning one of the most important patterns of myxozoan phylogenetic clustering, i.e. the invertebrate (final!) host [Holzer AS, Wootten R, Sommerville C (2007) Int J Parasitol 37, 1281-1295]. I am aware of the fact that the terms "marine clade" and "freshwater clade" have been established before the more recent discovery of further life cycles in the marine environment and that these terms are being maintained, but the fact that some myxozoan species clustering in the marine clade have invaded the freshwater environment without changing their invertebrate host group indicates that these clades would better be termed "polychaete clade" and "oligochaete clade" and that clustering according to invertebrate host is more important than the host´s environment, although changes between marine and freshwater habitats seem to have caused considerable expansion of the V regions in the ribosomal SSU sequences. Clustering according to the invertebrate host is one of the most important patterns of clustering devoid of exemptions, and it should be mentioned in this section.

PAPER I

This paper is extraordinary with regard to the sound approach taken in the techniques used. Phylogeny based on SSU and LSU ribosomal data has been investigated systematically by determining the most signal-rich rDNA region, the best set of data (single and concatenated gene region use, GBlocks), a combination of different phylogenetic algorithms and the analysis of tree topologies by tree indexes in order to come as close to the true phylogenetic history of the myxozoans as possible. Well done.

Remark with regard to the results: Due to the concerted evolution of the ribosomal gene tandem, one would not expect much difference when comparing the results from LSU and SSU ribosomal sequences. However, there are somehow conflicting signals with regard to some species, most importantly *Sphaerospora ranae,* which is not well supported in the analyses and differs in its position in the tree according to the ribosomal dataset used. Do you have any data from potein-coding sequences to support any of the positions observed in this study?

PAPER II

Apart from the confirmation of the phylogenetic tree based on ribosomal sequences by EF2 data, this paper represents a well-appreciated approach to map morphological and bionomical traits to the phylogenetic tree of Myxozoa as a first step to resolve the controversy between molecules and morphology observed in the Myxozoa.

Unfortunately, there is a major mistake in this paper related to the spore morphology of the bryozoans: As stated correctly in the introduction of this thesis, fishmalacaspores have FOUR spore valves, not TWO [Morris & Adams (2008) Parasitology 135, 1075-1092]. Together with the even higher number of valve cells in the bryozoan-malacospore (8 cells) this is supportive of a reduction of (possibly not only) the number of spore valve cells occurring in the myxosporeans. Probably the ancestor of all Myxozoa was multivalvular and not bivalvular/*Sphaerospora*-like. I saw that the paper is already published online so I don´t know if this can still be corrected or if the authors would want to publish an Errandum.

While I believe that the mapping of spore morphology to the phylogenetic tree is indeed very useful I also think that some other bionomical traits should have been included in such an approach, and an interpretation with regard to functionality is desirable for some spore characters:

- Missing bionomical character of definitive host.
- Furthermore, you did not include or at least comment on the spore stages produced by the definitive host although their morphology very strongly supports the phylogenetic clustering of the myxozoans into the major clades, with the large number of valve cells and radial symmetry in the malacosporeans, the triradiate symmetry of all actinosporeans, the "marine"=polychaete spores lacking any appendages, and with various patterns of appendages developing in the spores in the recent "freshwater"= oligochaete host species.

- Other missing bionomical character: Different lines of cellular presporogonic and sporogonic developmental stages as detailed in Morris & Adams (2008) Parasitology 135, 1075-1092.
- Character of polar filament: Detail is missing on the malacasporeans which have a very special polar filament, which is S-shaped in cross-section, occurring only in this clade [Morris & Adams (2008) Parasitology 135, 1075-1092].
- Ratio of dimensions of spore width to thickness: I don't understand this. Should length of the spore not be included here rather than thickness? Thickness is not even measured in all species. Please explain.
- While not important in the process of mapping morphological traits to phylogenetical trees, yet some characters of both, myxospores and actinospores seem to be adaptations to the habitat of the spores or for their transmission, e.g. surface ridges and striations enlarge surface area and occur only in coelozoic species, where these features allow the spores to float in the bile or the urine rather than depositing on the gall/urinary bladder wall where they might be encapsulated by host tissue. Same for spore projections which avoid rapid sinking in a non-moving water column (lakes, ponds) and enlarge the radius of dispersion. Functionality of the spore design is extremely important for successful parasite transmission and this should be included in the discussion.

PAPER III

This paper describes a new myxozoan species, for which a new genus (*Latyspora*) was established within the Sinuolineidae, and to which another, previously described myxozoan (*Sphaerospora lobosa*) is ascribed. Furthermore, a phylogenetic study includes *L. scomberomori* as well as *Sphaerospora testicularis*, and traces the morphological character of straight versus sinuous suture line.

I have seen a previous version of this paper but have refused its review due to lack of time, however, I have followed the paper's development. I am glad to see that the authors have decided to describe a new genus, given the evidence that too many morphological details differ from *Sphaerospora.*

At the current stage, the paper seems a bit disrupted and patched (especially the discussion jumps from one topic/species to another), and I think this is because the previous version of it included the new species *L. scomberomori* as a species of *Sphaerospora* and hence the lengthy discussion about the different origins of *Sphaerospora* and the lack of discussion of the newly established genus. As the authors are describing a new genus, a considerable part of the discussion should focus on this and elucidate morphological similarities with closely related species of *Latyspora*. There is a distinct feature which is extremely similar in *L. scomberomori* and some closely related species, e.g. *Z. hildae* [Auerbach M. (1919) Zool Anz 35:57–63] or *Z. lophii* [Freemann, Yokoyama, Ogawa (2008) J Fish Dis 31, 921–930] which is the differing orientation of the coiling of the polar filament in the two polar capsules and the orientation of the openings of their discharge channels, a striking feature which unites the related *Zschokkella* species with *Latyspora*, besides the sinuous suture line. This feature has not been described for *S. lobosa*.

With the regard to *S. lobosa*, I strongly disagree with moving or reassigning this myxozoan or any other to another genus without providing molecular data as it is possible that the species may have to be moved again in the future. In the same way, I disagree with the demise of *Leptotheca* based exclusively on (non comprehensive!) morphological comparisons [Gunter & Adlard (2010) Syst Parasitol. 75:81-104]. As the authors state themselves, it is necessary to find a solution for the molecular-morphological controversy but not before enough data have been compelled. This includes molecular data on the species to be reassigned. However, you should of course make a statement on the morphological similarity of *L. scomberomori* and *S. lobosa* and its unjustified transfer to *Sphaerospora* in the same way as you do for *S. armatura, S. glomerosa, S. koreana* etc.

Remark on the "surprising" position of *S. testicularis* within the marine urinary species: Like the urinary myxozoans, *S. testicularis* is a coelozoic species and the male reproductive organs or ducts, in their ontology, have the same origin as the urinary system: They are formed by somites of the nephrogenous strand, hence the term "urogenital system" for the coelozoic system of urinary and male genital ducts. Urinary and genital ducts are of the same origin and due to their function, it is likely that their surface is similar. In the same way, the position of *S. dicentrarchi*, a true histozoic species with non-spherical PCs and overlapping shell valves (=unique character for all *Kudoa* and for *Unicapsula*!) should cluster amongst other histozoic species which share these specific characters.

The discussion is too long and extensively orientated in the direction of the different origins of *Sphaerospora*. Especially the section on *P. minibicornis* is rather confusing and I suggest this part to be omitted or shortened considerably as this species does not fit the morphological description of *Sphaerospora*, neither are you ascribing it to this genus.

Small comments:

- Use *L. scomberomori* n. gen. n. sp. throughout the text (missing in last paragraph of introduction).
- Material & Methods: Tracing character evolution: "Curved sutural line is defined as non-straight from at least one of the views (apical, sutural)" – change sutural to lateral.
- Etymology: change "wide shape" for "large/pronounced width"
- Results on phylogenetic analyses and tracing character evolution: "The curved form of sutural line arose once in the ancestor of other marine myxozoan clades" – this is not clear, what OTHER? Clarify. Mention also the occurrence of a sinuous sutural line in some members of the freshwater clade.

PAPER IV:

Redescription and phylogenetical analysis of *Chloromyxum careni* from amphibians. Methods, analyses and discussion are sound and the images from TEM and SEM are of very high quality. No criticism or points of further discussion here.

PAPER V:

On the existence of cryptic species assemblages of several myxosporeans.

In this study, the molecular part has received most of the attention in order to resolve the standing issues of cryptic species assemblages in different species belonging to *Zschokkella* and *Chloromyxum*. The developed multiplex PCR assay designed for the differentiation of mxyozoans in mixed infections in the bile, is certainly very useful.

While molecular differences between isolates of myxozoans from different hosts and sites were determined on the basis of SSU and LSU ribosomal sequences, differences with regard to the variability of the spore morphology in different hosts/sites were not evaluated. This would have considerably aided the determination of the relation between molecular and morphological plasticity of myxozoan species and thus be extremely useful for the definition of the species concept.

Other remarks:
Discussion on surface pattern of spores: "The monitored surface pattern of *C. fluviatile* from silver carp isolates accords to the surface pattern observed in the type host…." This observation together with the varying surface ridge pattern in *C. careni* suggests that the striations are not a good character for analysis of interspecific differences. The authors should state this at some point.
Eliminate NA from table in *C. cristatum* SSU as you are actually providing this sequence.

Review dated 17th August 17th August 2010
Signed:

Dr. Astrid S Holzer

Prague, August 31, 2010

Review of doctoral thesis of Pavla Bartošová "Phylogenetic Analyses of myxosporeans based on the molecular data"

PhD thesis of RNDr. Pavla Bartošová is focused on the evolution of Myxozoa. This metazoan group is interesting from several points of view. Myxozoa represent the most reduced animals whose relationship to other metazoan phyla has not yet been satisfactorily elucidated and even the current best hypothesis is controversial for many scientists. The taxonomy of Myxozoa is based primarily on the morphology of one stage of the life cycle. However, this system substantially contradicts results of phylogenetic studies and the whole group needs a taxonomical revision at almost every levels. In addition, some myxozoan species cause serious diseases of economically important fish. The research on Myxozoa is, therefore, highly desirable.

In the introductory chapter the author presents Myxozoa and summarizes current and historical information on their morphology, life cycles and taxonomy. A separate section "Relationships of the Myxozoa" deals with the evolution of this group, both on inter- and intraphylum level, and also with the evolution of morphological characters. At the end of the chapter the author presents guidelines for taxonomic revision of Myxozoa on the genus level. Both introductory chapters are of a high quality and the author obviously has a deep knowledge of the biology of Myxozoa.

The main part of the thesis consists of two articles that have already been accepted by renowned international journals (Molecular phylogenetics and Evolution, BMC Evolutionary Biology, IF 4.294 and 3.556, respectively), and three manuscripts submitted to international journals. I believe that these manuscripts will be, or, possibly, some of them have already been, accepted for publication after minor revisions. Pavla Bartošová is the first author of three of the studies, and the corresponding author of two of them. The first two papers deal with the internal evolution of the Myxozoa as a whole. I consider these studies particularly important for the whole "myxozoology". The other three manuscripts dealing with the morphology, phylogenetic position and taxonomic revision of particular Myxozoa species and lineages I consider also successful. I appreciate the high quality of the documentation of results.

The final chapter summarizes the results of the thesis. In addition to the summary of particular papers, the author surprisingly states that she has examined the phylogenetic relationships between different groups of Metazoa to determine the position of the phylum Myxozoa. However, the previous chapters do not show evidence of such investigation. The chapter "Relationships of the Myxozoa with the metazoan groups" in the introduction, which covers the topic, is theoretical and the following text does not imply that the author worked on the relationships between Myxozoa and the other Metazoa. Supplements 1 and 2 containing phylogenetic trees of Metazoa based on concatenates of different genes appear suddenly in the end of the thesis and it is unclear how the data were obtained and analyzed. Could the author explain this discrepancy? Personally, I would rather omit this part of the thesis, as the enclosed publications quite adequately demonstrate author's abilities.

I have the following comments and questions:

1. The secondary structure of SSU rDNA was mentioned several times in the text, for example: "subhelix E43" (p. 17), "secondary SSU rDNA structure characteristics", "helix E43 in the V7 region of SSU rDNA" (Article 3). The author probably meant the secondary structure of SSU rRNA. In this case, seemingly unimportant difference leads to quite different interpretations. Secondary structure of DNA and RNA molecules is quite different. In the native state they differ by the number of strands, base pairing (for example, the common presence of the important wobble pair GU in RNA molecules). In any case, it makes no sense to speak about the helix E43 (or almost any helices at all) in the secondary structure of DNA molecules.

2. At page 17 the author states that there is "an interesting correlation between the length of [ribosomal DNA] sequences and host environments". Why is this correlation interesting? As far as I understand it, the insertion in the SSU rDNA sequence was present already in the last common ancestor of Myxozoa from fresh-water fish. This is supported by the species *Parvicapsula minibicornis*, which, although escaped to fresh water, phylogenetically belongs to the marine Myxozoa and does not possess the insertion. Such examples, when ribosomal RNAs of particular lineages are of a special length, are common in almost all groups of eukaryotic organisms. It is also usually easy to find a morphological, ecological, bionomial etc. feature which "correlates" with the length of the sequences. For instance, it is more or less true that amoeboid trichomonads possess a long SSU rRNA. However, it naturally does not mean that their amoeboid nature is anyhow connected with the length of the SSU rRNA. Accordingly, it is unsurprising that the group of amoeboid trichomonads is monophyletic. There are also amoebae with a short SSU rRNA sequence, e.g. the genus *Entamoeba*. The case of Myxozoa is, therefore, nothing special or particularly interesting. On the other hand, the possibility that the insertion arose several times independently in connection with the switch of the host between different environments, would be immensely interesting. Such a scenario is, however, difficult to imagine and obviously does not apply to Myxozoa.

3 In the paper 4 the authors state that SSU rDNA of *Chloromyxum careni* contains a long GT-rich insertion. Is it not true that the GT content in DNA is always 50%? I believe the the GU-rich sequence in the SSU rRNA molecule is the correct expression.

4. What is the author's opinion on the phylogenetic position of Myxozoa within the animals? From the thesis I got (perhaps mistaken) impression that the author inclines rather to the hypothesis of Myxozoa being basal Bilateria and that she welcomes the fact that the phylogenomic study by Jiménez-Guri et al., which placed Myxozoa within Cnidaria, was recently challenged by Evans et al. How important, according to the author, are SSU rRNA genes? Although their analyses convincingly support the hypothesis of basal Bilateria, they are still only two genes. There are many historical examples, when analyses of SSU- and LSU rRNA genes led to the formulation of monumental, but erroneous assumptions (e.g. Archezoa, the position of microsporidia outside the Fungi, etc.). Moreover, even though the phylogenetic analysis of Jiménez-Guri et al. was based "only" (according to the author) on 50 *Buddenbrockia* genes (how valuable would 50 genes be for the others!), it is still much more than two ribosomal RNA genes. Finally, the missing data is no longer such a problem as it was in the past.

5. The authors of the paper 5 deduce from the great sequence variability of *Zschokella nova* that it represents, in fact, a complex of cryptic species. Accordingly, from low sequence variability of *Chloromyxum cyprini* they conclude that this organism represents a single species. I believe that such statements are premature, especially that one about the existence of cryptic species. It is difficult, if not impossible, to infer the number of species solely from the degree of sequence variation. Could the authors present also other evidence which would support their opinion?

In conclusion, RNDr. Pavla Bartošová has clearly demonstrated her ability of excellent scientific work. Her Ph.D. thesis, in my opinion, fully meets the requirements for the doctoral theses and it is my pleasure to recommend it for acceptance.

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Examiner's report on the PhD thesis of Pavla Bartošová

by Dr. Edit Eszterbauer

The PhD thesis by Pavla Bartošová focuses on the phylogeny of Myxozoa, a topic having remarkable influence on the taxonomy of this parasite phylum nowadays. The phylogenetic relationships were studied at three different taxonomical levels: the position of the phylum among metazoan taxa was analyzed along with the inter- and intraspecific studies within the Myxozoa. Besides the numerous phylogenetic calculations using different parameters and methods, several dozens of new DNA sequences were obtained in the course of the PhD study, which shows the great volume of the research work and the enthusiasm of the Candidate.

The position of myxozoans within the Metazoa was investigated using SSU and LSU ribosomal RNA genes (rDNA), and protein-coding genes like EF1, EF2 and alpha-tubulin. Phylogenetic analysis based on ribosomal genes confirmed the previous theory that Myxozoa is a sister taxon to Bilateria. However, the analysis of protein-coding gene sequences revealed a basal placement of the phylum on the phylogenetic tree, contradicting with the findings based on rDNA data. Interestingly, none of the analyses supported the close relationship to Cnidaria as it was reported by several authors previously.

The second taxonomical level studied was the interspecific relationship within the Myxozoa. The Candidate aimed to confirm the evolutionary trends recognized with the analysis of SSU rDNA, and intended to involve LSU and protein-coding sequences to the phylogenetic analysis. By detailed and accurate examination of various parameters, the Candidate and her co-workers found that the 5'-end of the D5 region of LSU in combination with SSU rDNA sequence data supplied the most reliable and robust myxosporean phylogeny to date. Besides, their findings confirmed the reliability of the single-gene analysis using SSU rDNA data. HSP70 and EF2 sequences were also involved in the phylogenetic analysis, however, the low number of examined sequences does not allow to draw conclusions from the analysis.

The comparative analysis of morphological and bionomical characters in correlation with SSU-based phylogenetic trees brought very interesting new findings. The analysis involving 20 characters predicted the main features of the common ancestor of myxozoans (which might infect the renal tubules of a freshwater fish and had a sphaerosporid shape).

Studying the intraspecific genetic variations in some *Chloromyxum* and *Zschokkella* spp. originating from different hosts and geographic regions, the Candidate detected that *Chloromyxum fluviatile* and *Zschokkella nova* are cryptic species, and that *C. cyprini* and *C. cristatum* are synonym species.

It is positive that the Candidate and co-workers are cautious and moderate in most cases concerning the taxonomical revision, and are willing to avoid premature taxonomical rearrangements before extended sampling as well as taxonomical and morphological examination has not been done on "problematic" taxa. The only exception is the case of *Latyspora lobosa* (formerly called *Leptotheca lobosa* then *Sphaerospora lobosa*) that was moved to the genus *Latyspora* based on morphological data available in the original description only. My opinion is that the given species should not be moved to another genus without molecular characterization and without the examination of additional samples by the Candidate.

The thesis contains 54 numbered pages separated in three chapters. Five papers form the basis of the thesis: one published paper, one in press, and three submitted manuscripts. Unfortunately, the papers and MSs are not numbered, which makes it difficult to search and hampers the orientation in the thesis. The format of the thesis is not uniform. It mainly follows the requirements of the journals, thereby the line spacing (sometimes double-spaced, sometimes 1.5 line-spaced), the format of the text and the references differ in Chapter 3. The language of the thesis is acceptable, although several phrasing errors can be found in text. Pictures, line draws, photomicrographs are of outstanding quality.

Chapter 1 (General introduction) and Chapter 2 (Relationship of the Myxozoa) summarize the state-of-art in myxozoan taxonomy, and give a general overview about the nature of myxozoan parasites. The two chapters involve a high number of references (over 100), and most of the relevant papers and theories concerning the myxozoan phylogeny are cited in the introduction. However, the reader has the feeling that several important findings reported by other authors are not explained well because of space limitations. Maybe, it would have been better to cite less papers, but explain the trends in myxozoan taxonomy and phylogeny in more details. Curiously, the papers of the Candidate, which create the basis of the thesis, are cited and their findings are already mentioned in the General introduction in details. I think this should have been avoided and the results of the thesis should have been mentioned in the Summary of Results only.

The Summary of Results summarizes the findings of the PhD work, which was already mentioned separately in the respective Results of the manuscripts. Three and a half pages appear to be rather short for such a large amount of results, and I would have included the data mentioned in Chapter 2 in this part. Also, I would have gone into more details concerning the results and their scientific relevance, during the Summary.

Finally, I would like to congratulate the Candidate and her supervisor for the outstanding work. And concerning the amount and quality of the research work as well as the number and relevance of the results, I fully recommend the Candidate to be awarded the degree of PhD.

Questions to the Candidate:

1. Several authors reported that most of the eukaryotes have a mosaic genome arrangement. Studying the genomic organization of a sea anemone, Putman et al (2007, Science 317:86-94) found that the genome of these "simple" animals is rather complex, possessing a gene repertoire, which is more similar to vertebrates than to flies and nematodes. They also pointed out that different genes may have separate evolutionary history, and thereby they might show diverse phylogenetic locations. What is your opinion about this theory? What do you think, how the analysis of further myxozoan genes will influence the phylogeny of the phylum?

2. Molecular biological studies unveiled several cryptic species among the Myxozoa, which were either poorly identified previously, or their characterization was confined to spore morphology only. Based on which characters do you think a cryptic species should be differentiated from a valid species? What is your opinion, how large DNA sequence difference is "allowed" at intraspecific level in SSU and LSU rDNA respectively?