52nd Jírovec's Protozoological days

CONFERENCE PROCEEDINGS

24–28 April 2023
Modrava, Czech Republic

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11:00-11:15		Vít Dohnálek*	Taming of mitochondria with LYRM proteins
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14:15-14:30		Michael Hammond	Comprehensive sub-mitochondrial protein map of the parasitic protist *Trypanosoma brucei* reveals novel aspects of organellar biology
14:30-14:45		Ignacio Durante	Extensive identification of novel kinetoplast associated proteins in *Trypanosoma brucei*
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11:30-11:45		Dorsaf Ennaceur*	Candidate transport proteins for guanine in *Chromera velia*
11:45-12:00		Tamas Dobai*	Production and 3D structure prediction of SmolCathL, a cysteine protease of the ancient endoparasite *Sphaerospora molnari*
12:00-14:00		Lunch	
14:00-14:15		Daria Tashyreva	First whole-cell three-dimensional reconstruction of diplonemid ultrastructure and cell division
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Friday 28th		
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TALKS

Martin Benda, Ronald Malych, Luboš Voleman, Pavel Doležal

The role of late ISC pathway in the formation of cytosolic 4Fe–4S clusters in *Giardia intestinalis*

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Iron-sulfur clusters (Fe–S) are important cofactors involved in many cellular pathways in all domains of life. In eukaryotic cells, the biosynthesis of Fe–S clusters begins in mitochondria by a multiprotein assembly system called the ISC pathway. First, proteins of the ”early” ISC pathway assemble 2Fe–2S clusters, which can be either transferred to mitochondrial 2Fe-2S dependent apoproteins, transported to the cytosol, or passed on to the “late” ISC pathway, which assembles 4Fe–4S clusters for use within mitochondria. However, this mechanism has been described based on thoroughly studied model organisms and may not be universal. In this work, we decided to explore the assembly of Fe-S clusters in the much less studied pathogenic protist *Giardia intestinalis*. *Giardia* does not have canonical mitochondria, which have been reduced during the evolution to an organelle known as a mitosome. The sole role of mitosomes is believed to be the assembly of 2Fe-2S clusters for further use in other cellular compartments, as mitosomes lack respiratory chain complexes and are not involved in energy metabolism. Intriguingly, however, *Giardia* also contains proteins of the late ISC pathway, despite the apparent absence of client 4Fe-4S dependent apoproteins in the mitosome. Thanks to our recent advances in molecular genetics of *Giardia*, we were able to fully knock out all four late ISC pathway genes (i.e. grx5, iscA2, bolA3, and nfu1) and characterize the respective mutant strains. While all the deletion strains are viable, they exhibit different phenotypes, with the most severe one associated with the loss of the IscA2 protein. By using EPR spectroscopy, radio isotope incorporation, as well as by assessing the activity of cytosolic 4Fe–4S dependent proteins, we were able show that the mitosomal IscA2 affects the formation of cytosolic 4Fe-4S clusters. Although the exact mechanism of cluster formation in *Giardia* remains unclear, our data

suggest a unique link between the late ISC pathway and cytosolic cluster formation in this organism.

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Role of MICOS in mitochondrial maturation during *Trypanosoma brucei* differentiation

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Cristae are infoldings of the inner mitochondrial membrane that come in a variety of forms. They compartmentalise electron transport chain complexes (ETCs) and provide diffusion barriers via cristae junctions (CJ), making them vitally important for energy generation. The mitochondrial contact site and cristae organising complex (MICOS) found at these CJs plays a role in organising cristae and providing contact sites between inner and outer membrane. Additionally, MICOS participates in lipid metabolism and protein import into mitochondria.

While the mammalian bloodstream form (BSF) of the *Trypanosoma brucei* parasite has a tubular mitochondrion with tiny, stub-like cristae the insect procyclic form (PCF) possesses an elaborately branched, reticulated organelle with fully-developed discoidal cristae harbouring ETCs. Differentiation between these two life cycle stages can be induced in vitro, making the parasite an ideal model system to study crista development. *T. brucei* PCF MICOS is formed from two subcomplexes that differ in their localisation and function. The membrane-embedded MICOS subcomplex is important for CJ formation and maintaining cristae while the peripheral subcomplex is vital for protein import into the intermembrane space.

We show here that the subunit composition of MICOS plus interactions with SAM50 and ATP synthase are conserved in BSF. Interestingly, novel interactors were also discovered, perhaps indicating additional or divergent functions of BSF MICOS. In support of this notion and in contrast to the PCF situation, gene deletion mutants of peripheral complex subunits are viable in the BSF. Furthermore, the conserved trypanosome Mic10 homolog, which is part of the integral complex, is also dispensable for differentiation. However, ablation of peripheral trypanosome-specific MICOS components Mic34 or Mic40 compromises differentiation and mitochondrial morphology, as well as ETC assembly. This is most likely due to their previously established role in protein import in PCF. This interdependency between mitochondrial protein import and morphology is also exemplified by the effects of depletion of Mic34 and Mic40 in PCF, in which the highly branched PCF mitochondrion is rendered more simplistic and tubular like its BSF equivalent. This clear division of labour between the two trypanosome MICOS subcomplexes as well as the dispensability of the Mic10 homolog sets *T. brucei* MICOS apart from its opisthokont counterpart.

Anzhelika Butenko[1,2,3], Julius Lukeš[1,3], Dave Speijer[4], Jeremy G. Wideman[5]

Mitochondrial genome evolution: views and news

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Mitochondria are organelles of bacterial origin possessing their own genome and performing a variety of important cellular functions including but not limited to ATP generation. Mitochondrial genomes are highly diverse in terms of gene content. Several hypotheses aimed at explaining the retention of mitochondrial genes and persistence of mitochondrial genomes throughout evolution were put forward. At least two of them, co-location for redox regulation (CoRR) and constraints hypotheses, are widely debated and have a certain level of experimental support. However, there is no widely accepted explanation for a number of observations regarding the mitochondrial genome evolution, including the widely variable sets of mitochondrion-encoded genes and the emergence of peculiar mitochondrial gene and genome structures in certain eukaryotic lineages.

We use a phylogenetically equilibrated dataset of ~90 mitochondrial and nuclear genomes/transcriptomes of the representatives of major eukaryotic lineages to explore the patterns of gene retention in the mitochondrial genomes and gene transfer to the nucleus. We observe ~30-fold differences in the content of protein-coding genes in mitochondrial genomes across eukaryotes and highlight the cases of parallel evolution, when a similar pattern of mitochondrial gene retention and transfer to the nucleus is observed in lineages only very distantly related to each other. In opisthokonts, a core set of proteins are retained encoded in mitochondrial genomes, which includes proteins constituting central, often highly hydrophobic, components of electron transport chain (ETC) complexes. Some components of the translational machinery are retained universally across eukaryotes (SSU and LSU rRNA), whereas other components can be lost (5S rRNA) or transferred to the nucleus (tRNAs). The mitochondrial gene content of most eukaryotic lineages is larger than that of opisthokonts and includes, for example, variable sets of genes encoding ribosomal proteins and additional ETC subunits. By linking our analysis of mitochondrial gene content to recent advances in dating eukaryotic divergences, we can see that major mitochondrial gene content alterations occur rarely and often correspond to the origin of major eukaryotic lineages. Based on the obtained data, we critically re-evaluate the existing hypotheses aimed at explaining the evolution of mitochondrial gene content and emphasize that population genetics has to be taken into consideration.

Lawrence Rudy Cadena, Ignacio Durante, Michaela Svobodová, Michael Hammond, Julius Lukeš

Cutting the cord: Identification of a vital protein involved in the segregation of kinetoplast DNA

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The mitochondrial genome of *Trypanosoma* is contained in a specialized structure called the kinetoplast. Kinetoplast DNA (kDNA) is organized into a catenated network of minicircles and maxicircles that divide and segregate once each cell cycle. Although a number of proteins involved in kDNA replication and segregation are known, various key steps in the replication mechanism itself remains uncharacterized at the molecular level. An enigmatic structure, known as the *nabelschnur*, appears to connect the daughter kDNA networks prior to their complete segregation. To date, only a single protein (TbLab1) has been identified to localize to the *nabelschnur*.

Here, we identify a second protein, termed TbNab70, which localizes to both the kDNA disk and the *nabelschnur*. We show that TbNab70 is

required for correct segregation of kDNA, with depletion leading to delayed cytokinesis and decreased cell proliferation. We propose that TbNab70 is required for efficient kDNA division and specifically participates in the separation of daughter kDNA networks.

Prashant Chauhan[1,2], Lukas Muller[3], Johnathan Espinoza Wong[1,2], Alena Zíková[1,2], Ondřej Gahura[1]

Assembly factor mtSAF24 in the biogenesis of small mitoribosomal subunit in trypanosomes

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Mitochondria harbors mitoribosomes, macromolecular nanomachines composed of RNA and proteins, which translate mRNAs encoded in the mitochondrial genome. Biogenesis of mitoribosomes requires coordinated assembly of mitoribosomal proteins (mtRPs) and RNA components of both subunits, and relies on several assembly factors (AFs). While some mtRPs and all mt-rRNAs are encoded mitochondrially, most mtRPs and all AFs are nuclear-encoded and posttranslationally imported into mitochondria. Assembly of small mitoribosomal subunit (mtSSU) in *Trypanosoma brucei* involves three structurally characterized precursors, in which numerous AFs provide enzymatic activity and structural framework essential for remodelling of immature rRNA and assembling of mtRPs. The earliest characterized mtSSU precursor is known as the mtSSU assemblosome. It contains immature rRNA complexed with an incomplete set of mtSSU proteins and 34 AFs. The assemblosome features protrusion on the immature intersubunit side, capped with a low-resolution disc-shaped structure, which may represent a part of the lipid bilayer. The structure contacts solely the N-terminal domain (NTD) of AF mtSAF24. Our results show that suppressing mtSAF24 expression by RNA interference or knockout by CRISPR-Cas9 produces strong growth defect accompanied by a loss of mtSSU rRNA, documenting its essential role in mtSSU assembly. Furthermore, upon ectopic expression of an RNAi-resistant recoded C-terminal domain (CTD) of mtSAF24, the growth phenotype was rescued, which suggests that the association of assemblosome with the peripheral disc is not essential in vivo under cultured conditions. Sub-fractionation of mitochondria shows that mtSAF24-V5 is predominantly present in the peripheral membrane fraction, in accordance with its possible lipid binding ability. We recombinantly expressed and purified full length mtSAF24 and its CTD and NTD. We aim to utilize these recombinant proteins for in vitro phospholipid binding assays (co-flotation or co-sedimentation assays with liposomes; PLIMAP) to test the capacity of mtSAF24 to bind to lipids. Our study aims to illuminate whether the assembly of mtSSU in *T. brucei* occurs in the association with the inner mitochondrial membrane, which would be an unprecedented feature of mtSSU biogenesis.

Tamás Dobai[1], Zdeněk Franta[1], Astrid S. Holzer[2,3], Pavla Bartošová-Sojková[2]

Production and 3D structure prediction of SmolCathL, a cysteine protease of the ancient endoparasite *Sphaerospora molnari*

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Parasite-derived proteases are major players in parasite's pathogenesis. Cathepsins, the most abundant lysosomal cysteine proteases, are crucial molecules participating in many important biological processes including digestion or immunomodulation. *Sphaerospora molnari* is an ancient myxozoan endoparasite of common carp *Cyprinus carpio*. The parasite massively proliferates in carp blood, the site where *S. molnari* likely acquires its nutrients. However, the exact molecular mechanism of parasite's feeding is yet to be identified. Data mining of the *S. molnari* transcriptome revealed the presence of three cathepsin L isoenzymes. One of these, *S. molnari* cathepsin L (SmolCathL) is highly expressed in all stages across parasite intrapiscine development including its high abundance in blood stages. Our aim was to produce an active recombinant protein (recSmolCathL) to allow its biochemical characterisation and future exploration of its digestive roles during parasite's feeding. Its 3D structure predictions and comparison with digestive enzymes of other parasites was also performed to provide insights into the structural and functional properties of the enzyme studied.

A gene encoding SmolCathL proenzyme was cloned into pASK-IBA37+ plasmid containing N-terminal 6x His-tag and the tetracycline inducible promoter. recSmolCathL was produced using ArcticExpress(DE3) competent *Escherichia coli* cells and purified via a combination of affinity and size exclusion chromatography. The activity of recSmolCathL was tested under different pH, temperature, and buffer conditions using Z-Phe-Arg-AMC and E-64 as a generic cathepsin L substrate and inhibitor, respectively. We have observed that recSmolCathL possesses a broad pH optimum peaking at pH 4.1 and at 30˚C. The comparative structural analysis between the Alphafold-predicted SmolCathL and *F. hepatica* cathepsin L crystal revealed high homology of both enzymes with differences in the ERFNIN and GNFD motifs in the propeptide region.

Vít Dohnálek, Pavel Doležal

Taming of mitochondria with LYRM proteins

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LYRM superfamily comprises small, highly conserved proteins that are essential participants of various mitochondrial processes, such as iron-sulfur cluster assembly, oxidative phosphorylation or mitoribosomal translation. We have established that their mitochondrial interactors are of prokaryotic origin, while the LYRMs have no apparent bacterial homolog and thus, are most likely a eukaryotic invention.

The activity of LYRMs is regulated by the Acyl Carrier Protein (ACP). The ACP is a highly negatively charged protein that is able to carry the acyl chain which needs to be inserted into the hydrophobic core of the LYRMs in order to activate them. The source of the acyl is mitochondrial fatty acid synthesis pathway of type II (mtFASII) that is present in the majority of eukaryotes as well as prokaryotes. We hypothesise that the main reason for the preservation of this pathway in eukaryotes is its linkage to the regulation of LYRMs which themselves are crucial regulators of energy metabolism. LYRMs together with ACP and mtFASII constitute a sophisticated regulation system that might have been one of the key tools that the eukaryotic cell had developed to interact with its newly acquired endosymbiont. To support our hypothesis, we focused on various eukaryotic lineages and their versions of the system, particularly on the anaerobic protists that had often reduced their mitochondria to hydrogenosomes or mitosomes. In many cases, these organisms lost certain parts of the system and their LYRMs contain unusual features and have low sequence conservation.

We have conducted thorough bioinformatic analyses of LYRMs and their interactors. While the phylogenetic analysis revealed a very diverse distribution of LYRM subfamilies across the eukaryotic tree of life, examination on structural level showed us various adjustments of LYRMs

to specific changes of mitochondria in different eukaryotic lineages. Using the AlphaFold2, we were also able to model interactions of LYRMs and their hypothetical interactors which helped us to elucidate their interrelationships. Altogether, we believe our findings bring important new insight into the role of LYRMs in mitochondrial biogenesis and evolution.

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Extensive identification of novel kinetoplast associated proteins in *Trypanosoma brucei*

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The single kinetoplast containing mitochondrial DNA (kDNA) is the defining feature of Kinetoplastids, and the tripartite attachment complex (TAC) connects the basal body with the kDNA coupling its synchronized segregation during cell division. Both these complex subcellular structures are appealing targets of intervention against trypanosomatids' driven neglected diseases. Here, we screened the tryptag localization repository and initially prioritized 10 previously undescribed proteins displaying putative kinetoplast proximal enrichment (KEP) and therefore chosen for further validation. By means of a standardized experimental screening workflow, expression and KP or TAC localizations were firstly verified by WB of crude mitochondrial fractionations and IFA analysis of alternate endogenously tagged cell lines. KP and TAC newly identified proteins were subsequently subjected to co-IP and complexome data analysis and novel interactors were retrieved. The subcellular localization of these new KP and TAC interacting proteins was also verified by alternate endogenous tagging. Additionally, the essentiality of these proteins was assessed by RNAi knock-down and kDNA associated phenotypes were explored by cell cycle and maxi- and minicircle relative abundance analysis. As a result of these dedicated investigation, new KP and TAC interacting proteins were identified and partially characterized regarding their essentiality and effects in kDNA replication, resulting in an enrichment of proteins associated with KP and TAC copartments. Moreover, this work provides a methodological pipeline for the identification of novel KP/TAC associated targets for intervention against trypanosomatids' caused diseases.

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Candidate transport proteins for guanine in *Chromera velia*

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Nitrogen is often a limiting nutrient for photosynthetic organisms, impelling them to take up and store N beyond the required amount. One of the forms of N storage is accumulation of guanine as crystals which was observed in several microalgae species. Among the microalgae accumulating guanine, is *Chromera velia*, a unicellular autotrophic alga, and the closest known phototrophic relative to apicomplexan parasites. The synthesis and consumption of the guanine and the mechanism behind

the storage of the N and its remobilization is yet to be fully understood. Microscopy and cell fractionation experiments highly suggest that guanine crystals are present inside special vacuoles. This means that guanine or related molecules need to be transported across membranes for crystallization and dissolution to occur. In search for possible transport proteins, we identified homologues of transporter proteins for purines or derivatives like nucleotides from plastids of plants and algae, in the genome of *C. velia*. Based on presequences analyses, we selected the non-plastid transport proteins for further studies. We want to investigate the transport properties of these proteins via heterologous expression in *Escherichia coli*. Therefore, we designed expression constructs for two homologues of Equilibrative nucleoside transporter proteins (ENT) and three of solute carrier family 43 member 3 proteins (SLC43a3). To ensure an efficient protein expression in *E. coli* and prevent any biased codon usage, the gene of interest were codon optimized and inserted in-frame with the histidine tag into isopropyl-β-D-thiogalactopyranoside (IPTG) inducible expression vector. *E. coli* cells were harvested during the exponential growth phase and the protein extract of each construct was analyzed in a discontinuous, denaturing system on 10%, 12% separating polyacrylamide gels. In the following phase of the project, expression of the proteins will be verified also via western-blot using anti his-tag primary antibodies. To analyze transport properties of the recombinant proteins, the *E. coli* cells harboring the different expression plasmids with the appropriate recombinant proteins will be supplemented with radiolabeled guanine and guanine nucleotides. In a parallel approach, guanine crystals were isolated with the goal to retrieve and identify proteins that could be present in the same compartment as the crystals, and hence have a function in the crystal formation and consumption.

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Preliminary studies of striated fibres and assemblins in Preaxostylans

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The flagellar apparatus, comprising the basal bodies and the microtubular and fibrous structures directly associated with them, is the core of the cytoskeleton in most protists. The similar attachment, positioning, and paths through the cell of the non-microtubular structures suggest the possibility of homology in different lineages. The system I fibres of green algae and the microribbons of the gut parasite *Giardia* are composed respectively of striated fibre assemblin (SFA) and giardins, which are known to be homologous non-microtubular components. Recent work has greatly extended the number and diversity of SFA in both taxonomic diversity and paralogy, however, SFAs have only been characterized in a few species. We had previously found putative SFAs in the anaerobic preaxostylans *Paratrimastix pyriformis* and oxymonad *Monocercomonoides exilis*. To investigate their possible roles in the cell we constructed hemagglutinin (HA) tagged proteins of putative SFAs in *M. exilis* and *P. pyriformis* for antibody generation, and two of the raised antibodies against *P. pyriformis* proteins exhibited sufficient targeting specificities to SFAs. The preliminary results from IFA and expansion microscopy confirmed these two *P. pyriformis* SFAs are closely associated with the entire length of the right, left and singlet microtubular roots attached to the posterior basal body. The results suggest that the SFA are basic components of the cytoskeleton of excavates.

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Dynamics and isolation of guanine crystals from *Chromera velia*

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Nitrogen availability is often limiting the growth rate of photosynthetic eukaryotes. *Chromera velia*, a photosynthetic microalga closely related to apicomplexan parasites, contains a high abundance of crystalline inclusions, which we have shown to consist of the nitrogen-rich compound guanine via Raman micro spectroscopy and HPLC analyses.

Counting of the crystals via polarizing light microscopy showed that in the nitrogen replete cultures, the number of crystals did not change throughout the experiment. In contrast to this, in the nitrogen depleted culture, the number of crystals decreased, until no crystals could be observed at day five. Simultaneous observation via transmission electron microscopy showed that in the cells from the nitrogen depleted cultures, crystals are still present, but are smaller than the ones in cells from the nitrogen replete cultures. When the cells were transferred back to normal f/2 medium with nitrate, crystals could be detected with the light microscope again, and after 3 days, numbers and sizes of the crystals were the same between previously nitrogen-starved cells and cells that had been maintained in nitrogen replete medium throughout the experiment. From the results of this experiment, we conclude that the nitrogen stored in the guanine can be re-mobilized by the cells, when external nitrogen cannot be assimilated, and that nitrogen starved cells react to the addition of nitrogen by forming guanine crystals.

Furthermore, to identify the proteins associated with the guanine crystals, we modified the established protocol of isolation of plastids and mitochondria from the *C. velia* cells and successfully isolated the guanine crystals. Currently, we are trying to observe the morphological appearance and localization of the isolated guanine crystals by using freeze fracture technique along with Serial block face Scanning electron microscopy (SBF-SEM).

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Evolutionary and physiological insights gained from the N- and C-terminal prediction of intracellular protein locations in cells with complex plastids

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Complex plastids evolved by eukaryote-eukaryote endosymbiosis, which lead to extensive rearrangements of the intracellular distribution of metabolic pathways. These rearrangements are an inevitable consequence of the evolution of protein targeting systems, and of the transfer of genes from the organelle to the nuclear genome.

Comparing the intracellular location of proteins between different groups of organisms can therefore help elucidating phylogenetic relationships between these organisms. Furthermore, studying, the intracellular distribution of metabolic pathways in the cells, can also give important physiological insights.

A variety of targeting signals can be used for genome wide prediction and allow for interspecies comparison. Proteins targeted via the secretory pathway are of crucial importance for such analyses, because unlike in cells with primary plastids, in cells with complex plastids, also the plastid proteins enter the endoplasmic reticulum (ER), en route to the plastid. Unlike proteins that pass through the ER, ER resident proteins are often characterized by C-terminal signals. I will present characteristics of these signals, and discuss how the different prediction approaches can be integrated.

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Comprehensive sub-mitochondrial protein map of the parasitic protist *Trypanosoma brucei* reveals novel aspects of organellar biology

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In this study, we have generated a high confidence mitochondrial proteome (MitoTag) of the *Trypanosoma brucei* procyclic stage containing 1,239 proteins, of which 337 have not previously been identified as mitochondrial. We used the TrypTag dataset as a foundation and took advantage of the properties of the fluorescent protein tag that caused aberrant but fortuitous accumulation of tagged matrix and inner membrane proteins near the kinetoplast (mitochondrial DNA). Combined with transmembrane domain predictions, this characteristic allowed categorization of 1,053 proteins into mitochondrial sub-compartments, the detection of unique matrix localized fucose and methionine synthesis and the discovery of new kinetoplast proteins, which revealed kinetoplast-linked pyrimidine synthesis. Moreover, disruption of targeting signals by tagging allowed mapping of the mode of protein targeting to these sub-compartments, identifying a set of C-tail anchored outer mitochondrial

membrane proteins and mitochondrial carriers likely employing multiple target peptides. This represents the single most comprehensive mapping procedure of a mitochondrion to date.

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FeS cluster machinery in the amitochondriate eukaryote *Monocercomonoides* exilis composes of a single large complex

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Monocercomonoides exilis is the first eukaryotic organism described as a complete amitochondriate, yet it shares common features with heterotrophic anaerobic/microaerophilic protists, some of which bear divergent mitochondrion-related organelles or MROs. It has been postulated that the retention of these organelles stems from their involvement in the assembly of essential cytosolic and nuclear FeS proteins, whose maturation requires the evolutionarily conserved mitochondrial ISC and cytosolic CIA machineries. The amitochondriate *M. exilis* lacks genes encoding the ISC machinery yet contains a bacteria-derived SUF system (MeSuf), composed of the cysteine desulphurase SufS fused to SufD and SufU, as well as the FeS scaffolding components MeSufB and MeSufC. Here, we show that expression of the *M. exilis* SUF genes, either individually or in tandem, can restore the maturation of the FeS protein IscR in the *Escherichia coli* double mutants of sufS, iscS and sufB, iscUA. In vivo and in vitro studies indicate that purified MeSufB, MeSufC and MeSufDSU proteins interact suggesting that they act as a complex in the protist. MeSufBC can undergo conformational changes in the presence of ATP and assemble FeS clusters under anaerobic conditions in the presence and absence of ATP in vitro. Altogether, these results indicate that the dynamically interacting MeSufDSUBC proteins may function as an FeS cluster assembly complex in *M. exilis* thereby being capable of replacing the organelle-enclosed ISC system of canonical eukaryotes.

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Intra-genomic diversity of the V9 hypervariable region of the 18S rRNA gene in eukaryotes and its impact on metabarcoding

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High-throughput amplicon sequencing (metabarcoding) is an important tool for studying the diversity and ecology of microorganisms in various habitats. However, it is also associated with several inherent biases that affect our interpretation of results. One arguably serious but poorly documented problem is the intragenomic diversity of the molecular barcode. Here we investigate this phenomenon using the V9 region of the 18S rRNA gene. We compare cross-genome diversity in 19 eukaryotic phyla abundant in marine plankton, with a particular focus on eupelagonemids, a protist group that emerged as one of the most diverse lineages in the marine plankton based on V9 metabarcoding studies. At the level of barcodes, denoised barcodes with sequencing errors removed, and operational taxonomic units (OTUs), the intragenomic variability of the

V9 region is approximately the same across diverse eukaryotic taxa. Nearly all genomes and transcriptomes we investigated are dominated by one V9 sequence and one OTU, whereas the remaining OTUs are much less abundant. For this reason, intragenomic polymorphism in the V9 region has little effect on the overall outcome of metabarcoding studies. Our results suggest that most of the sequence variability observed at the barcode level is caused by sequencing errors and that the *SWARM* OTU definition algorithm commonly used in metabarcoding studies is not optimal for collapsing main sequences and erroneous sequences derived from them into one out, leading to an overestimation of diversity in metabarcoding data such as those from the *Tara Oceans* study. Eupelagonemids did not exhibit exceptionally high level of intra-genomic V9 sequence variability in our study, and we showed that the *SWARM* algorithm overestimates their diversity in V9 metabarcoding data much more (by a factor of five) than that of nearly all other eukaryotic taxa (1.5-fold, median for 70 taxa).

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Exploring the mitochondrial genetic code diversity

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During translation, the ribosome uses the genetic code consisting of nucleotide triplets to assemble a functional protein. However, this code is not invariant – one eukaryotic organism might even employ different codes in its nucleus, mitochondria, and plastids. A particularly intriguing aspect is the relationship between the usage of codons as translation terminators and the composition of release factors which recognise them. Typically, three stop codons are present in a genetic code: UAA, UAG and UGA. In mitochondria, their recognition is carried out by mitochondrial release factors mtRF1a (UAA and UAG) and mtRF2a (UAA and UGA). The former has been independently lost from different eukaryote lineages, underpinning reassignment of UGA as a sense (usually tryptophan) codon, whereas an unprecedented case of mtRF1a loss recently uncovered by our lab relates to a stop-to-sense reassignment of UAG. Furthermore, our team has already shown a correlation in changes in stop codon recognition, including sense-to-stop-reassignments, with mutations of specific amino acids in mitochondrial release factors in the green algal order Sphaeropleales and the stramenopile group Labyrinthulea. However, a comprehensive picture of mitochondrial genetic code diversity and evolution is lacking due to both lack of analyses of existing sequence data and poor sampling of particular eukaryote lineages, such as Rhizaria or Amoebozoa. We have initiated a systematic exploration of mitochondrial genetic code diversity and the underlying molecular mechanisms, such as alterations to the mitochondrial release factors across the eukaryote phylogeny. In multiple cases of protist taxa with no mitochondrial genome sequence available, we could obtain information on their mitochondrial genetic code by analysing sequences of mitochondrial transcripts extracted from transcriptome assemblies. As a result, we have discovered multiple new cases of stop-to-sense, sense-to-stop, and sense-to-sense codon reassignments in mitochondria as well as new independent instances of mutated mitochondrial release factors. These correlate with specific mitochondrial genetic code changes, reinforcing the notion of the significance of the mutations in altered termination codon recognition. Specific examples will be discussed.

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Queuosine-tRNA modification as a means for gene expression regulation in *Leishmania mexicana*

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All kinetoplastid parasites including leishmania have polycistronic transcription, hence regulation of gene expression is mediated mostly by post-transcriptional mechanisms. One of the post-transcriptional steps is represented by tRNA modifications that directly regulate translation by modulating codon–anticodon interactions. The Queuosine (Q) modification is found at the wobble position 34 of tRNAs containing the GUN anticodon, leading to the change of GUN into QUN anticodon. Consequently, the efficiency of the translation of NAU and NAC codons by Q-tRNA is symmetrical. Here, we examine the role of the Q-tRNA modification in *L. mexicana* differentiation and infectivity. Increased abundance of Q-tRNAs in the amastigote stage, when compared to the insect promastigote stage, suggests an important role for this modification in the mammalian-infective stage. The production of Q-tRNAs is catalysed by guanine transglycosylase (TGT1/2). We employed CRISPR/Cas9 to generate a gene knock-out (KO) for TGT2 subunit in *L. mexicana*, which resulted in the depletion of Q-tRNAs as expected. We did not observe any growth phenotype in TGT2 KO when cultured as promastigotes, or when differentiating promastigotes into amastigotes. Although the TGT2 KO amastigote stage did not show any growth defect in culture, the KO cells exhibited reduced infectivity in macrophages in vitro as compared to WT. Most importantly, mice infected with *L. mexicana* TGT2 KO developed significantly smaller lesions than with WT. The decreased infectivity could be a consequence of alteration in the host's immune response. However, the immune analysis of the infected mice showed no difference in the levels of IgG1 and other markers, but a decrease in IgG2a, which corresponds with a lower parasite abundance in the lymph nodes after TGT2 deletion. In order to explain the observed phenotypes, we performed a proteomic analysis and assessed the abundance of the NAU codons, decoded by Q-tRNAs, in genes encoding the depleted proteins in the KO strain. Among the hits was Gp63, an important virulence factor of leishmania, or numerous enzymes of lipid metabolism, suggesting remodeling of the cell surface. We conclude that the Q modification is required for *L. mexicana* infectivity, and most likely, the defect is a consequence of altered protein expression in leishmania. Altogether, the Q modification represents another way how the parasite can regulate the gene expression, and adapt to different hosts and conditions.

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Peroxisomes in anaerobic fungi Neocallimastigomycota

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Peroxisomes are multifunctional and dynamic organelles that could be found in all major groups of aerobic eukaryotes. The most conserved metabolic pathways of peroxisomes are involved in the oxygen-dependent oxidation of fatty acids, which results in hydrogen peroxide formation. Due to the key role of oxygen in peroxisomal metabolism, it had been widely accepted that the adaptation to anaerobic lifestyle led to the loss of peroxisomes. To our surprise, we recently identified anaerobic peroxisomes in Archamoebae members. The findings break the paradigm "no oxygen no peroxisomes", and opens a new field for evolutionary research of anaerobic eukaryotes. Our recent homology searches for peroxins across anaerobic eukaryotes suggested presence of peroxisomes

in members of anaerobic fungi Neocallimastigomycota, including *Neocallimastix lanati*, *N. californa*, *Anaeromyces robustus*, and *Piromyces finnis*. We identified 13 most common peroxins in these species, together with fungus-specific peroxins, that cover all functional categories of peroxisomal biogenesis. We also identified putative peroxisomal matrix proteins. The targeting to peroxisomes of seven *N. lanate* candidates was verified, using heterologous model *S. cerevisiae*. The functional annotation revealed presence of typical components of fatty acid beta-oxidation, which are conserved in all investigated species, while catalase and peroxisomal acyl-CoA oxidase are absent. The homology searches also suggest presence of enzymes involve in carbohydrate metabolism, redox-balance maintenance, and ROS metabolism, and other enzymes with no clear biochemical context. In conclusion, anaerobic fungi are another eukaryotic group that possess anaerobic peroxisomes.

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The first filasterean parasite illustrates holozoan protist diversity

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Holozoan protists include members of four distinct groups that encompass diverse morphologies, habitats and ecological niches. Here, we report the metagenome-assembled genome of *Txikispora philomaios*, the first parasitic member of the holozoan group Filasterea. The *T. philomaios* genome encodes homologs of animal developmental transcription factors and the integrin adhesome—proteins that play key roles in animal cell-cell adhesion, signaling and development. In contrast to its free-living relatives, *T. philomaios* has undergone losses of metabolic genes, including biotin and glycogen metabolism pathways. I discuss how our results add to our understanding of the genomic repertoire of the protist ancestors of animals and provide insights into the independent adoption of a parasitic lifestyle in holozoan protists.

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In the spotlight, losing IR region: the complexity and convergence in genome evolution of algal secondary plastids

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Plastids have a rich evolutionary history, whose main chapters include: their transformation from free-living cyanobacteria into organelles permanently integrated with host cells, their spreading across the diversity of eukaryotes through secondary and higher-order endosymbioses, and their repeated losses of functions commonly associated with parasitism.

However, even with the broad availability of genomic techniques, studies of the molecular aspects of plastid evolution have been largely concentrated on primary plastid-bearing plants and algae, as well as secondary plastid-bearing parasites, such as Apicomplexa. In contrast, the free-living algae carrying complex plastids, documented as the key contributors to oceanic primary production, remain poorly understood from the molecular evolutionary standpoint.

To fill this void, we investigated the plastid genomes (ptDNA) of two algal lineages carrying plastids obtained in independent endosymbiotic events: Euglenophyta, which possess secondary green alga-derived plastids, and Dictyochophyceae, which possess secondary red alga-derived plastids. Having obtained a total number of sixteen complete plastid genome sequences from the representatives of the aforementioned groups, we analysed their organization, coding contents and rates of evolution, which enabled us to come to rather surprising conclusions.

We observed that in case of euglenophytes, the rate of evolution of plastid protein-coding genes does not correlate with the organization of plastid genomes. This stands in contrast with the conclusions of past studies focused on genomes of primary plastids, where the loss of inverted repeat regions was shown to correlate with substantially increased genome-wide rate of gene evolution. Furthermore, we demonstrated that the process of endosymbiotic gene transfer in dictyochophytes has continued long after the establishment of the new organelle in this lineage, leading to diversified gene content of the ptDNA in contemporary species. On the other hand, the coding contents of euglenid plastid genomes are strictly conserved, with diverse composition observed only in one gene family. Finally, we detected a possible lateral gene transfer event between the ptDNA of euglenids and dictyochophytes, involving a group II intron maturase gene previously identified only in a single euglenophyte species. These findings demonstrate that the evolutionary paths of endosymbiotic organelles and their genomes still hold major mysteries to be uncovered.

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A surprisingly diverse population of dsDNA viruses colonizes a bicosoecid with a non-standard genetic code

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The genetic code is conserved among the vast majority of living organisms; however, the massive growth of genomic and transcriptomic data in the past few years yielded the discovery of several variant codes. Except of systematic analyses of ciliate genomes, most instances describing departures from the standard genetic code in eukaryotic nuclear genomes were encountered by chance. Inspired by our own accidental discovery of novel code variants, we screened a large and phylogenetically diverse collection of transcriptome assemblies and detected a contaminant in algal transcriptome assembly with a stop-to-sense codon reassignment, specifically decoding UAG as glutamine. The contaminant was identified as the recently described marine bicosoecid *Bilabrum latius*, which our phylogenomic analysis placed as a deeply diverged sister lineage of *Cafeteria burkhardae* (previously misidentified as *C. roenbergensis*). It has been described that *C. burkhardae* is infected by a nucleocytoplasmic large DNA virus (NCLDV) and its specific virophage called Cafeteriavirus-dependent mavirus. Virophages, small dsDNA viruses, parasitize on giant viruses of the NCLDV group, and, as has been shown for Mavirus, can

provide an adaptive anti-giant virus defense mechanism in unicellular eukaryotes. Besides that, a more recent study has shown that the genome of *C. burkhardae* is also colonized by numerous endogenous mavirus-like elements (EMALEs). Motivated by these finding, we scanned our genome assemblies of *B. latius* for the presence of endogenous viral elements (EVEs). Our analyses unveiled an abundant population of EVEs reminiscent of EMALEs that, remarkably, have adapted to the biology of their host by employing UAG as a glutamine codon. The EVEs in the *B. latius* genome represent two different viral subgroups. Most of them correspond to virophages (formally classified as the family Lavidaviridae, or most recently as the class Maveriviricetes) like the *C. burkhardae*'s EMALEs, but display a larger phylogenetic diversity when compared to the EMALEs. The second subgroup of EVEs in the *B. latius* genome consists of only two elements united by closely related genes of the virion morphogenesis module, yet differing by their replication module. Notably, this pair of *B. latius* EVEs represents a novel isolated subgroup of polinton-like viruses (PLVs) not closely related to any of the thousands of PLVs or PLV-derived EVEs found in other taxa. These findings suggest that the community of viruses associated with *B. latius* is broader that the one associated with its relative *C. burkhardae*, questioning the notion put forth by others that an altered genetic code may serve as a mechanism protecting the organism against viruses. Furthermore, the presence of the putative provirophages points to the existence of a NCLDV virus hosted by *B. latius*, which should, by definition, exhibit the same genetic code adaptation.

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Unveiling the diversity of the anaerobic class Odontostomatea

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Anaerobic ciliates thrive in sulfidic, microoxic habitats and have been studied for over a century. Because of the difficulty in their cultivation and the limited methods available at the time, most original descriptions were based only on observations of living cells from fresh samples, which lead to imprecise morphological characterizations and underestimation of their species richness. In the last two decades, details of the morphology and phylogenetic position of anaerobic ciliates from several different lineages have been revealed (e.g., Metopida, Clevelandellida, Caenomorphidae, Muranotrichea, Parablepharismea, Plagiopylea) showing enormous diversity of these groups. Nevertheless, the well-known anaerobic class Odontostomatea has been largely overlooked, likely due to their highly divergent 18S rRNA gene, which makes it difficult to sequence using universal eukaryotic primers. So far, only three of the more than 20 described species, *Saprodinium dentatum*, *Discomorphella pedroeneasi*, and *Mylestoma* sp., have been studied using modern morphological and molecular methods. We sampled mud sediments from freshwater, brackish, and marine habitats, established cultures, and partially unveiled the diversity of the class Odontostomatea through morphology based on in vivo, silver-impregnated specimens, and electron microscopy. Due, in large part, to the design of more taxon-specific primers for several groups of odontostomatids, we successfully conducted phylogenetic analyses of previously unstudied species and genera based on partial 18S rRNA gene sequences, and subsequently improved also their morphological characterization. We also studied the symbiotic archaeal methanogens of particular species using molecular and microscopical methods, such as TEM, FISH, and Sanger sequencing.

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The early evolution of dinoflagellates in the light of transcriptome data from *Eudubosquella* and *Ichthyodinium*

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The earliest environmental surveys of the eukaryotic 18S ribosomal RNA gene revealed a remarkable diversity of unidentified taxa related to dinoflagellate algae. One of the unidentified sequence clusters, called MALV-I (or MAG-I), has consistently come up as a major component of ocean planktonic samples. Later studies showed that MALV-I clade includes two distinct endoparasites of marine protists and fish embryos - *Euduboscquella* and *Ichthyodinium*. The two parasitic species lack stable laboratory cultures and have no sequence data available other than the 18S rRNA gene. To better understand their biology, we sequenced transcriptomes from four *Euduboscquella* cells and a transient *Ichthyodinium* culture. We discuss how these data contribute to our understanding of the MALV-I relationship to dinoflagellates and their molecular make-up: proteins involved in DNA condensation, metabolism in non-photosynthetic plastids, predicted host interactions and other important evolutionary traits in one of the most abundant parasitic groups in the oceans.

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The guided entry of Tail-anchored proteins pathway in *Giardia intestinalis*

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Approximately one-fourth of all cellular proteins represent integral membrane proteins. Their transport requires precise timing and regulation to reach their final destination. Tail anchored (TA) proteins represent a special class of integral membrane proteins characterized by the presence of a single C-terminal transmembrane domain that contains the targeting information same as anchors TA proteins to organelle membranes. This topology enables TA proteins to mediate interaction among the compartments in processes such as vesicular transport, apoptosis and protein translocation. Most of the TA proteins are targeted post-translationally to the endoplasmic reticulum membrane by the Guided Entry of TA proteins (GET) pathway, which is well studied in mammals and yeast. Some of GET pathway components were identified in plants and recently in *Plasmodium falciparum* and *Giardia intestinalis*. We identified all missing components of the GET pathway in *Giardia intestinalis*, including divergent Bag6 homolog, which was considered to be present only in metazoans until now. Our data show that last eukaryotic common ancestor (LECA) possessed all the GET pathway components (Sgt2, Get1-Get5, Bag6) and therefore Bag6 was secondarily lost in some lineages of eukaryotes including yeast. Moreover, the interactome of *Giardia* GET components revealed the involvement of GET proteins in the

other cellular pathways such as protein degradation. Our results from both experimental and bioinformatical studies suggest that the GET pathway is an ancestral eukaryotic pathway and has ancient and crucial role in cellular function.

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Implementation of live-cell imaging tool to visualize *Trichomonas vaginalis*

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Anaerobic protist *Trichomonas vaginalis* is a medically challenging human parasite that causes sexually transmitted disease. To study the pathogenicity and virulence of this parasite, we used a transfection system for the expression of tagged recombinant protein of interest and more recent methods of reverse genetics. Currently, the monitoring of phenotypes in trichomonads and other anaerobes on the level of fluorescence microscopy is dependent on studies of fixed cells with anti-tag antibody detection. The live-cell imaging is a challenging issue because broadly employed green fluorescent protein (GFP) and its derivatives require oxygen molecules for fluorochrome maturation and cannot be used for anaerobic parasites. Previously, we tested the haloalkane dehalogenase-based Halo tag, however, in trichomonads, this system suffers from very fast bleaching. Here we established new tools, which allow live-cell imaging studies in anaerobic eukaryotes based on yellow fluorescence-activating and absorption shifting tag (Y-FAST). This system does not require oxygen molecules, but it binds with the chemical substrates such as 4-hydroxy-3-methylbenzylidine-rhodanine (HMBR) or 4-hydroxybenzylidene-rhodanine (HBR) for fluorescence. This substrate is highly target-specific and non-toxic for cells. With this approach, we have visualized hydrogenosomes, endoplasmic reticulum, cytoskeleton, and lysosomes using specific proteins targeted to these compartments. Also, native promoter and linker (2xGGGGS) was helpful for some of the protein of interest to visualize in the expected place and have correct folding. Thus, we established and optimized the protocol for live-cell imaging of *T. vaginalis* using oxygen-independent labeling.

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Deciphering modification of 3' termini in secondary plastids of euglenids

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Secondary plastid of euglenids exhibit various unorthodox features. For example, in *Euglena gracilis*, polyadenylation of small portion of plastid transcripts was documented by Zahonova et al. (2014). This is so far the only documented polyadenylation of transcripts in secondary plastid. In our project we aim on understanding the molecular mechanism underlying this type of modification in *Euglena* plastid transcripts. We have identified four proteins, putatively involved in 3´terminus mRNA modifications, in plastid proteome of *E. gracilis* (Novak Vanclová et al., 2020) and one more

putative mRNA 3´end modifying enzyme in *E. gracilis* transcriptome. Each of the identified genes is nuclear encoded and possesses typical plastid targeting pre-sequence. In the same time cytoplasmic targeted versions of these genes were identified, mostly (with one exception) encoded as separate genes. Specifically, we have found: 1. one gene for poly(A)-specific ribonuclease (PARN), in which the plastid-targeting pre-sequence is added via alternative splicing; 2. one gene for polynucleotide phosphorylase (PNPase); 3. one for phosphodiesterase (PDE12), and 4. two genes coding for TRF family ribonucleotidyl transferases, ptTNT1 and ptTNT2. All of these genes, except for ptTNT2, were found in *E. longa* transcriptome. We have performed knock-out (KO) of the PDE12, PARN, PNPase, ptTNT1 and ptTNT2 by CRISPR/Cas9 method. The phenotype of homozygous KO mutants in ptTNT1 or ptTNT2 is macroscopically recognizable, cultures being yellowish instead of green. The phenotype of PNPase mutants is even more profound – the culture being almost whiteish in colour. Interestingly, homozygous KO mutants in PDE12 or PARN, or KO mutant in control gene GSL2 do not exhibit such phenotype. Moreover, analysis of ptTNT1 and ptTNT2 KO mutants showed that they are not able to perform photosynthesis in vivo. Thus, ptTNT1 and ptTNT2 seem to play a role in proper function of *E. gracilis* plastid either by direct modification of plastid transcript 3´termini or indirectly. The same might be true for PNPase. Currently we are working on understanding the observed phenotypes.

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Syntrophic symbioses in anoxia: Studying freshwater metopid ciliates and their methanogenic endosymbionts

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Anaerobic ciliates are predominant bacterivores in oxygen-depleted environments, playing an indispensable role in microbial food webs. Importantly, most if not all, anaerobic ciliates harbor prokaryotic symbionts, including methanogenic Archaea or various Eubacteria, and thus represent a particular habitat themselves. The mitochondria of anaerobic ciliates are adapted to anoxic conditions and produce hydrogen that serves as a metabolic substrate for the symbionts. Although the existence of syntrophic relationships between anaerobic ciliates and methanogenic Archaea is well known, little is known about the symbiont identity and other aspects of the symbiosis.

We have studied 45 isolates of freshwater metopid ciliates (class Armophorea) representing 30 species. Maintenance of free-living metopids in long-term cultures has enabled to study these ecologically important symbioses in detail. We have investigated the symbioses using a variety of methods, including Sanger sequencing of the 16S rRNA gene, Illumina amplicon sequencing, fluorescence in-situ hybridization, and transmission electron microscopy. We identified the methanogenic symbionts in all of the studied isolates and were able to compare the symbiont diversity both on intra- and interspecies levels. Sanger and Illumina amplicon sequencing revealed that each of the studied ciliate strains harbor a single dominant methanogenic archaeon belonging to either *Methanobacterium* or *Methanoregula*. Fluorescence in-situ hybridization and transmission electron microscopy revealed that the symbionts are distributed all over the

host cytoplasm and found in close association with host mitochondria, further corroborating the syntrophic nature of the symbioses. The relationship between the host and the symbionts seems to be stable, the symbionts persist over generations in long-term cultures, and the ciliates do not exchange the symbionts when two species with different symbionts are co-cultured.

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Circadian rhythms and circadian clock gene homologs of secondary alga *Chromera velia*

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Most organisms on Earth are influenced by periodic changes in their environment. The circadian clock is an endogenous device that synchronizes behavior, physiology, or biochemical processes to an approximately 24-hour cycle, allowing organisms to anticipate the periodic changes of day and night. Although circadian clocks are widespread in organisms, the actual molecular components differ remarkably among the clocks of plants, animals, fungi, and prokaryotes. We examined the presence of daily activity in *Chromera velia*, the closest known photosynthetic relative of apicomplexan parasites. *Chromera velia* has a motile stadium – zoospores, which formation was described to be connected with the light period of the day. Here we showed this periodical release of zoospores that continues rhythmically for the first days in constant darkness. We also showed that the presence of blue light spectra is necessary to trigger the process of zoosporogenesis. Our genomic search identified six cryptochrome-like genes, two genes possibly related to CCA/LHY, whereas no homolog of animal, cyanobacterial or fungal circadian clock gene was found. Our results suggest that *C. velia* contains a functional circadian clock of yet unknown type.

Alastair G. B. Simpson

The enduring mystery of ‘excavates’

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Excavates are a collection of, minimally, three protist taxa that include small heterotrophic flagellates with similar suspension feeding systems incorporating a complex longitudinal groove together with a vane-bearing posterior flagellum— Discoba, metamonads and malawimonads. The question of whether excavates are monophyletic, polyphyletic, or (deeply) paraphyletic is profoundly important for understanding the overall tree and early evolutionary history of crown eukaryotes. Yet despite being articulated > 20 years ago, it remains unresolved. Most molecular phylogenetic analyses over the last decade do not recover excavates as monophyletic, yet give mutually inconsistent alternatives. Meanwhile, recent morphological examinations reinforce the idea of special homology of the typical excavate cell architecture across major excavate groups, but

are limited in scope and could be explained by either monophyly or paraphyly. The recent history of taxon discovery inspires optimism that molecular phylogenetic analyses may well be more robust in the nearer future. Meanwhile, the door is open for comparative examinations of morphology that incorporate a wider range of information.

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First whole-cell three-dimensional reconstruction of diplonemid ultrastructure and cell division

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Diplonemid protists (Euglenozoa) are among the most species-rich and abundant eukaryotes in the Worlds Ocean. In the past decade, multiple studies have focused on the diversity and ecology of diplonemids, their metabolic capabilities, and complex mitochondrial and nuclear genomes. However, to date, the knowledge on diplonemid cell structure has been limited to the use of classical transmission electron microscopy, providing rather sketchy information about the cellular architecture of this ecologically important group of organisms. Detailed knowledge of cell biology of this eukaryotic lineage is essential for correct interpretation of ecological and molecular data.

We provide the first comprehensive study of diplonemid cellular architecture as well as ultrastructure of cell division based on whole-cell 3D reconstruction of a new representative, *Lacrimia vacuolata* sp. n., assembled from serial block-face scanning electron microscopy images and complemented with light, fluorescence, and transmission electron microscopy. Out of all euglenozoans, similar research is available for two model species – parasitic *Trypanosoma brucei* (kinetoplastids) and photosynthetic *Euglena gracilis* (euglenids), which hardly represent the bulk diversity of euglenozoans, which unlike the abovementioned species, are mostly free-living phagotrophic biflagellated cells. Here, we illustrate the position, morphology, and fine structure of the feeding and flagellar apparatuses as well as common diplonemid organelles and identify peculiarities previously not reported in this clade. We further present the first ultrastructural description of diplonemid cell division, including degradation and reassembly of complex feeding and flagellar apparatuses and partitioning of organelles into daughter cells.

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The ancestral shape of the access proton path of mitochondrial ATP synthases revealed by a split subunit-a

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The passage of protons across membranes through F1Fo-ATP synthases spins their rotors and drives synthesis of ATP. While the principle of torque generation by proton transfer is known, the mechanisms and routes of proton access and release and their evolution are not fully understood. Here, we show that the entry site and path of protons in the lumenal half-channel of mitochondrial ATP synthases are largely defined by a short N-terminal alpha-helix of subunit-a. In *Trypanosoma brucei* and other Euglenozoa, the alpha-helix is part of another polypeptide chain that is a

product of subunit-a gene fragmentation. This alpha-helix and other elements forming the proton pathway are widely conserved across eukaryotes and in Alphaproteobacteria, the closest extant relatives of mitochondria, but not in other bacteria. Thus, the proton entry site, the shape of the access half-channel, and the proton transfer mechanism involving a chain of ordered water molecules, predate eukaryotes and possibly originated in the lineage from which mitochondria evolved by endosymbiosis.

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Mitochondria targeting histone in *Chromera velia*

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Histone proteins are the critical component of chromatin across eukaryotes and archaeans, serving as a molecular sprocket to pack millions of DNA nucleotide bases inside the nucleus. Recent reports on metazoans demonstrated the presence of histones in mitochondria and revealed that the histone protein involves in the regulation of gene expression in the organelles. Studying a phototrophic alveolate, *Chromera velia*, we found in the genome and transcriptome, a canonical histone H2A protein has an N-terminal presequence which resembles mitochondrial transit peptide. To investigate the subcellular localization of putative mitochondrial histones H2A (Cvel_mtH2A) in *C. velia*, we made an ectopic expression of Cvel_mtH2A fused with a green fluorescent protein (GFP) in a diatom *Phaeodactylum tricornutum*. The transgene (Cvel_mtH2A-GFP) is found co-localized with a mitochondrial marker in *P. tricornutum* under a confocal laser microscope. In contrast, an N-terminal 50 amino acids truncated of Cvel_mtH2A (ΔCvel_mtH2A-GFP) is localized in the nucleus. This suggests that the presequence of Cvel_mtH2A likely leads the protein into the mitochondrion, where it could interact with mtDNA. Currently, we are working on developing a stable transformation system in *C. velia* and a specific antibody for Cvel_mtH2A to verify the mitochondrial localization of the protein in *C. velia*. In a broad search of similar histone sequences within apicomplexans, we only detect a histone H2A and a few histone H3 in *Eimeria* that could possibly target the proteins to apicoplast and mitochondrion, respectively. Our finding shed the light on the possible organellar histones in chromerids and apicomplexan parasites.

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On the relationship between protist metabarcoding and protist metagenome-assembled genomes

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Current studies focused on protist communities are based largely on marker gene metabarcoding and whole genome analysis through metagenomics. Gaining a deeper understanding of the correspondence between the data produced by these two approaches has the potential to reveal the advantages and disadvantages inherent in each technique and to integrate information between datasets.

We investigated the correspondence between V9 metabarcoding OTUs from the 18S rRNA gene (V9 OTUs) and metagenome-assembled genomes (MAGs) from the Tara Oceans expedition (2009-2013) and made an attempt to match them based on their relative abundances across samples. We evaluated the performance of several methods for detecting correspondence between features in the compositional dataset and developed a series of controls to filter artefacts of data structure and processing. After selecting the best-performing correspondence metrics, ranking the V9 OTU-MAG matches by their proportionality/correlation coefficients and applying a set of selection criteria, we identified candidate matches between V9 OTUs and MAGs.

Our findings suggest that V9 OTUs and MAGs of some taxa can be successfully matched with one another, in which one MAG and one V9 OTU likely represent the same biological entity. However, there are also scenarios in which: i) a single MAG matches many V9 OTUs; ii) a single V9 OTU matches many MAGs; iii) a set of V9 OTUs matches a set of MAGs. Notably, different MAGs within the same genus do not necessarily all belong to the same scenario; that is, some MAGs from a genus might match only a single OTU, whereas other MAG/OTU matches might fall within one of the 3 aforementioned non-1-to-1 scenarios.

We applied thorough procedures to filter potential artefacts, permitting an interpretation of the complex relationships between MAGs and V9 OTUs in terms of the genomic and ecological diversity of the underlying biological entities that they represent. These results illustrate that it may be possible to relate the OTUs of metabarcodes with OTUs of metagenome-assembled genomes from the same samples, but that the correspondence between the two datasets can be more complex than a direct 1-to-1 OTU match.

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Wanted: Alive – Using citizen science to explore the diversity of trypanosomatids in invasive true bugs (Hemiptera: Heteroptera)

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Citizen science (i.e. public involvement in research) has proven itself to be a powerful tool in monitoring invasive or endangered species of both animals and plants. In contrast, protists have rarely been subjects of such surveys due to their small size and difficult identification. In this ongoing project, we explore the possibility of utilizing the citizen science-based monitoring program “Najdi.je” to survey trypanosomatid diversity in economically important invasive bugs *Leptoglossus occidentalis*, *Nezara viridula*, and *Halyomorpha halys*. So far, *Phytomonas serpens* and an undescribed species of *Obscuromonas* have been found in the digestive tract of *L. occidentalis*. The surprisingly frequent occurrence of *P. serpens*, a dixenous parasite of tomatoes, is particularly peculiar since its host feeds almost exclusively on conifers. In the further course of this project, we aim to continue collecting data from citizen scientists, especially after the invasive bugs become established in natural or seminatural habitats and might become infected with native species of trypanosomatids. Furthermore, we hope to elucidate the enigmatic life cycle of *P. serpens* in *L. occidentalis*.

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Trichomonas vaginalis and the vaginal microbiome: Interactions in the early steps of infection

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Trichomonas vaginalis (TV) is the most common non-viral sexually transmitted human pathogen. It feeds on vaginal epithelial cells and microbiota through phagocytosis. Subsequent destruction occurs in lysosomes, eukaryotic organelles with an acidic pH and a set of degradative hydrolases. The secretion of virulence factors by TV plays a fundamental role in the host-parasite interaction prior to phagocytosis. Active secretion of a variety of proteins has been observed in axenic TV cultures and includes phosphatases, amylases, and proteases. However, a comprehensive profile of the secretome under more natural conditions, namely in company with vaginal bacteria, remains elusive. A large subset of the healthy vaginal microbiota is comprised of Lactobacilli. Here we investigated the influence of *L. jensenii* (LJ) on the secretion of virulence factors by TV.

TV and LJ were co-cultivated for multiple time frames. Phagocytosis was confirmed through several means of microscopy including transmission electron microscopy, scanning electron microscopy, and fluorescence microscopy using Rab7 as a lysosomal marker and DAPI to visualize both LJ and TV nucleus. To analyze the trichomonad secretome upon interaction with LJ, the cells were removed and the proteins in the supernatant were analyzed by label-free quantitative mass spectrometry (MS). Gene expression levels of selected differentially secreted proteins were examined through real-time quantitative PCR. Then, selected candidates were overexpressed in TV under a strong promoter to investigate the candidates' role in TV virulence.

Microscopy suggests an active interaction of TV with LJ as phagocytosed LJ was observed in TV lysosomes. MS results indicate that a variety of peptidases and surface proteins are involved in the early steps of the interaction including Cathepsin-L like cysteine peptidases such as TvCP2, a single Leishmanolysin-like metallopeptidase, GP63-like metallopeptidases, and *Trichomonas* beta-sandwich repeat (TBSR) proteins, a heterogeneous group of surface transmembrane proteins of yet unknown function.

As decolonization of *Lactobacillus* has been observed in vaginal TV infections, our analysis aims to provide a comprehensive set of secreted proteins that may be involved in parasite-bacteria interactions and potentially responsible for the impaired microbial community.

POSTERS

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Biochemical properties, crystal structure and localization of Smolstatin, a cystatin-like stefin of the myxozoan parasite *Sphaerospora molnari*

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Parasite-driven cystatins, in addition to their internal housekeeping role in regulating protein degradation, are recognized as essential molecules involved in host-parasite interactions. These protease inhibitors have been used as effective therapeutic targets against various parasitic infections. Myxozoa, previously classified as protists, are evolutionarily ancient fish parasites belonging to the phylum Cnidaria. They possess phylogenetically

distinct cystatins of the stefin type, which are structurally similar to the cystatin-like stefins found in flukes. Here, we biochemically and structurally characterize the cystatin-like stefin of *Sphaerospora molnari* (Smolstatin), a myxozoan pathogen of the common carp *Cyprinus carpio*. Additionally, we investigate the gene expression, protein abundance, and localization in different stages of parasite development in its fish host.

Smolstatin was produced as an active recombinant protein (recSmolstatin), against which polyclonal antibodies were raised. Biochemical properties were examined using inhibitory activity assays against a set of cysteine and serine proteases. Protein abundance in different parasite stages was assessed using western blotting. The localization of smolstatin mRNA in *S. molnari*-infected carp gill tissue and in parasite blood stages was assessed by RNA in situ hybridization. The native protein localization within different parasite stages and on the blood stage surface was determined by immunoconfocal fluorescent and TEM/SEM immunogold microscopy. Protein crystallization was performed under several conditions, and data were collected at the BESSY-II synchrotron. The crystal structure was solved at a resolution of 1.99 Å.

Smolstatin is highly abundant in sporogonic parasite stages, and it localizes both to the compartment and to the surface of parasite blood stages. In spores, the protein concentrates inside the capsulogenic cells, sporoplasm, and the spore valves. RecSmolstatin effectively inhibits enzymatic activities of cysteine cathepsins, mainly cathepsin L. The protein crystallizes as a domain-swapped dimer. Smolstatin is an evolutionarily and structurally unique protein, and its role in host-parasite interactions is currently being elucidated."

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Mitochondrial calpain in *Euglena gracilis*

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Calpains are an ancient and large superfamily of cysteine proteases responsible for the cleavage and irreversible modification of a large variety of substrates. They are involved in many cellular processes and are responsible for several human pathologies; therefore, calpains have been intensively studied in humans and other mammals. However, information about calpains in protists and other microorganisms is scarce. In this study, we have investigated the presence of mitochondrial calpain (in humans, calpain 10) in *Euglena gracilis*. *Euglena gracilis* is a unicellular eukaryotic flagellate with many applications in biotechnology, cell, and evolutionary biology. We show that calpains, defined by possessing the CysPC core domain, are present in *E. gracilis*, and we found for the first time a homolog of a mitochondrial calpain in this flagellate. Investigating calpains in unicellular flagellates might bring new insights into the origin and evolution of this family of proteolytic enzymes.

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Highly variable gene arrangements in myxozoan mitochondrial DNA

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Mitochondrial (mt) gene order is generally well conserved within metazoans, including anthozoan and medusozoan cnidarians. Myxozoans, highly reduced cnidarian parasites, have been found to have fast evolution of their mt genomes, leading to problematic annotations of mt genes, as evidenced by the identification of only two protein-coding genes in *Myxobolus squamalis*. Gene order can only be documented in closely related *Kudoa* species with the identical arrangement of their mt genes. Here we present high variability in the gene order of newly obtained myxozoan mt genomes.

Prior to mtDNA extraction, the mitochondrial fraction was isolated from the four myxozoans *Myxidium lieberkuehni*, *Nephrocystidium pickii*, *Zschokkella nova*, *Sphaerospora molnari*, and *Zschokkella* sp. from *Gadus morhua*. Long reads of mtDNA were sequenced using Oxford Nanopore technology. The reads were assembled into mtDNA contigs using Flye assembler. Specific primers were designed to obtain long overlapping PCR products covering the entire mtDNA molecule. These products were sequenced using Barcode-Tagged Sequencing on an Illumina sequencer and mapped to mtDNA to correct nanopore sequencing inaccuracies.

We sequenced the whole circular mtDNA molecule of *Z. nova* and *S. molnari*, and the partial mitochondrial mtDNA molecule of *M. lieberkuehni*, *N. pickii* and *Zschokkella* sp. Because of the fast evolutionary rates, we were able to identify only a few mitochondrial protein-coding genes (cox1, cox2, nadh1, nadh2, nadh3, and cytB) and 12S and 16S ribosomal RNA genes. In contrast to the mt genomes described so far, where the genes were arranged together and away from the rest of the mtDNA molecule containing the non-coding region, the genes in the genome of *Zschokkella nova* were distributed throughout the molecule. Interestingly, the order of the protein-coding genes in the mtDNA of the closely related species *M. lieberkuehni* and *N. pickii* appears to be different according to their partial sequences. Interestingly, cox1 gene was found fragmented into three parts in *Zschokkella* sp. The absence of tRNAs, and extremely fast evolutionary rates was the common feature for all sequenced mitochondrial genomes.

In the metazoans, mtDNA gene rearrangements might have acted to rearrange the tRNAs. In the myxozoans, all tRNAs were found to be absent from the mt genome, so the reason for the translocations of their mt genes remains unknown.

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eDNA metabarcoding as a promising approach for understanding the microsporidian diversity

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Endoparasites, such as Microsporidia, are often overlooked due to their microscopic size and hidden lifestyle within their hosts. Microsporidia exhibit a remarkable diversity, with approximately 1500 species across 187 genera, infecting a wide range of hosts from protists to vertebrates, and

invading all known organ and tissue systems. Many microsporidia are host-specific parasites that have evolved and co-speciated with their original hosts, suggesting that the known species diversity represents only a fraction of their true diversity. Traditional research on parasite diversity relies on parasitological studies of hosts, which is critical for accurate species description, but can be laborious and may not reflect their full diversity. However, eDNA metabarcoding is a promising approach that can overcome this obstacle. This method involves detecting parasite DNA from host or environmental samples and we assume this method successful in detecting microsporidia due to their resistant spores that can persist in the environment and thus can be detected in eDNA.

To evaluate the effectiveness of this method for Microsporidia, we collected and isolated DNA from specimens of three invertebrate taxa reported as hosts of Microsporidia, including Bryozoa (*Fredericella sultana*), members of Trichoptera, and amphipod crustaceans (*Gammarus* spp.) from several locations. In addition, we used eDNA from aquatic sediments from Hostačovka brook in Central Bohemia, Czech Republic. We selected the V1-V3 region of SSU rDNA as the suitable marker for metabarcoding. We performed metabarcoding using specific microsporidian barcoded primers, and the resulting amplicons were paired-end sequenced on an Illumina MiSeq (250 bp). We used a bioinformatic pipeline to demultiplex reads and cluster microsporidian operational taxonomic units (OTUs), and then performed maximum likelihood analysis to reveal microsporidian OTU relationships.

Our preliminary survey of microsporidian metabarcoding revealed over 60 microsporidian OTUs in the three invertebrate taxa screened, with 14 microsporidian OTUs detected in Bryozoa, 31 in Trichoptera, and 25 in gammarids. While several OTUs were assigned to known species parasitizing the screened hosts, the majority represented unknown microsporidia. Our eDNA preliminary survey revealed 69 microsporidian OTUs in Hostačovka brook, demonstrating the potential of this method for eDNA-based diversity assessment of these parasites. Our pilot study offers a promising approach for understanding the diversity, life cycles, and host-parasite interactions of these unique parasites and can serve as a starting point for future research.

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Methanogenic symbioses in Psalteriomonadidae

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Psalteriomonadidae is a group of free-living anaerobic protists belonging to Heterolobosea, which inhabit various, mainly freshwater, anoxic sediments. Psalteriomonadids are ancestrally amoeboflagellates, but some species have lost the flagellate stage while some others have lost the amoeba stage. Three species of *Psalteriomonas*, the type genus of Psalteriomonadidae, are known to form syntrophic symbioses with methanogenic Archaea, which were thought to belong to the genus Methanobacterium (Methanobacteria: Methanobacteriales). The symbiotic methanogens presumably use hydrogen and possibly other metabolites of the host‘s mitochondrion-related organelles (hydrogenosomes), for methane production, a potent greenhouse gas. Although the symbioses of anaerobic protists and methanogens are quite common in anoxic environments, this phenomenon was almost exclusively studied in ciliates and Archamoebae. To extend the knowledge about the identity and diversity of methanogenic symbionts of psalteriomonadids, we Sanger-sequenced the partial 16S rRNA gene of the symbionts of ca. 20 strains of long-term cultivated *Psalteriomonas* spp., including several undescribed species, and verified the obtained data by Illumina amplicon sequencing. Unlike in the previous works, the symbionts were identified exclusively as members of the genus *Methanoregula* (Methanomicrobia:

Methanomicrobiales). Sanger and Illumina data consistently show a single dominant methanogen in each *Psalteriomonas* strain. Phylogenetic analysis shows distinct host-specificity of the symbionts. Methanogenic symbionts and their arrangement within the host cell were further studied using transmission electron microscopy.

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The analysis of lateral gene transfer in symbiont-bearing diplonemids

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Lateral gene transfer (LGT) is an important mechanism facilitating the acquisition of novel genes and metabolic pathways in prokaryotes and unicellular eukaryotes. The recent discovery of the presence of endosymbionts in diplonemid protists prompted us to investigate the extent of LGT in the genomes and transcriptomes of five *Rhynchopus* strains possessing symbionts. Analysis of 16S rRNA genes revealed that these bacterial symbionts belong to the Holosporaceae and Chlamydiaceae. We have performed a gene prediction and a semi-automatic phylogenetic analysis of predicted proteins. To evaluate possible LGTs, we have built phylogenetic trees for each of the protein-coding genes of *Rhynchopus* spp. Each gene with a putative LGT signal was manually confirmed and then thoroughly annotated. Finally, we recovered 151 horizontally transferred genes from prokaryotes in diplonemid genomes. Surprisingly, we have found no compelling evidence for endosymbiotic gene transfer in any of the *Rhynchopus* hosts. This may indicate that either acquisition of the symbiont was a recent event and may have acted as a speciation trigger, or that the host possesses inner mechanisms that prevent gene transfer. The majority of laterally transferred genes have been identified as enzymes, particularly hydrolases, oxidoreductases, transferases, lyases, and other enzyme classes. The metabolic pathways mainly affected are starch and sucrose metabolism, and fatty acid biosynthesis and degradation. The main sources of LGT in the observed diplonemids were the representatives belonging to marine bacterial lineages (such as α-proteobacteria, γ-proteobacteria), followed by the Terrabacteria group, the PVC group or Bacteroidetes.

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RNA viruses in the trypanosomatid genera *Blastocrithidia* and *Obscuromonas*

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Multiple instances of RNA viruses have been reported in trypanosomatids; however, due to the vast diversity of these protists, not all trypanosomatid lineages have been screened. Here we describe three groups of viruses infecting the subfamily Blastocrithidiinae, which is a monophyletic taxon comprising two genera: *Blastocrithidia* and *Obscuromonas*. Remarkably, *Blastocrithidia* spp. are the only known trypanosomatids possessing a unique genetic code where UGA stop codon has been reassigned to tryptophan and UARs code either glutamate or a stop signal. We were interested in comparing the viromes of *Obscuromonas* and *Blastocrithidia* in the context of the latter's unique genetic code, which could present a barrier for virus infection. Interestingly, no RNA viruses were found in any of the three *Blastocrithidia* isolates screened. Nevertheless, a Tombus-like endogenous viral element was detected in the genome of *B. triatomae* suggesting possible past infections with this virus. On the other hand, representatives of three distinct virus families were discovered in *Obscuromonas*: (i) Narnaviridae, broadly infecting trypanosomatids, (ii) Mitoviridae, which is the first encounter in trypanosomatids, and (iii) Qinviridae previously described only from insect metatranscriptomes. Conversely, we did not detect any Leishbunyaviridae which are rather prevalent in the family Trypanosomatidae. Consequently, our results increase our knowledge on the diversity of viruses in trypanosomatids and support the hypothesis of an immunity mechanism to RNA viruses in *Blastocrithidia* based on its deviant genetic code.

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Quantum efficiency (Fv/Fm) and performance of retained plastids in an oligotrich mixotroph and its prey

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Mixotrophic organisms have both heterotrophic and autotrophic capabilities. The mixoplankter *Strombidium rassoulzadegani* is an oligotrich ciliate with the ability to retain plastids from its phytoplankton prey. It has been shown to grow on a variety of phytoplankton, but it appears to grow best on the unicellular green alga *Tetraselmis chui* (PLY-429) and the cryptophyte *Rhodomonas lens* (RHODO). The ciliate cannot generate new plastids, and they are replaced with newly ingested ones every 24-48 hrs. Little is known about plastid photophysiology within the ciliate during that time. We present a novel way to examine the performance of the chloroplasts within the ciliate cell and its food using a Fluorescence Induction and Relaxation fluorometer (mini-FIRe) to measure the quantum yield of photochemistry in Photosystem II (Fv/Fm, i.e., quantum efficiency). We find that Fv/Fm can be used as a consistent measurement for the health of the chloroplasts in both the original phytoplankton cell and within the kleptoplastidic mixotroph. The Fv/Fm of the retained plastids was found to decline when the ciliate was deprived of replacements, suggesting a decline in the kleptoplast functionality. Further, when exposed to the known PSII inhibitor DCMU, the Fv/Fm of the kleptoplasts was reduced along with the growth rate of the mixotroph. This suggests that as Fv/Fm declines, so does the ability of the kleptoplasts to contribute to the growth of the mixotroph. This was corroborated with a light saturation curve where average growth rates across irradiances were found to correlate with Fv/Fm values further supporting the idea that Fv/Fm, as measured via the FIRe technique, can deliver information about the kleptoplast health and therefore the overall contribution of the plastids to the growth of the mixotroph.

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Green or white: Knock-in of HA-tags in plastid protein Derl-1 results in an unstable bleached phenotype of *Euglena gracilis*

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The secondary plastid of the photosynthetic protist *Euglena gracilis* is surrounded by three membranes. Hundreds of proteins with the function in the plastid are nucleus-encoded, thus the transport machinery for these proteins through the plastid envelope is needed. The translocon of *E. gracilis* seems greatly reduced in comparison to other species with secondary plastids. Of the known components of the TIC/TOC (translocon of inner/outer chloroplast membrane) complexes that are in plastids responsible for transport across the two innermost membranes, *E. gracilis* possesses only Tic21 subunit of the TIC complex and the TOC complex is probably lost. Two potential candidates for the transport across the second membrane were found in the chloroplast proteome of *Euglena gracilis* – Derlin-like pseudoproteases Derl-1 and Derl-2. These two distant homologues of the ERAD system of the endoplasmic reticulum could be the main part of the protein-conducting channel in the second plastid membrane.

We performed RNAi on *E. gracilis* genes for Derl-1 and Derl-2 and obtained knock-downs with visible yellow phenotype in both cases which points out the importance of these proteins for the function of the chloroplast. Subsequently, we accomplished the C-terminus HA-tagging in Derl-1 and obtained two clones – Derl-1-HA-A6, and Derl-1-HA-H5. Derl-1-HA-A6 contained one Derl-1 allele with a correctly inserted 2xHA-tag, in the other allele the C-terminus was modified by an extension of one amino acid and two amino acids were modified. A wild-type allele was not detected in this clone. In the Derl-1-HA-H5 clone, one allele contained a correctly inserted 1xHA tag, and the other allele remained wild-type. Derl-1-HA-A6 exhibits bleached phenotype and growth retardation after inoculation into Cramer-Myers media. This phenotype is, however, not permanent and the culture turns green completely after 8 days. Derl-1-HA-H5 shows no differences from the wild type, neither in colour nor in growth rate. Expression of tagged Derl-1 was not detected in any clone.

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Bioavailable Iron and Metabolism in *Babesia divergens*

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Transition metals are involved in numerous crucial biological processes. The most abundant metal in enzymatic processes is iron. It is crucial for almost all known organisms and is toxic in excess. Iron is an essential nutrient for parasites, and they have developed various mechanisms to acquire iron from the host. Limiting the amount of available iron and other metals is one of the mammalian defensive strategies against invading

pathogens called nutritional immunity. There are various approaches that mammals, including humans, use to limit the amount of iron available for pathogens. Generally, these mechanisms regulate iron homeostasis and prevent iron toxicity.

Babesia is a genus of parasitic protozoa that infects red blood cells in various animals, including humans. Parasites are living in a hemoglobin-rich environment, which can be used as a source of nutrients. In general, Apicomplexa can import iron by two main mechanisms: the parasites could ingest and recycle host iron-containing proteins or directly access the cytosolic labile iron pool. The molecular mechanisms underlying these iron uptake processes in *Babesia* parasites are not fully understood, and further research is needed to clarify the details of these processes. Here, we aimed to test whether *Babesia divergens* free merozoites use a reductive mechanism to take up iron from ferric complexes. *B. divergens* 2210A G2 was cultivated in a bovine erythrocyte suspension obtained from a parasite-free cow by a previously described procedure.

In total, 15 µl of pelleted merozoites were incubated with 55Fe(II)-ascorbate, 55Fe(III)-citrate and transferrin at 37 °C for 1 hr and washed 3 times by centrifugation. The level of cell-associated radioactivity was determined by liquid scintillation counting. The uptake rate for 55Fe(II)-ascorbate – 313,7±104,0 fmolFe/min per 1 µg of protein, for 55Fe(III)-citrate was 218,6±28,2 fmolFe/min and transferrin – 59,7±12,8 fmolFe/min. In cells incubated on ice, the uptake rate significantly decreased from that observed at 37 °C, demonstrating that iron uptake is an active process.

To determine whether iron acquired from ferrous ascorbate, ferric citrate and transferrin is metabolized intracellularly, we performed blue native polyacrylamide gel electrophoresis (BN-PAGE) of proteins isolated from *B. divergens* merozoites incubated as described above. The radiograph showed several distinct high-molecular-weight bands, indicating that iron was incorporated into protein complexes.

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Comparison of two differently cultivated cultures of *Euglena gracilis* using proteomic tools

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Euglena gracilis is a unicellular protozoan microorganism belonging to an extremely variable group of eukaryotic microalgae, which are an important part of aquatic ecosystems. It represents an alternative source of variable proteins, which makes it a permanent object of scientific interest. Due to its metabolic versatility, *E. gracilis* is a widely studied microalgae. Metabolic adaptability is ensured thanks to its genome, which encodes a number of enzymes. Most of these enzymes are regulated post-transcriptionally, which allows cells to quickly adapt to changes in the environment. The aim of our study was to monitor cell growth in different cultivation conditions (light and dark) and to investigate adaptation mechanism of *E. gracilis* to dark cultivation using a quantitative high-throughput proteomics. Cells were cultivated in Cramer-Myers culture medium in the light and in the dark conditions. We collected the cells after 7 days of cultivation, when they reached the lg phase. A total protein fraction was isolated from *E. gracilis* cells with a SDS-containing buffer. 76 proteins were differentially abundant by strict statistic criteria. These

proteins are involved in energy metabolism, in disease and defence mechanisms and in protein synthesis. Six proteins were detected only in cells growing in the dark. The aim of our study was to investigate the adaptive metabolism of *Euglena* in different cultivation conditions (light and dark) using quantitative high-throughput proteomics. Due to the change in cultivation conditions, we can influence the biochemical processes in the cell, which can be used for the benefit of biotechnological applications.

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This is the (distal) end (of the eukaryotic flagellum)

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The distal end, also known as the flagellar tip, is one of the most important regions of the highly evolutionary conserved eukaryotic flagellum. Despite the known functions of the flagellar tip (the site of the axonemal assembly and disassembly, IFT turnover, release of extracellular vesicles, attachment to the host, and signaling), little is known about its protein composition. We leveraged data of the Tryptag.org project available for the unique flagellated model organism *Trypanosoma brucei*, and identified 75 proteins localizing to its flagellar tip. Some of the identified tip proteins are specific for kinetoplastids, while others have orthologs in other eukaryotic flagellated organisms. Interestingly, we found that 2 of mammalian orthologs localized to the tip of a mammalian cilium, suggesting a conserved role for these proteins across eukaryotes. We then performed a comprehensive characterization of the flagellar tip proteins in *T. brucei*, including their detailed localization, and assessment of their function by measurements of flagellar length, swimming velocity, and growth rates on RNAi depleted cultures. Our findings show that there are proteins that promote flagellar growth, limit the growth of mature flagella, or limit the growth of all flagella. In addition, we assessed which of the flagellar tip proteins in *T. brucei* are structural, which is an important step toward understanding the flagellar tip structure (for more information see the poster of M. Zelená). In summary, our study provides a comprehensive characterization of the complete set of flagellar tip proteins in *T. brucei* and sheds light on the molecular mechanisms underlying the processes occurring in this crucial region of the eukaryotic flagellum. Hence, this is the end of the initial characterization of the flagellar tip in *Trypanosoma brucei*.

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Nuclear genome of *Cafileria marina*

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Unlike autotrophic stramenopiles, heterotrophic representatives from this group are mostly overlooked and underrepresented in phylogenetic studies and databases despite the fact that heterotrophic flagellates play important

role in aquatic ecosystems serving as nutrient remineralizers by bacterial consumption.
Cafileria marina is a novel bacteriophagous representative of one of heterotrophic stramenopile basal groups from family Bicosoecida (Heterokonta). Examination of *C. marina* ultrastructure revealed tight connection between mitochondria and nucleus which is rather unusual and unique phenomenon. Our previous work on *C. marina* mitochondrial DNA sequencing and mitochondrially encoded tRNAs gave us some ideas about the reason of the mitochondria and nucleus connection but without nuclear genome we could not move forward.

Therefore, to better understand the full conjunction between these two compartments we have decided to sequence whole nuclear genome. However, isolation of pure genomic DNA was challenging due to the nature of cultivation process because *C. marina* requires continuous co-cultivation with bacteria as its food source. Moreover, physical size of the bicosoecid is quite similar to the bacteria and prevented us from sufficient separation of the desired sample before DNA isolation. To overcome these problems we have took advantage of eukaryote-specific methylations and fished out the eukaryotic DNA from the mixed isolation using methylation specific binding magnetic beads. The main advantage of this isolation is a quick and easy protocol that selects DNA fragments greater than 15kb, thus the DNA sequencing can be run on any Illumina platform; moreover, the length of the DNA molecules can also be sequenced by Nanopore technology.

This successful approach of the eukaryotic DNA enrichment and isolation provided 30–50% eukaryote-specific sequencing reads. In order to remove all remaining bacterial contamination we have devised custom pipeline that allowed us to assemble first clean draft of nuclear genome usable for annotation. Evaluation of the genome by BUSCO revealed that our draft assembly is on par with closest known representative genome of *Cafeteria roenbergensis* both on completeness and number of annotated genes and therefore suitable for phylogenomics. This analysis confirmed position of *C. marina* and could allow us to get more insight into unique features of *C. marina* in future.

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Inside the gut: An unexpected genetic diversity of morphologically uniform ciliates of family Nyctotheridae (Armophorea)

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The intestines of almost every animal are swarming with immense numbers of anaerobic protists. Among them, ciliates are usually the first to catch the eye of anyone exploring that diverse fauna mainly because of their size (some are even macroscopic) and sometimes bizarre shapes. One group of intestinal ciliates, Nyctotheridae (Ciliophora: Intramacronucleata: Armophorea: Clevelandellida) is well known for its hydrogen-producing mitochondrion-related organelles that provide ATP for the cell along with hydrogen and other waste products utilized by various endosymbiotic methanogens. Despite their notoriety, information about the diversity, phylogeny, and host specificity of Nyctotheridae is surprisingly scarce. The goal of this project is to extend our knowledge of morphological and molecular diversity of Nyctotheridae from invertebrates. We started by sampling ciliates from cockroaches housed in the collection owned by our faculty and we are currently broadening the range of hosts to other insect groups and various other invertebrates. We study protargol-impregnated specimens to determine morphology of the ciliates and we use Sanger sequencing of the 18S rRNA, ITS1-5.8S-ITS2 and 28S rRNA genes for phylogenetic analyses. Early results show that the genus *Nyctotherus*, despite being morphologically quite uniform, is very diverse genetically and it is not as host specific as previously thought. However, the majority of its species have been described only based on morphology and host organism. We plan to use morphological data from our preparations to identify the known ciliates to species level and molecular data to show the phylogeny of the group, providing a more comprehensive picture of the family Nyctotheridae.

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Cnidarians in South Bohemia? Revealing hidden diversity on the Malše River with eDNA metabarcoding

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Myxozoa represent a unique group of endoparasites that mainly infest fish and whose diversity is still largely unexplored. Traditionally used detection methods requiring investigation of host tissue are an invasive technique that encounters difficulties such as access to rare, threatened, or endangered host species or the seasonal occurrence of Myxozoa in the host. The eDNA metabarcoding of infectious myxozoan spore stages present for transmission in the aquatic environment is a promising non-invasive method for assessing myxozoan biodiversity at a given site.

We performed eDNA analysis on both water and sediment samples from 15 localities along the course of the Malše River. Using nine myxozoan clade-specific and barcoded primer sets targeted on the V4 region of SSU rDNA we generated amplicons that were pooled and used for high-throughput sequencing (Illumina 250bp, pair-end). The primer sets are designed to amplify the entire diversity of the oligochaete-infecting freshwater myxozoans. We used a bioinformatics pipeline to demultiplex our samples and generate sets of unique sequences with an OTU radius of 3%. Phylogenetic trees were constructed using Bayesian inference and maximum likelihood.

We detected a high myxozoan diversity on the Malše river. Based on the metabarcoding approach and seasonal sampling we compared the biodiversity of myxozoans on the upper and lower course with taking seasonality into account. In the entire river course, 136 myxozoan OTUs were detected within 9 taxonomic groups. A subsequent phylogenetic analysis of gained OTUs revealed their phylogenetic relationships, diversity and varying distribution of each group of freshwater myxozoans across the river. A comparison of myxozoan diversity at each site using different variables (such as abundance, number of all OTUs or captures) revealed interesting patterns in the distribution of each myxozoan group, which are influenced by human activities, among other factors.

Our results show that our metabarcoding eDNA analysis is a powerful tool for the non-invasive and sensitive detection of myxozoan biodiversity suitable for large-scale monitoring applications. This suggests that only the tip of the iceberg of myxozoan diversity may be known and that myxozoans represent the most diverse cnidarian group even in places where we wouldn't expect such enormous biodiversity.

Daryna Zavadska, Daniel J. Richter

Hypotheses on the (co)evolution of nuclear and mitochondrial genome features in kinetoplastids

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Kinetoplastids are a group of euglenozoans with peculiar nuclear genome features: high gene density, few canonical spliceosomal introns, and polycistronic gene clusters [DOI: 10.1038/s41598-021-82369-z, DOI: 10.1016/j.pt.2020.10.001, DOI: 10.1016/j.pt.2006.02.006]. Kinetoplastids are also well known for the complex maintenance and organisation of their mitogenome with a huge kinetoplast DNA (kDNA) content. Here, we would like to discuss hypotheses on the evolution and function of mitochondrial and nuclear genomes of kinetoplastids and approaches to test them.

1.1. Reduction in the number of introns and denser gene arrangement is a result of natural selection for reduced genome size. Specific reduction in the size and number of non-coding sequences relative to coding sequences might point to the presence of selective pressure acting to reduce overall genome size.

We plan to obtain transcriptomes of diverse kinetoplastid representatives. Transcriptome data combined with the estimates of genomic DNA content (see 1.2) will allow us to estimate the proportion of coding sequences in their nuclear genomes. Knowing the phylogeny of the sequenced representatives and evolutionary distances among them, we will estimate the rate of coding versus non-coding region losses in kinetoplastids and compare them to the rates in other lineages that are known for having extremely expanded or reduced genomes.

1.2. Natural selection for reduced genome size can be connected with the presence of kDNA in the lineage.

Measuring the DNA content in diverse kinetoplastids would allow us to test whether there is any correlation between the DNA content of the nuclear and mitochondrial genomes. Combined with the data on the relative fraction of non-coding nuclear DNA (1.1), we could test whether the genome reduction at the expense of non-coding nuclear DNA could have been the evolutionary compensation for the increase in kDNA content.

2. kDNA content and functionality depends on nutritional status.

We plan to investigate the functional or structural changes in kDNA of kinetoplastid cultures grown in a defined medium depleted of phosphorus/nitrogen-containing nutrients. Comparing these observations to cultures grown in nutrient-replete medium and following changes over a time course could shed light on the functional plasticity of kDNA and its hypothetical structural or resource storage functions (as proposed in [DOI: 10.1002/iub.1894]).

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Virulence factors of pathogenic trichomonas: The role of proteins with cadherin repeats

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Trichomonas vaginalis is the most prevalent non-viral sexually transmitted human pathogen. It possesses various virulence factors needed for host infection. Among these are numerous adhesins, which are indispensable for the first step of infection. Adhesins are located on the trichomonad surface and they are released in exosomes, or directly to the cell microenvironment (secretome). Among these are cadherin domain containing *Trichomonas vaginalis* beta sheet repeat proteins (TBSRs). Cadherin family proteins can form homo and heterotypic interactions, which makes them potentially important for both host-pathogen and pathogen-pathogen interaction. Importantly, they are upregulated upon contact of trichomonads with human ectocervical cells as well as with lactobacilli. These observations suggest their role in host but also lactobacilli cell interactions. Furthermore, cadherin proteins associate with alpha and beta-catenin, which interact with the actin cytoskeleton and function as a transcription factor, respectively. Thus, TBSRs may function as adhesins as well as signalling proteins. Here we investigated their potential interacting partners using bioinformatical analysis

All potential TBSRs from the *Trichomonas vaginalis* genome were identified via an HMM of known TBSRs with HMMER. Potential homotypic partners were identified by searching with the same HMM model against the human proteome. Interactions were modelled via ColabFold/AlphaFold. Visualization of interacting surfaces was done in PyMOL.

Around 220 potential TBSRs were identified, from which 57 have a transmembrane domain and possess either two cadherin domains or one cadherin and one *Trichomonas vaginalis* specific beta repeat domain. Through homology search and AlphaFold/ColabFold analysis, several potential interacting partners were identified. All of the identified interactions are potentially important for *Trichomonas vaginalis* pathogenicity. However, it is necessary to experimentally validate the interacting partners.

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This is not the end, it's just the tip of the iceberg: structural analysis of *Trypanosoma brucei* flagellum tip by ExM and Cryo-ET

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The flagellum is an organelle essential for motility, infectivity and morphogenesis of the human parasite *Trypanosoma brucei* and many other protists. The assembly of the microtubule-based flagellar axoneme occurs exclusively at the distal end of the flagellum, also known as the flagellum tip. In addition to axonemal assembly, the flagellum tip is also the place of cell signaling, turnover of intraflagellar transport proteins and the release of extracellular vesicles. Two prominent structures have previously been identified at the tip of the mature flagellum in *T. brucei*: the axonemal capping structure and the microtubule plugs. Despite the biological importance of the tip, these structures remain poorly characterized.

We set out to study the composition and function of the microtubule plugs and the capping structure. Leveraging the Tryptag.org project, we have identified 75 proteins localizing to the distal tip of the trypanosome flagellum. Further characterisation revealed that 29 of these proteins are detergent-insoluble, indicating their association with the axoneme. To further sort these candidate proteins we sub-localized them with expansion microscopy (ExM). Using cryo-electron tomography (Cryo-ET) on whole cells we were able to resolve the microtubule plugs and a filamentous structure distal to the axoneme, possibly corresponding to the axonemal capping structure. Next, we plan to precisely localize the constituents of these structures by combining Cryo-ET with immunogold labelling and by performing Cryo-ET on cells depleted of individual constituents.