Norbert Nowotny, PhD
Professor of Virology

Evaluation of the PhD thesis entitled
„Molecular evolution of flaviviral genes“,
submitted by Mgr. Jiří Černý

Flaviviruses are a worldwide distributed, large family of viruses, which contain very important human and animal pathogens. These viruses gained a lot of attention when West Nile virus emerged fort he first time in 1999 on the American continent; also the currently in South America heavily dispersing Zika virus is a member of this virus family. Although a lot of research activities have been focussing on flaviviruses, nonetheless gaps remained in our understanding of certain aspects of this virus family.

One of these gaps is our very limited knowledge on the evolution of different flavivirus genes. This aspect was intensively tackled by Mgr. Jiří Černý in his Ph.D. study, and I wish to congratulate him and his supervisors for choosing this topic.

Mr. Černý investigated various aspects of flaviviral gene and genome evolution. One of the model viruses he used for his study is tick-borne encephalitis virus (TBEV), a virus which is highly prevalent in the Czech Republic and therefore also of great local interest. Among other outcomes, this part oft he study revealed a novel mutation in the NS5 polymerase subdomain of TBEV, which may play an important role in TBEV pathogenicity.

Mr. Černý also investigated in great detail the upstream open reading frame (ORF) of TBEV. He could show that this ORF does not code for any peptide.

He investigated several flaviviral genes in order to establish their evolutionary histories. By doing so, he could demonstrate that the flaviviral evolution was characterized by multiple recombination events, which formed today's flaviviruses containing i) flavivirus-specific proteins, ii) general viral proteins, and iii) cell-derived proteins.

A major part of his study dealt with viral and cellular polymerases. He identified that especially the polymerase genes are excellent marker genes for reconstructing the evolutionary history of RNA viruses as well as for understanding virus-cell evolutionary relationships.

Department of Microbiology and Immunology
College of Medicine and Health Sciences
Sultan Qaboos University
P.O. Box 50, Muscat 123, Oman
Phone +968 94103480
nowotny@squ.edu.om, www.squ.edu.om

Institute of Virology, Department of Pathobiology
University of Veterinary Medicine Vienna
Veterinaerplatz 1, 1210 Vienna, Austria
Phone +43 1 25077 2704, Fax +43 1 25077 2790
Norbert.Nowotny@vetmeduni.ac.at, www.vetmeduni.ac.at

The study, which led to the Ph.D. thesis, is comprehensive and well-designed. Mr. Černý used state-of-the art technology and methods in carrying out the different tasks of the study. Mgr. Černý managed to publish the results of the studies in three high-impact Journals, and he prepared two further manuscripts, which will be submitted for publication soon.

In his Ph.D. thesis, Mgr. Jiří Černý obtained new and highly interesting results. Also the thesis itself, written in perfect English, is excellently compiled, well-structured, nicely illustrated, and easy to read.

In case I have to give a mark, the thesis deserves the best possible mark.

Please join me in congratulating Mgr. Jiří Černý and his supervisors to this significant achievement. I wish Mr. Černý the very best for his personal and professional future!

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Prof. Norbert Nowotny, PhD

Veterinärmedizinische Universität Wien
Institut für Virologie
Veterinärplatz 1, 1210 Wien
T +43 1 25077-2303
F +43 1 25077-2390

Faculty of Science
CHARLES UNIVERSITY IN PRAGUE

Review of dissertation thesis of Mgr. Jiří Černý

Thesis supervisors:
Doc. RNDr. Daniel Růžek, PhD (Institute of Parazitology , Biology Centrum,CAS)

Prof. RNDr. Libor Grubhoffer, CSc. (Faculty of Science,University of South Bohemia ; Institute of Parazitology, Biology Centrum,CAS)

Thesis "Molecular Evolution of Flaviviral Genes" of Mgr Jiří Černý is based on five manuscripts, two of them yet not published. Two papers are devoted to evolution of virus RNA dependent polymerases because they can serve, for their high degree of conservation, as markers for studies of evolution of RNA viruses. Further research focuses on evolution of *Flaviviridae,* namely Flavivirus genus (1 manuscript) and then, specifically on evolution of Tick Born Encephalitis virus (TBEV) (2 manuscripts).

To explain virus evolution is a big chalenge. The fast evolution and high mutation rates make it difficult especially for RNA viruses. Sequence based phylogenetic analyses do not operate satisfactorily. Mgr Černý (and his co-workers) combined different approaches, including those based on protein tertiary structure similarities in their evolution studies.

He presented evolution of viral (and cellular) polymerases, used them as markers for construction of RNA virus evolution history and showed that RNA viruses are very ancient life forms (publication 1 and 3). Another manuscript describes sequence and structure – based search of distant viral and cellular homologues of Flavivirus proteins. Using Bayesian algorithms, author (and co-aoutors) reconstructed the evolution of selected proteins and showed that individual flavivirus genes have different evolutionary history (Manuscript 5).

Very important part of Mgr Černý´s research is focused on TBEV. He has sequenced genomes of TBEV isolated from patients with severe forms of TBE in effort to detect genome areas relevant for TBEV pathogenicity and virulence. He characterised also upstream ORF of TBEV Neudoerfl strain and showed that it does not encode any peptide (Publications 2 and 4).

Alltogether, the thesis represents an impressive amount of work and the candidate provided his ability to exploit various approaches in the field of virus evolution research.

The thesis is written in shortened form. Texts of 5 manuscripts are accompanied by chapters of - i) Introduction to evolution studies (17 pages) , - ii) Introduction to used methods (3 pages), - iii) Discussion (5 pages) , - iv) Conclusions and future perspectives and - v) References. More attention should be paid to this part of thesis.

Chapter" Introduction" is a review including - current knowledge of the biology of flaviviruses, - evolution of virus proteins and genomes, - evolution of virus polymerases and evolution of viruses and life from the perspective of polymerase evolution.

Unfortunately, the text is accompanied by numerous grammaticall and punctuation mistakes (e.g. - page 4 "Flavivirus are" or "Particles has..." "bounding of viral particles" etc.). Sometime, the information is redundant (as in Fig- 2 and page 4 of the text). Not all abbreviations are explained and there is not abbreviation list in the thesis.

Figure 3 is not signed and its legend is missing.

On page 12 – terms: genetic drift and shift are interchanged - accumulation of mutations should be named drift while recombination event of viruses with segmented genomes is antigenic shift.

Questions:

Polymerases of minus RNA viruses were not involved into your RNA virus evolution studies because of absence of structural data. The lack of information is surprising. (Incidentally very recently, the crystal structure of Influenza C polymerase was published in Nature 2015. How distant seems to be this group of viruses from plus RNA viruses according to data (if they exist) obtained by other approaches?

Faculty of Science
Charles University in Prague
Albertov 6, 128 43 Praha 2

Why do you think that found mutation 3203 Ile→ Thr or Ser in NS5 protein is important for TBEV pathogenicity? Do you intend to prove it experimentally?

Recently, very interesting paper appeared "A phylogenomic data-driven exploration of viral origins and evolution" authors, Nasir A. and Caetano-Anollés G. in Sci.Adv. 2015. Can you comment the results of the paper based on structural and functional data of proteomes - establishing an ancient origin of the viral supergroup and the concept of reduction of modern viruses from ancient cells - proto-virocells which coexisted with ancestors of modern cells.

In conclusion:

Mgr. Černý demonstrated skills in work with bioinformatics databases, computational processing of biological sequences, protein structure modelling and in work with phylogenetic programs. He made a valuable research on evolution of flaviviruses. The high level value of the thesis is proved by three publications in recognized scientific journals (where Mgr Černý is twice the first and ones the second author) and in two other submitted manuscripts (in both as the first author).
Thus, the thesis fulfilled and exceeded the criteria for obtaining a Ph.D. award.

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Prague, December 30, 2015

Doc. RNDr. Jitka Forstová, CSc.

Faculty of Science
Charles University in Prague
Albertov 6, 128 43 Praha 2

www.natur.cuni.cz

INSTITUTE OF VIROLOGY
Slovak Academy of Sciences

RNDr. Boris Klempa, PhD.
Department of Virus Ecology

Referee review of the doctoral thesis:

Mgr. Jiří Černý: „Molecular Evolution of Flaviviral Genes"

The doctoral thesis of Mgr. Jiří Černý is written in a "cumulative" way and is based on three published articles and additional two manuscripts which are currently „in revision". The thesis is focused on various aspects of virus evolution on an impressive scale, from point mutations of the tick-borne encephalitis virus (TBEV) up to virus-cell evolutionary history. The thesis thereby covers considerable range of knowledge associated with a spectrum of applied laboratory and bioinformatical methods and skills. I appreciate this remarkably broad coverage as well as the collection of rather "non-orthodox" topics. Obviously, the student had to learn and perform not only common molecular biology laboratory techniques but also bioinformatic and phylogenetic methods dealing with genetic as well as structural data.

Because of the cumulative character, the thesis is written in an easy-to-read style. The first part, consisting of the first thirty pages, contains general introduction and introduction to used methods together with the Discussion and Conclusions and future perspectives chapters. The actual results of the thesis are then presented in the form of the particular manuscripts which were the outcome of the study. I found it a pity that the published articles were not presented with the published graphical layout but as "submitted manuscripts" with all the tables and figures at the end. I assume that this formal structure is given and could not be changed but I found it more difficult to follow than a regular style of published scientific articles.

Altogether, the thesis consists of 220 pages divided into seven chapters. This extra-ordinary extent is clearly the consequence of five manuscripts being the part of the thesis. The thesis is written in English. Even though it is obvious that it is not author´s mother-language, the readability is very good. The text is easy to follow also for uninvolved reader despite of the difficult topics.

As mentioned above, I appreciate the different levels at which flavivirus evolution was analyzed. It considerably extends genomic data of TBEV from Central Europe. The study of the short upstream open reading frame of TBEV is also very useful even though the expression of this ORF could not be detected. The combination of sequence and structural data to analyze viral RNA dependent polymerases or even right-hand polymerases in general not only provided unique and valuable insights to viral evolution but also represents a framework which can be further developed and extended when analyzing distantly related proteins.

I have few questions for the student which should stimulate the discussion and do not in any way doubt or criticize the obtained data or conclusions.

Dúbravská cesta 9, 845 05 Bratislava 45, Slovak Republic 02 59302 465 02 5477 4284
boris.klempa@savba.sk

INSTITUTE OF VIROLOGY
Slovak Academy of Sciences

RNDr. Boris Klempa, PhD.
Department of Virus Ecology

- In the analysis of the patient-associated TBEV sequences, you claim that the novel mutation I3203S/T may play an important role in TBEV pathogenicity. What does the author assume? Does this mutation (or other patient-associated mutations in general) occur during the course of infection in humans? Or is this mutation pre-existing in the ticks but leads to more severe clinical course which in turn increases the chance for its detection?
- In the abstract of the manuscript dealing with TuORF, you claim that the TuORF sequence is very stable and it therefore should have a biological function. How would you then explain that not all TBEV strains have the TuORF? As the artefact of the various virus isolation procedures? This statement also seems to be in contrast with the final conclusions of the very same article stating that one can assume minor or no role of TuORF in flavivirus infection.
- Since significant part of my work is focused on hantaviruses, I am very sorry that no RNA dependent RNA polymerases of the negative stranded RNA viruses were included into the analyses. If I understood it correctly, the reason was the lack of structural data. Nevertheless, could you speculate where the position of these polymerases could be on your tree? Do you think that the fact they are large multidomain proteins reflects that they are evolutionarily younger or the other way round?
- In the ms. dealing with the righ-hand polymerases, you present as Fig. 4 an unrooted phylogenetic tree but a putative root is indicated with question mark. How was this rooting estimated? Or is it only your assumption? If your concept is correct and the RNA-dependent RNA polymerases are the most ancient group, it seems that retroviral RNA-dependent DNA polymerases are then evolutionary older than the DNA polymerases

In my opinion, the thesis fulfills all requirements for a PhD thesis. I fully recommend the thesis for the defense and, after the successful defense, to award Mgr. Jiří Černý the academic degree of „Philosophiae doctor" ("PhD.").

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RNDr. Boris Klempa, PhD.
reviewer

Dúbravská cesta 9, 845 05 Bratislava 45, Slovak Republic 02 59302 465 02 5477 4284
boris.klempa@savba.sk