



To
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Evaluation of the PhD thesis by Filip Husník
“Genomic and Cellular Integration in the Tripartite Nested
Mealybug Symbiosis”

Insects are associated with a diversity of microbial symbionts that can confer a broad range of novel ecological traits to their hosts. Across several insect taxa, such mutualistic interactions can be highly intimate, with the symbionts being localized intracellularly in specialized host cells and exhibiting severely reduced genomes. These associations are the result of long-term host-symbiont coevolution, and both partners are dependent on each other for successful development and reproduction. In his PhD thesis work, Filip Husník has investigated such intimate symbiotic associations in plant sap-sucking insects in order to understand the molecular interplay between host and symbionts and the evolutionary fate of intracellular symbionts.

In particular, Filip studied the fascinating tripartite symbiosis between mealybugs, their intracellular betaproteobacterial symbiont *Tremblaya princeps*, and a gammaproteobacterial symbiont that lives within the *Tremblaya* cells. In an excellent genomics-based study, Filip was able to demonstrate that the metabolic capabilities of the two symbionts are perfectly complementary, and that both *bona fide* host genes and genes that were horizontally transferred from putative facultative symbionts to the host genome fill in existing gaps in metabolic pathways of the dual symbionts. He concludes that at least six lineages of organisms contribute or have contributed to the functioning of the mealybug association (host, two symbionts, and horizon-

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tally acquired genes from at least three different sources), rendering the central metabolic pathways of the symbiotic union a complex metabolic patchwork.

Comparing the genomic composition of tripartite symbioses across different mealybug species, Filip discovered that, surprisingly, the intrabacterial symbiont, but not *Tremblaya*, has been repeatedly replaced over the course of evolution. Interestingly, most of the replacements originated from a clade of *Sodalis*-allied bacteria that are well-known symbionts of tsetse flies and weevils. Again, detailed metabolic analyses based on genomic data demonstrate metabolic complementarity between host, the two symbionts, and horizontally acquired genes from different sources. These analyses shed light on a complex history of gene loss and retention in the mealybug symbiosis that differs between host taxa, and they reveal the diverse gammaproteobacterial symbionts to be in different stages of genome erosion.

In a third paper, Filip Husník contributed to a study spearheaded by Alex Wilson's lab on amino acid transporters involved in host-symbiont interactions in plant sap-sucking insects. The combination of genomic and transcriptomic analyses revealed that two amino acid transporter gene families have undergone large expansions in the Sternorrhyncha, and that gene duplications likely allowed for the recruitment of amino acid transporters to the insect-symbiont interface in the bacteriomes, providing insights on how metabolite exchange between hosts and symbionts can be streamlined.

Filip Husník's work not only transforms our understanding of how intimate multipartite symbioses function on the molecular level, it also blurs the distinction between symbionts and organelles. His studies have resulted in three publications in the highest-ranking journals (*Cell*, *Proceedings of the National Academy of Sciences of the USA*, and *Molecular Ecology*) and a number of presentations at international conferences. In summary, Filip's thesis is an outstanding piece of work that I enthusiastically recommend to be defended. In fact, if this were a PhD thesis here in Germany (where PhD theses are graded), and I would not hesitate to give it the highest possible mark (with distinction), considering its outstanding quality.

Sincerely,

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REVIEW

On the PhD thesis "Genomic and Cellular Integration in the Tripartite Nested Mealybug Symbiosis"

by RNDr. Filip Husník

Bacterial endosymbionts are widespread across eukaryotic phyla. These endosymbionts are recruited from diverse bacterial lineages and provide their hosts with various novel physiological functions, including nitrogen fixation, photosynthetic and chemosynthetic carbon fixation, or production of specific amino acids and cofactors. The metabolic functions gained through endosymbiosis can enable a host eukaryote (including crop and pest species) to thrive in new habitats or feed on new diets. Hence, the phenomenon of endosymbiosis is of both, ecological and economical relevance. Furthermore, bacterial endosymbionts can contribute significantly to eukaryote complexity and evolution. This is most prominently exemplified by the acquisition of mitochondria and plastids. But also vertically transmitted endosymbionts that co-evolve with their hosts (often for dozens of millions of years) can become highly integrated on cellular and metabolic level.

A consequence of long term co-evolution between host and vertically transmitted endosymbionts is the reductive evolution of the endosymbiont genome. The fact that endosymbiont genomes are therefore typically small and thus relatively easy to sequence and assemble resulted in a very one-sided understanding of the biology of endosymbiosis. Since the first insect endosymbiont genome (that of the aphid endosymbiont *Buchnera*) was published in the year 2000, dozens of endosymbiont genomes have been sequenced. Hence, endosymbiont genomic features and the process of genome reduction are quite well characterized. But we only start to understand how endosymbiosis shapes the corresponding host genomes and the molecular mechanisms that underlie the establishment and maintenance of a stable endosymbiotic associations with bacteria with extremely reduced genomes.

The fascinating work of Dr. Filip Husník provides valuable insights into the evolution of host genomes and the evolutionary dynamics of endosymbiotic bacteria. The models that he uses are plant sap feeding insects of the Hemiptera, in particular the tripartite nested mealybug symbiosis. In a series of three papers (two as a first author and one as a contributing author) he provides fascinating insights (1) into the complementarity of functions encoded on host and endosymbiont genomes in the mealybug *Planococcus citri*. Genes encoded on all three genomes, of the host and the two nested

endosymbionts, are needed to give rise to intact metabolic pathways of chimeric phylogenetic origin. Interestingly, some of these genes that localize to the host genome apparently were acquired through horizontal gene transfers from bacteria other than the endosymbionts. (2) He sheds light of the role of gene duplications for the recruitment of amino acid transporters to the bacteriocytes. (3) And he explores the stability of the tripartite mealybug symbiosis with the surprising result that the innermost γ -proteobacterial endosymbiont in different mealybug species results from multiple replacements at different points in time.

The informational content of the three publications is high and the scientific insights novel. The methods used are overall rigorous and figures are carefully arranged and illustrate very well the essential findings.

For an outside reviewer it is of course inherently difficult to judge the contribution of the PhD candidate vs. ideas and writing skills of his supervisor, when a thesis is represented by a collection of published papers. However, the introduction and summary part written by Dr. Husnik demonstrate his deep interest in the scientific questions related to the evolution of endosymbiotic associations. He provides a broad, profound, and very up to date review of the relevant literature. He explains convincingly, why he feels that studying more recently established bacterial endosymbionts contributes also to our understanding of the evolution of plastids and mitochondria. Furthermore, introduction and summary are well structured, formally of high quality, clearly written and thus very pleasant to read.

Minor points of criticism: The discussion of the symbiotic interface in the mealybug remains a bit vague. Also the discussion of protein import into bacterial endosymbionts should be more precise. It is important to clearly differentiate protein import into and protein targeting to a bacterial endosymbiont (the latter may include protein translocation across the symbiont membranes but could also result from an extracellular protein association with the endosymbiont)

Final judgement: Overall, I rate the PhD thesis submitted by Dr. Husnik excellent. I find his work fascinating and technically well done with only very minor points of criticism. I strongly recommend to offer Dr. Husnik the opportunity to defend his thesis in front of the committee.

Sincerely,



Dr. Eva Nowack

Questions

- You mention the widely distributed thioautotrophic endosymbionts only very briefly. Could you give a few examples of such symbionts and describe their physiological and ecological role?
- As you point out correctly, a next important step towards understanding the biology of insect endosymbioses (in particular with nested endosymbionts) would be to determine localization of specific proteins encoded by the various partners. Could you suggest experiments to address this question? What would be the technical difficulties?
- Could you speculate on the molecular mechanisms that allow the three partner organisms in the mealybug symbiosis to exchange metabolites. In which membrane of the host/symbiont interface exactly do you expect bacteriocyte-specific host-encoded transporters to localize? And how do you imagine metabolic fluxes over the *Tremblaya/Moranella* interface to be regulated?



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March 10, 2017

Re: Ph.D. thesis "Genomic and cellular integration in the tripartite nested mealybug symbiosis" by RNDr. Filip Husník

It is an extremely rare situation when I do not have much to say. Yet, this is exactly the case here. I really think not much is needed to be said when a Ph.D. thesis stylishly includes 3 "short" pieces – a first-authored paper in *Cell*, a first-authored paper in *PNAS USA*, and a paper in *Molecular Ecology*. The current cumulative impact factor of these 3 journals is over 44!!! As a matter of fact, Filip has published **5 other papers** during his graduate studies (including a first-authored paper in *BMC Biology*), enough material for another PhD thesis on a related subject.

The work under review ("Genomic and cellular integration in the tripartite nested mealybug symbiosis") includes a comprehensive introduction to the topic, a collection of 3 published papers, and a summary. The overall structure of the thesis is well organized and the work is presented in a concise and comprehensive manner. The published papers represent a truly significant step forward in our understanding of evolutionary forces driving endosymbiosis.

The following questions are of the general nature and intend to provoke some discussion.

- 1) On general applicability of metagenomic or metatranscriptomic approaches to identify endosymbionts. Can they be misleading in documenting the true host of the symbiotic bacteria? Insects, for example, harbour numerous parasites. Is it possible to discriminate endosymbionts of insects from endosymbionts of their parasites? In my opinion, this question is crucial because it may influence all the calculations of precise timing of bacterial acquisition, etc.
- 2) Can you elaborate on potential experimental approaches (novel or old) which would allow studying these questions in the wet lab? *In silico* inferences are beautiful, but they need to be validated *in vitro*.
- 3) On sampling in a search for novel endosymbionts. I have been puzzled by one strange observation for years. Routinely, field sampling includes cultivation in a medium containing a mix of several antibiotics. This may be the reason for the relatively low number of endosymbiotic cases

documented thus far. Yet, in some circumstances, bacteria survive just fine. How this discrepancy can be explained?

The presented thesis is the best I have ever reviewed and I strongly believe that RNDr. Filip Husník deserves to be awarded a Ph.D. title.

Sincerely,

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TO WHOM IT MAY CONCERN

Opponent's report on the dissertation thesis "*Genomic and Cellular Integration in the Tripartite Nested Mealybug Symbiosis*" by Filip Husník, School of Doctoral Studies in Biological Sciences, University of South Bohemia

10/03/2017, Ostrava

It is an honour and a great pleasure to serve as an opponent of the dissertation thesis by Filip Husník. Let me directly confess this is the best PhD thesis I have ever evaluated. Suffice it to state that two of the three papers included in the thesis were published in the journals *Cell* and *PNAS* with Filip as the first author. The latter paper is in fact co-authored only by Filip's supervisor, leaving little doubt on the fundamental role Filip played in designing the study, gathering the data and preparing the manuscript. Filip's contribution to the third paper included in the thesis (again published in a very good journal – *Molecular Ecology*) was apparently less extensive, but still significant, judging from the specification of his role in the respective study provided in the introductory part of the thesis. All three papers are seminal contributions to the field of evolution and function of bacterial endosymbionts of insects, with direct implications for understanding the process of endosymbiosis in general.

I see no point in providing a detailed review of these papers, as they were already peer-reviewed by others and their significance is further attested by a growing number of citations in scientific literature (presently 103 for the paper in *Cell*). I read two of these papers immediately after their publication (i.e. long before I was invited to evaluate Filip's thesis) and I very much enjoyed them both. I was actually so much excited by Filip's *Cell* paper that I immediately invited him to give a talk at our seminar series at the University of Ostrava, even though I had not met him before and his name was new for me. He delivered an excellent presentation and I still remember our subsequent stimulating informal discussion on various topics related to endosymbionts and technical aspects of their study.



The thesis is not limited only to the three papers mentioned above, but includes a very significant introducing text that has not been published before. Let me, therefore, concentrate on this part in my report. I do not know whether this introduction was written solely for the thesis or whether Filip's intentions were broader, but the structure of the text and its style suggest that this is a manuscript of an opinion paper ready for submission to a journal (if not, it should be submitted and published anyway!). Rather than going into too much detail about bacterial endosymbionts of insects as such, it provides a fresh and highly original perspective on the process of endosymbiosis in general, specifically addressing the question of the emergence of what we usually call "organelles". Notably, this year we celebrate a 50-year anniversary of the publication of the seminal paper by Lynn Sagan (Margulis) that revived the idea that some structures of the eukaryotic cell may be of a symbiogenetic origin. Since then the phenomenon itself has become one of the foundations of modern evolutionary thinking and a lot has been learned about the actual evolutionary history of eukaryotic endosymbions and organelles. However, people are still striving to understand many fundamental aspects of the process of how endosymbionts become integrated with the host cells and how this may ultimately lead to the origin of what is considered to be an organelle. And Filip has a lot to say to this.

In the *Introduction* of his thesis Filip builds on his own results and on the current literature, and paints a general framework of the "organellogenesis". His knowledge of the relevant literature is perfect (he has managed to integrate even the most recent papers published just a few weeks ago) and I applaud to his ability to synthesize the various findings concerning different types of endosymbiont-host systems into a concise and persuasive. I think he was able to precisely identify the most salient questions of the endosymbiont-organelle transitions and to emphasize the most important insights, or even paradigmatic changes, that have recently been achieved in this field. To be at least somewhat critical, I will mention three points that in my opinion could (or should) have been dealt with, but are omitted from the *Introduction*.



First, I think it would be highly relevant to comment on the "symbionelle" concept proposed by Reyes-Prieto et al. (*Environmental Microbiology* 2014). May be Filip does not agree with the authors or finds the concept useless or superfluous, but I believe it should not be ignored completely in the context of his writing. Second, I would probably explicitly mention the so-called "shopping bag" model proposed by Larkum et al. in 2007 to explain the apparently diverse evolutionary origin of plastid proteins but applicable as well to the mitochondrion and at least some other endosymbionts/organelles, such as the chromatophore of *Paulinella*. Filip does cite the paper in passing, but I would dedicate more space to presenting this catchy idea. Finally, Filip does explain the role of horizontal gene transfer (HGT) in the endosymbiont-host integration, but he speaks about gene acquisition by the genome of the host cell. However, there is also a growing list of well-documented examples of genes moved by HGT into the organellar genomes, including mitochondrial and plastid ones. In my view this is a somewhat neglected ingredient of the endosymbiont evolution with a potentially underestimated significance. For example, one can imagine this as one of the ways to cope with the "symbiotic rabbit hole" problem Filip outlines in Box 2 in the *Introduction*. For example, there are intriguing examples of a replacement of original cyanobacterial genes by equivalent genes from another bacterial source in plastid genomes (e.g. the *rpl36* gene in the plastid genome of haptophytes and cryptophytes; Rice & Palmer, *BMC Biology* 2006). I believe this aspect needs to be better reflected in the general discussion on endosymbiosis. This leads me to a specific question Filip may want to answer during the defence of his thesis: What is known about the role of HGT in shaping the genomes of bacterial endosymbionts of insects?

Altogether, I can only repeat that Filip's thesis is an exceptional achievement that fully testifies to his scientific competence exceeding substantially an ordinary PhD candidate. I would like to add that outcomes of his research are not restricted to the papers included in the thesis. In fact he has co-authored five more papers, all in international peer-reviewed journals. One of them is especially important – Filip's phylogenetic study published in 2011 in *BMC Biology* (i.e. even before he started his PhD study) and demonstrating via application of sophisticated phylogenetic methods that several lineages of insect



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endosymbionts emerged separately rather than from a single endosymbiosis event. Hence, despite the early stage of his scientific carrier Filip has already established himself as a significant figure in the field of biology of endosymbionts. I wholeheartedly recommend he be awarded the title *philosophiæ doctor*.

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