Ludek Koreny - Ph.D. Thesis, 2011

Review and Questions: Andrew E Allen

Evolution of the tetrapyrrole synthesis in eukaryotes

In general I found this dissertation to be of exceptional overall quality. Varied techniques, computational and wet lab, were used to uncover numerous original and penetrating insights related to heme biosynthesis and metabolism throughout the eukaryotic tree of life. Once of the many strengths of the work is in the overall holistic treatment of eukaryotes such that interrelationships within the entire eukaryotic tree of life are handled. It is a very impressive piece of work. Congratulations. Since the entire thesis, other than chapter 1, is published or submitted I have very few specific critiques. I present below some minor points concerning chapter 1 (*review*) followed by some questions that arise from considering the entire thesis.

Chapter 1 (*review* chapter):

1) Fig. 3 : labels for inner and outer mitochondria and the mitochondria in general could be helpful

2) Pg 13 (thesis page 13): The long paragraph on page 13 should be broken up a bit into more than one paragraph and there are some points that need to be further clarified. For example, the terms "certain phototrophs" and "few primary algae" and "the CPOX of this putative origin" are vague and need to be sharpened by providing specific examples.

3) On page 16 the abbreviation PPIX begins to appear and I don't think it is written out the first time. Since it refers to a metabolic intermediate and not an enzyme it is confusing.

4) The first sentence on thesis page 17 is confusing and wordy; re-word.

5) There is a statement in the second paragraph on page 17: "apoptosis makes little sense in unicellular algae". This is a flammable statement to say the least. I urge you review the literature on this topic; especially papers by Berges, Falkowski, Vardi, and Biddle. The subject of PCD and cell death has become a highly fashionable topic in marine microalgae and its occurrence in these organisms has certainly been established.

6) Bottom paragraph, pg. 17: reword the first sentence to avoid beginning with "besides", which is a preposition. For example: All eukaryotic phototrophs, other than Archeaplastida,....

7) On page 18 in the third paragraph, the term "primary heterotrophs" is used and this is not its first usage. This term needs to be better established and defined in the beginning of the chapter. It is a critical concept for the chapter and needs to be handled more precisely.

8) The long paragraph on page 20 (beginning) should be split up.

9) Page 21, second paragraph; informal phrases like "dangerous business" should be avoided. Similarly, the first sentence of the second paragraph on page 25 is highly informal and should be reworded. For example, "However, one eukaryote, *P. serpens*, is capable of robust growth in the absence of heme."

10) The long paragraph on page 26-27 should be split up.

Questions:

1) Throughout the thesis a division is implied between trophic mode and cellular localization of heme synthesis components. Of course photosynthetic eukaryotes also require heme in the mitochondria where O2 is consumed and CO2 evolved despite opposite overall cellular fluxes. What is known about management of heme for respiratory processes in photosynthetic eukaryotes? Further, many marine microalgae are commonly Fe limited for growth and of course not all Fe can be eliminated from photosynthetic processes (although through usage of plastocyanin and flavodoxin Fe cell quota reduction occurs) --- do you think this puts further pressure on cells to reduce mitochondrial heme demand ? It has been shown for example that mitochondrial AOX activity is important for iron limited cells, but this has been interpreted to be in response to a demand for increased electron sinks. Do you think active mitochondrial AOX activity can reduce overall cellular Fe demand ? How much ?

2) In the genome of the polar diatom *Fragilariopsis cylindrus* we have observed the occurrence of genes encoding the enzyme neuroglobin. As far as I am aware such genes have not been found in photosynthetic eukaryotes to date. Could you please speculate on a possible function ? The Southern Ocean (origin of this diatom) is highly Fe limited.

3) Through the thesis, you commonly refer to primary heterotrophs and certainly I generally understand what you mean with this term. However, trophic strategy is highly polyphyletic. Is it known very well that heterotrophic stramenopiles were in fact early diverging ? It is possible that some heterotrophic stramenoiples diverged much later? There is a very abundant group of marine strameoplies known to date only form 18S rRNA data called the MAST group. It seems unlikely that they share a common line of descent with other clades of heterotrophic stramenopile (such as phytophthora). Once more genomes of stramenopiles are available, what do you predict to find in terms of diversity in heme synthesis ? Also many marine protists are "mixotrophic" and derive nutrition from heterotrophic and photosynthetic processes. Considering that a great deal of marine protist biomass employs this strategy can you comment on possible physiological, regulatory, or evolutionary constraints or features you might look for in the genomes of such organisms ?

4) What is known about co regulation and or tradeoffs in physiological regulation of heme and chlorophyll synthesis in photosynthetic eukaryotes. Is this simply controlled at the last step or is there some feedback between Mg and or Fe chelatase activity and demand for precursors. In

the Fe limited marine environment where Fe limitation demands overall remodeling of pigment metabolism it seems that there must be some interesting regulatory cross-talk between regulation of heme and chlorophyll synthesis. Please speculate ?

5) Given the emergence of large metagenomic and metatranscriptomic datasets for marine eukaryotes can you please comment on what might be some data mining strategies to elucidate any interesting patterns diversity of heme synthesis pathways and strategies? In other words, what might make good biomarkers to target for analyses (relative abundance, diversity, expression level) aimed at uncovering interesting features related to variation in heme metabolism?

6) Lastly, could you speculate further on the physiological and evolutionary selection behind the apparent loss of heme a requirement in *Phytomonas*. You speculate that life in a carbohydrate rich/high sap environment has perhaps driven this. Is this environments also known to be Fe poor? Relative to other plant parasites is the niche of *Phytomonas* known to be different? Do you speculate that other Fe requiring functions have been compensated for at the genome level. In ultra Fe limited regions of the ocean or in other environments would you expect similar adaptations? If so (and related to the last question) which genes would you look for (or absence) to perhaps reflect such adaptations ?

Oponentský posudok na dizertačnú prácu:
"Evolution of the tetrapyrrole synthesis in eukaryotes", autor Mgr. Luděk Kořený

Predkladaná dizertačná práca sa zaoberá evolúciou syntézy tetrapyrolov, predovšetkým však hému u eukaryotických organizmov.

Odrazovým mostíkom pre takúto ucelenú evolučnú štúdiu sú originálne experimentálne práce a analýzy údajov, ktoré doktorand uskutočnil na jednobunkových eukaryotických organizmoch reprezentujúcich dve fylogeneticky vzdialené línie (Apicomplexa, Euglenozoa); vysoko atraktívne z evolučného hľadiska, a to či už z pohľadu archaickosti alebo spôsobu života.

Zvolená téma práce je aktuálna a významná. Výskum na tomto poli dynamicky napreduje a riešenie problematiky si vyžaduje originálny a komplexný prístup. To všetko je v práci zakomponované, pretože umne zužitkováva a ďalej tvorivo rozvíja bohatstvo myšlienok, materiálu a dlhoročné skúsenosti pracoviska na poli štúdia parazitických prvokov, vrátane medzinárodných kontaktov.

Ciele práce boli vytýčené jasne – sú navzájom dôvtipne prepojené čo dokumentuje do detailov premyslenú koncepciu celého projektu, podčiarknutú jasnou experimentálnou stratégiou.

Z hľadiska metodického si rozsah a úroveň dokumentovanej experimentálnej práce zasluhuje nielen uznanie, ale aj obdiv. Doktorand nielenže zvládol veľké množstvo moderných mikrobiologických, molekulárno-biologických, biochemických techník a bioinformatických postupov, ale viaceré z nich aj tvorivo rozpracoval, resp. skĺbil do nových celkov.

Výsledky sú originálne a vyvodené závery sú diskutované nielen v kontexte dostupných poznatkov z tejto problematiky, ale sú sformulované aj nové originálne hypotézy, ktoré sú vzápätí nápadito testované. Jasne načrtnutý je aj prínos pôvodných zistení autora pre ďalší rozvoj vednej disciplíny, aktuálne otázky, na ktoré sa treba sústrediť a smery, ktorými je záhodno sa uberať.

Z formálneho hľadiska práca pozostáva zo štyroch originálnych experimentálnych štúdií (dve uverejnené, jedna v tlači – všetko v prestížnych kvalitných časopisoch; jeden rukopis článku zodpovedajúcim spôsobom pripravený na zaslanie opäť do dobrého časopisu); vo všetkých prácach je doktorand prvým autorom. Týmto štyrom experimentálnym štúdiám je predradená prehľadová teoretická štúdia o evolúcii syntézy tetrapyrolov u eukaryotických organizmov (cca 30 strán), plus krátky úvod a stručné záverečné zhrnutie.

Práca je napísaná v anglickom jazyku, dobrou angličtinou, čo je praktické nielen vzhľadom na doteraz uverejnené práce doktoranda v renomovaných časopisoch a rukopis pripravený na uverejnenie , no odráža aj úzku, operatívnu spätosť doktoranda, resp. domáceho riešiteľského kolektívu, so zahraničnými spolupracovníkmi.

Teoretická štúdia o evolúcii syntézy tetrapyrolov u eukaryotických organizmov je napísaná veľmi pútavo, zohľadňujúc rozmanité aspekty skúmanej problematiky, a to nielen v celej ich hĺbke a šírke, ale pokiaľ sa len dá, aj proporčne. V tomto review sú starostlivo zapracované a patrične akcentované aj originálne zistenia doktoranda (detailne prezentované v štyroch nasledujúcich experimentálnych štúdiách – tri z nich pred uverejnením v časopisoch prešli serióznym oponentským procesom, ktorého spochybňovanie by bolo nenáležité). Keďže práve teoretické review nebolo doposiaľ nikde uverejnené, a teda nebolo predmetom oponentského posudzovania, moje otázky, pripomienky, námety do diskusie sa budú dotýkať hlavne tejto časti dizertačnej práce.

V prípade apikomplexných parazitov, sa v súvislosti s lokalizácia niektorých významných metabolických procesov (syntéza lipidov, izoprenoidov, hému) v ich rudimentárnych plastidoch už dlhší čas uvažuje (a v práci to opakovane spomína aj doktorand, no bez bližšieho upresnenia), že tieto dráhy môžu predstavovať perspektívne terapeutické ciele v boji proti týmto parazitom. Kým v prípade atakovania syntézy lipidov a izoprenoidov sú už známe aj konkrétne inhibítory ako aj miesto a spôsob účinku týchto látok, špecifické atakovanie syntézy hému v apikoplastoch považovali mnohí autori donedávna za menej nádejný spôsob terapie. Nastal v tomto smere v posledných rokoch nejaký významnejší pokrok? t.j. je možná špecifická inhibícia syntézy hému v apikoplastoch, ak áno, akými látkami a v ktorom kroku?

V teoretickom úvode autor opakovane používa termín „ancient eukaryotes", akési starodávne, prastaré eukaryota, na označenie prvotných ekaryotických buniek, ktoré sa neskôr stali hostiteľskými bunkami pre predkov mitochondrií, za ktoré sa všeobecne považujú alfa-proteobaktérie (na obr. 2 znázornená bunka, ktorá prijala alfa-proteobaktériu, už má zakreslené jadro – znázornené rovnako ako u neskorších „pravých" eukaryotov). Mohol by uchádzač bližšie ozrejmiť ako si takéto archaické eukaryota predstavuje, ktorým organizmom a z akých vývojových línii, domén, by boli najpríbuznejšie. V práci použité formulácie vedú k záveru, že podľa jej autora eukaryotické bunky existovali už pred vytvorením endosymbiózy medzi alfa-proteobaktériou a hostiteľskou bunkou (ktorá by ešte mala prokaryotickú organizáciu - podľa definície minimálne „jadra"); žeby Archezoa?

Je známe, že niektoré kmene bičíkovca *Euglena gracilis* sú schopné života aj za anaerobných podmienok. Vtedy dochádza k dramatickým zmenám v niektorých metabolických procesoch, napr. v syntéze lipidov, kedy sa produkujú estery voskov. Sú známe údaje, ako sa za takýchto podmienok mení syntéza tetrapyrolov, ak nie, mohol by sa doktorand (s hlbokou znalosťou problematiky) zamyslieť, k akým zmenám v syntéze hému by mohlo dochádzať.

Na strane 18, prvý odsek autor uvádza, že: „ it is generally accepted that there were two independent secondary endosymbioses that involved green algal endosymbionts. One of them led to the origin of photosynthetic euglenids, while the second gave rise to chlorarachniophytes (Rogers *et al.* 2007)." Pre úplnosť by sa žiadalo dodať, že tretí príklad existencie sekundárnych zelených plastidov nachádzame u dinoflagelát (rod *Lepidodinium*).

Autor na strane 17/18 trochu úskočne (citujúc Archibald 2009) uvádza: „There are many alternative hypotheses explaining the evolution of plastids in eukaryotes that imply several secondary or tertiary endosymbioses and it is currently impossible to decide, which one is the best (Archibald 2009). Predsa by ma však zaujímal názor doktoranda na závery komparatívne genomických prác fotosyntetizujúcich organizmov, ktoré naznačujú, že zástupcovia súčasných chromalveolát obsahujúce červené plastidy, prešli v evolúcii štádiom, kedy mali zelené plastidy (neskôr ich stratili, nahradili zelenými) (Nozaki *et al.* 2009), resp. *vice versa* euglény s dnešnými zelenými plastidmi prešli v evolúcii štádiom, kedy mali červené plastidy (neskôr ich stratili, nahradili zelenými) (Maruyama *et al.* 2011) alebo že Rhizaria so zelenými sekundárnymi plastidmi mali predtým možno terciárne červené plastidy (Yang *et al.* 2011).

Uvedené otázky a pripomienky neznižujú vysokú kvalitu odvedenej a dokumentovanej práce.

Záverom možno konštatovať, že predložená doktorandská dizertačná práca dokumentuje schopnosť jej autora pracovať s odbornou literatúrou, premyslene organizovať experimenty a precízne ich realizovať, vypracovávať nové metodické postupy, formulovať originálne hypotézy a načrtávať spôsoby ich testovania, spracovávať výsledky a vyvodzovať z nich zodpovedajúce závery. Práca plne vyhovuje požiadavkám kladeným na doktorandské práce a odporúčam ju k obhajobe.

Na základe mnou posudzovanej dizertačnej práce navrhujem udeliť jej autorovi Mgr. Luďka Kořeného akademický titul "philosophiae doctor" ("PhD.").

Bratislava 13. 10. 2010

Prof. RNDr. Juraj Krajčovič, CSc.
Katedra genetiky
Prírodovedecká Fakulta
Univerzita Komenského v Bratislave

In Prague 19th of October 2011

The thesis by Luděk Kořený, MSc. presents his research activity during his Ph.D. study under the supervision of Dr. Miroslav Oborník on The Evolution of the tetrapyrrole synthesis in eukaryotes. The thesis is divided into seven chapters: author's overview, introductory review, which combines current state of the knowledge with author's own experimental work, four individual studies and author's comclusion.

The review is written in very good English and as a whole, it is a nice piece of text for a reader unfamiliar with the topic. With minor changes can be used as manuscript for submission. It describes the evolutionary history of heme distribution and very short insight into the functions of heme-containing proteins. Most of the body is dedicated to the tetrapyrrole biosynthesis in heterotrophs and phototrops. While heterotrophs seem to follow unified pathway divided between mitochondrion and the cytosol, with the acquisition of plastid the story gets a twist. The whole pathway is now localized in the plastid with some residual activities detected in mitochondria. However, major 'complication' comes with the secondary endosymbiosis. As a result various enzyme mosaics or two parallel coexisting pathways can be found in Apicomplexa, or Euglenida, respectively. The final part of the review is dedicated to the eukaryotes, which can live part of or entire life without heme. These are anaerobes, which lack cytochromes but also surprisingly aerobes such as *Phytomonas serpens*. Here, the review stresses out the highlights of ready-to-publish manuscript, which is Study IV of the thesis.

It is obvious from Luděk's professional development, reflected by the publications involved in the PhD thesis, that he has been strong in bioinformatics (such as data mining and topology predictions) and phylogenetics from the beginning and during the course of his PhD study the publications and the manuscript illustrate his involvement in functional biochemical and cell biology studies and perhaps Luděk's actual participation on 'wet' experiments could be described during the talk. Anyway, Luděk has a very good mix of skills which is, unfortunately, still quite rare in our academia. On all papers, Luděk is listed as first author.

The first publication (Study III) in International Journal of Parasitology reports on the reconstruction of heme synthesis in kinetoplastids. The study published in a Current Opinnion format builds on the gene identification and subsequent phylogenetic reconstructions of enzyme participating in heme biosynthesis. The study proposes clear evolutionary model for different set ups found among kinetoplastids. The complete loss of the pathway in trypanosomes, the acquisition of bacterial endosymbiont in non-Trypanosoma trypanosomatids as well as partial horizontal transfer of the pathway into Leshmania and Crithidia species.

The second publication (Study II) in Genome Biology and Evolution adds the bioinformatic identification of two parallel tetrapyrrole synthetic pathways found in Euglena. This photosynthetic protist was found to contain complete and C4 and C5 pathways and authors proposes with good bioinformatic support, that mitochondrial/cytosolic C4 pathway coexist with plastidial C5 pathway. From evolutionary perspective, this interesting arrangement may reflect early step in the mutual co-evolution of these two pathways.

The third manuscript (Study I) accepted on Plant Cell takes advantage of recently identified *Chromera velia*, a photosynthetic relative to obligatory parasitic apicomplexans, which contain rudimental but still essential apicoplast. Here, the authors created a conceptual map of *C. velia* tetrapyrrole synthesis and identified the involvement of initial C4 synthetic steps by metabolic labeling, unique observation for eukaryotic phototroph. This also makes *C.velia* potent model for apicomplexan organelle and the design of novel chemotherapeutics.

The ready-to-submit manuscript (Study IV) represents a major discovery on dispensable role of heme group in the biology of aerobic kinetoplastid *Phytomonas serpens*. By a series of bioinformatic and very elegant biochemical techniques author convincingly show that *P. serpens*, although still capable of integrating heme group into its apoproteins, can live without it with virtually no impact on its growth. The study id of great general importance and will certainly be accepted for publication is some high-profile journal.

To sum up, the thesis presented by Luděk Kořený is very consistent piece of scientific work of excellent quality and I am more than happy to recommend it for granting a PhD degree.

Notes>

Page 4 Perhaps not ideal to refer to a recent paper and saying the following: "The biochemistry of heme synthesis is one of the first metabolic pathways that have been studied (Heinemann et al. 2008)."

Page 8 I would be careful in saying "Localization of the particular enzymes often reflects their origin. Enzymes targeted to a given organelle likely originated from the bacterial ancestor of that organelle. Localization in a plastid suggests a possible cyanobacterial origin, whereas the mitochondrial localization hints to the origin of the enzymes from an α-proteobacterium. Similarly, the cytosolic location is usually compatible with an evolutionary origin in the nucleus of the eukaryotic host"

This is not entirely true, as for instance mammalian and yeast mitochondria contain more "eukaryotic inventions" than proteins with traceable alphaproteobacterial origin (22% in human and 32% in yeast, Gabaldon and Huynen 2003).

Page 9 ".... all the remaining enzymes of the heme
synthesis seem to be encoded by the ***ancient*** eukaryotic genes....." what are the ancient eukaryotic genes?

Page 9 - mitochondrial ***transit*** peptide – "transit peptide" works better for plastids.... mitochondrial targeting sequence/peptide is usually used in mitochondrial field

>Questions

1. Does heme itself (without a protein component) has any activity? Could heme exist as a bioactive molecule before its insertion into a apoprotein. And also do heme-less apoproteins have any function?

2. What is the role of mitochondrial pathway in land plants?

3.Why do you think all this variability in enzyme distribution is happening, could you hypothesize on the example of *C. velia* and *P. falciparum* on the possible metabolic advantages of the re-localization?

4.Is there any functional or evolutionary interaction between heme synthesis and iron-sulfur cluster synthesis in mitochondria and plastids?

Pavel Doležal, PhD