PhD thesis 'Genomics of Preaxostyla flagellates' by Lukáš Novák.

The Preaxostyla is a group of anaerobic or microaerophilic microbial eukaryotes that often can be found living in the intestinal tract of animals. Some species in the group are free-living but also in oxygen-poor environments. Until recently, they had received little attention although they are part of a much larger story about the evolution of all eukaryotes.

Some 40 years ago now, it was thought that eukaryotes arose by the gradual increase of complexity of a prokaryotic ancestor which became a simple ur-eukaryote. At one point, this ancestor engulfed a bacterium that became the modern day mitochondrion. This organelle is mainly known as the provider of large amounts of ATP. This theory, the Archezoa hypothesis, required that early on, there existed eukaryotes that had not acquired the mitochondrial symbiont and in fact, that some modern-day lineages of eukaryotes were descendants of these 'Archezoa'.

However, over the years, more and more evidence was uncovered that demonstrated these living 'relics' were not amitochondriate at all but had a mitochondrial organelle. Most of these mitochondria are highly derived and only contain small subsets of known mitochondrial enzymes and metabolic pathways. Some contain enzymes not found in textbook mitochondria. Up to recently, it seemed the only pathway that remained in these organelles was the one producing iron-sulfur clusters, essential redox-active cofactors found in many crucial enzymes of all living organisms.

The Preaxostyle are part of the Excavata that played an important role in the downfall of this Archezoa hypothesis.

This thesis presents data based on the discovery of a preaxostylid that seemingly has completely lost its mitochondrion and the work presented describes that discovery and the various subsequent follow-on studies.

The work presented is part of the international tradition to present scientific findings in the peer-reviewed literature and of a high standard. The applicant's part in these large international studies has been described and constitutes a genuine contribution to new knowledge. This thesis therefore is suitable for the award of the title of Doctor of Philosophy.

There are however a few points I would like to raise with the candidate and would appreciate getting an answer to.

The overall story, in relation to the message that mitochondria are not essential for eukaryotes as *M. exilis* does not have them, is interesting. The candidate discussed the Archezoa hypothesis, so much realise that up until the 90s 'most' people were comfortable with the notion that there actually were eukaryotes without mitochondria. The labs that discovered evidence for mitochondria in the Archezoa actually had a hard time convincing editors and reviewers that eukaryotes without mitochondria apparently did not exist. So, the field has gone full circle and it is peculiar now to see elaborate arguments put forward why mitochondria are not essential. What the applicant carefully avoided however is the hotly contested notion where there is amitochondrial requirement for eukaryogenesis or not. This is somewhat of an amiss in this thesis (the sole mention seems to be in the MBE paper, page 2306, and then only one sentence).

Section 1.1.2 describes various microbial eukaryotes and their unusual mitochondria. Most important organisms (*Trichomonas, Giardia*) are discussed and several others perhaps less extensively. It is surprising that the discussion about stramenopiles (section 1.1.2.9) does not mention *Blastocystis* at all. This organism has played a rather prominent role in the discussion about mitochondrial organelles and has had its genome sequenced in 2017 (Gentekaki *et al* PLoS Biology 15(9): e2003769) so there would have been ample time to include this in the thesis.

In section 1.2.1.6 the Archamoeba are being discussed in relation to their replacement of the ISC system with the NIF system. Although I am not assessing this thesis to check if my work is properly represented, it is a little peculiar that in the discussion of the replacement of ISC for NIF, the work of myself (van der Giezen *et al* 2004 BMC Evol. Biol. 4, 7.) and Ali *et al* (J. Biol. Chem. (2004) 279, 16863–16874) on *Entamoeba* is not mentioned but similar work by Nývltová *et al* 2013 is, considering the findings nine years earlier were somewhat more surprising. Similarly, the dual localisation for *Mastigamoeba* is taken as genuine while the dual localisation of NIF in the cytosol and mitosomes of *Entamoeba* (Maralikova *et al* 2010 Cell. Microbiol. 12, 331-342.) is ignored (text says 'likely localized exclusively in the cytosol').

In section 4.1.2 the suggestion is given that *M. exilis* cannot produce hydrogen because it, just like *Giardia* and *Entamoeba*, does not have hydrogenase maturases? *Giardia* has been shown to produce hydrogen (Lloyd *et al* (2002) Microbiology, 148, 727–733) irrespective of having these maturases so perhaps *M. exilis* might make hydrogen?

In the same section but perhaps relevant for the whole thesis (for example in the MBE paper page 2302). Why is iron-sulfur cluster assembly localised in most if not all mitochondria and its derivatives? Is there a physiological reason for this phenomenon? Is iron-sulfur cluster assembly using SUF biochemically/biophysically different from ISC assembly? Does *M. exilis* actually make iron-sulfur clusters?

Section 4.1.3.2 discussed the loss of the glycine cleavage system and suggests it is intimately linked with the loss of the mitochondrion or a prerequisite. Discuss this in relation to *Giardia* and *Entamoeba* please.

The Current Biology paper, page 1275 (aspartate/ornithine carbamoyltransferase and pyridine nucleotide transhydrogenase have no targeting signals)/page 1276-1277 (three proteins with predicted targeting signal)/page 1278 (PFOR and hydrogenase have no targeting signal/heterologous targeting)/page 1281 ('such a hypothetical organelle could not be recognized as a mitochondrion homolog by any available means'). Heterologous targeting is a powerful tool but how useful is this to convincingly demonstrate the absence of a mitochondrion in the organism where the genes are from? In addition, as it has been shown in *Trichomonas*, hydrogenosomal targeting seems rather unusual compared to mitochondrial targeting and the mitochondrial targeting signals might less important (see Garge *et al* (2015) Genome Biol Evol 7, 2716-26). What could have been done to more convincingly demonstrate that, for example, PFO is not organellar in *M. exilis*?

BMC Evol Biol paper, page 12. Unfortunately, a homologous transfection system could not be developed (and this is indeed a major task). However, like above, heterologous targeting is then used to provide an answer for the homologous system. This is understandable as *T. vaginalis* targeting is an available method. However, what else could have been used instead to give answers in the homologous system?

Mol Biol Evol paper, page 2292. It is mentioned mitochondria 'were considered indispensable due to their essential core function(s)'. As mentioned above, those familiar with the Archezoa hypothesis when it was still 'valid' had no issue with mitochondria being dispensable. Also, what is or are the essential core function(s) of mitochondria? This is an important issue and might explain the phenomenon in *M. exilis*.

Mol Biol Evol paper, page 2305. The single *Giardia* DRP is discussed but why not the 4 (or 5) DRPs in *Entamoeba*? The situation is quite confusing (conflicting publications on which DRP does what) but Herman *et al* 2017 (Sci. Rep. 7: 12854) describes more *Entamoeba* DRPs, are these in *M. exilis*?

Paratrimastix, page 154. Why is the energy metabolism (PFO and ACS) predicted to be cytosolic? No evidence is provided.

Outreach, page 161. You mention mitochondria have only one function, the production of iron-sulfur clusters. But earlier in your thesis you say that *Entamoeba* mitosomes are not involved in iron-sulfur cluster assembly? So, what is it? Are there more functions important/crucial or what is happening in the *Entamoeba* mitosomes if they don't do this important sole function?

It would be great to hear the candidate's response to the points raise above and look forward to the public defence.

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