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Dear professor Alena Zíková,

I am pleased to report back with my assessment, review, and comments on the PhD. thesis document submitted by RNDr. Jana Říhová titled "Genome evolution of endosymbiotic bacteria in blood-sucking insects".

Strict blood feeders have for long been overlooked in regards to their associations with microbial symbionts. Říhová's published work during her PhD. has moved forward our understanding of the nature of symbiotic associations between obligate blood feeders and microbes. Through both published manuscripts, she and her collaborators have uncovered novel associations between bacterial taxa previously not known as symbionts of arthropods. I found the most valuable and novel results to be those relating to the exploration of possible environmental sources of the symbionts, as well as that of broader exploration of nutritional symbioses across blood-feeding lice. Říhová's work thus contributes greatly to our understanding of not only the nature and evolution of nutritional symbioses in blood feeders but also to the process of the evolutionary transition from a free-living bacteria to an obligate host-associated one.

Following my detailed reading of RNDr. Říhová's thesis, **I recommend that the thesis be defended as planned.** I find the document well structured and the body of work to be commensurate to the expectations for a PhD. candidate.

As an annex to this letter, you will find my specific comments and questions arising from the thesis, which should promote further discussions.

I much look forward to hearing RNDr. Říhová's presentation and the discussion that will ensue.

Sincerely,

A large, solid red rectangular block used to redact the signature of Dr. Alejandro Manzano-Marín.

Dr. Alejandro Manzano-Marín, Ph.D.

ANNEX: Detailed review and comments

Introduction

I found the introduction to be broad enough to make the thesis readable and approachable to a larger biology-trained audience. I appreciate the effort taken in not only introducing the pre-existing knowledge relating to the microbial symbioses across blood-feeding arthropods, but also in covering the topics of genome reduction and other phenomena, such as horizontal gene-transfer and the role this can play in animal-microbe symbioses. In particular, I find the candidate covered well the topic of phylogenetic inference of symbiotic lineages, including the biases, problems, and methods to alleviate such problems (i.e. long-branch attraction). This is especially useful, as the candidate goes well into detail and effort to reconstruct the evolutionary relationships of the uncovered symbionts in the presented work.

A thing that I would have much appreciated are summary figures and diagrams condensing some of the most important information of the thesis. This could have been very useful for facilitating the reading of biologists outside of the field of animal-microbe symbioses and even outside of the blood-feeders' symbioses. Finally, a larger part could have been dedicated to the first two sections ("Symbiosis as a driving force of evolution" and "Basic characterization of symbiotic forms"), which I felt fall a bit short for providing a robust overview of the historical and state-of-the-art knowledge.

Aims and scope

I found the presentation of the aims and scope to be generally good and clear, particularly the summaries of the achievements, especially the direct mention to the research outputs. I did find the presentation of the starting hypothesis a bit convoluted, and would have appreciated a much shorter and concise statement of the hypotheses.

Manuscript #1

This first manuscript from the thesis, I found particularly interesting and serving as a base for a larger body of work sparking from the observation of *Neisseriaceae*-related bacteria and providing stronger support for the origin of symbionts from blood-feeding invertebrates from bacteria occurring as mammalian blood pathogens.

- **Questions for the candidate**

Regarding the "remarkable contrast" between the BLAST analysis for *L. polyplacis* vs. that of the *Neisseriaceae*-related symbiont, is this that "remarkable" (especially in the light of species diversity covered for *Legionella* vs. *Neisseria*)? What about undiscovered lineages between the two taxa?

Regarding the *Neisseriaceae*-related bacteria found in males, can you speculate as to their role? Do males feed? Is anything known about this organs or symbiont density throughout their life cycle?

In respect to the origin of the *Neisseriaceae*-related symbionts, is it possible those of *Hoplopleura* and *Polyplax* derive from similar free-living but symbiotically-prone lineages? I believe dismissing absolutely a "common symbiotic" origin might be too strong of a statement.XXXX

Manuscript #2

In this manuscript, the candidate and co-authors uncover a novel nutritional symbiotic bacteria associated to the chipmunk lice. What I found most interesting from this study is the uncovering of an additional *Sodalis*-related bacteria that has established an obligate nutritional association with a blood feeder. This is especially interesting in the light that this particular bacterial taxa is also widely found as a symbiont of, for example, phloem- and grain-feeders. This work sums to the body of knowledge about the propensity of the "*Sodalis*-like group" to establish symbiotic associations with arthropod hosts.

- **Questions for the candidate**

It is mentioned that N. pacificus rather forms a group of closely related species and the authors show phylogenies of both host and symbionts. However, not much is mentioned on the symbiont genome side. Are there patterns observed in the symbionts supporting the species separation (i.e. specific gene losses, low[er] nucleotide-level identity across the genome) that is comparable to, for example, different lineages of Buchnera or Blattabacterium in their respective hosts?

Based on the genomic analyses of Lightella and the Neisseriaceae-related symbiont, could possible collaboration (or dependence from the Neisseriaceae-related symbiont) in the production of B vitamins explain the co-occurrence of these symbionts?

Ongoing Research I

This work I believe provides one of the most exciting lines of research from the presented thesis work. The exploration of possible environmental sources of the symbiotic lineages is work that is rarely performed for a variety of technical and feasibility reasons. However, I believe the candidate succeeded in doing so, and as a result, provide very valuable information as to the nature and origin of likely "symbiotically-prone" lineages. This type of work has mostly been done in stinkbugs, and has the potential of providing valuable resources to experimentally modify and observe processes relating to the evolution of symbiotic lineages.

- **Questions for the candidate**

Why was the use of assembly graphs not used to insure genome completeness? This might be especially useful when dealing with pseudogene-rich genomes (such as the Neisseriaceae-related symbionts might have)? Without such methods, wouldn't you expect to miss non-coding regions?

Regarding the possible obligate nutritional symbiont (Neisseriaceae-related symbiont HaMa), can an accelerated rate of evolution be detected in (some of) the encoded genes that would support a life-style change?

Ongoing Research II

Being a strong supporter of discovery-based research, I found this work quite valuable. Here, the candidate took the challenge to explore the identity of nutritional symbionts of blood-feeders, finding support for the establishment of certain lineages before species diversification (i.e. those of *Polyplax*), the existence of *Francisella* symbionts in blood-feeding arthropods outside of ticks, and the much more common occurrence than expected of *Sodalis*-related symbionts across blood-feeders. I

believe this work will serve as basis for various future research lines exploring the nature and evolution of these associations.

- **Questions for the candidate**

While the origin of many of the symbiotic lineages can be expected to be the mammalian host, how do you explain the common presence of Sodalis-related bacteria?

What do you think about your results finding Riesia nested within Arsenophonus? Should this taxon be considered a synonym to Arsenophonus?

Why do you think Arsenophonus is recurrently evolving as a symbiont of blood feeders? What could be their ecological source?

Summary of the main results and conclusion

I find the summary of the results and conclusions good and concise. They present a useful summary of the main contributions of the thesis and drive clear connections among the different published and ongoing work.