Differences in mitochondrial NADH dehydrogenase activities in trypanosomatids

Petra Čermáková1, Anna Madarović1, Peter Baráth2, Jana Bellová3, Vyacheslav Yurchenko3,4, and Anton Horváth1

1Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia; 2Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovakia; 3Faculty of Science, Life Science Research Centre, University of Ostrava, Ostrava, Czech Republic and 4Martsinovsky Institute of Medical Parasitology, Tropical and Vector Borne Diseases, Sechenov University, Moscow, Russia

Abstract

Complex I (NADH dehydrogenase) is the first enzyme in the respiratory chain. It catalyses the electron transfer from NADH to ubiquinone that is associated with proton pumping out of the matrix. In this study, we characterized NADH dehydrogenase activity in seven monoxenous trypanosomatid species: Blechomonas ayalai, Herpetomonas tarakana, Kentomonas soroconicus, Leptomonas seymourii, Novymonas esmeraldas, Sergeiya podlipaevi and Wallacemonas raviniae. We also investigated the subunit composition of the complex I in dixenous Phytomonas serpens, in which its presence and activity have been previously documented. In addition to P. serpens, the complex I is functionally active in N. esmeraldas and S. podlipaevi. We also identified 24–32 subunits of the complex I in individual species by using mass spectrometry. Among them, for the first time, we recognized several proteins of the mitochondrial DNA origin.

Introduction

NADH:ubiquinone oxidoreductase [EC 1.6.1.1], eukaryotic complex I, is the largest and the most complicated enzyme of the respiratory chain. Its subunits are encoded by both the nuclear and mitochondrial genomes (Chomyn et al., 1985; Walker et al., 1992). It couples the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the mitochondrial inner membrane. The proposed mechanism includes conformational changes, electrostatic interactions and water molecules that constitute proton-translocation across the mitochondrial inner membrane. The hydrophilic arm contains two enzymatically distinct regions: the N-module involved in the oxidation of NADH and subsequent electron transport, forming a tip of the arm, and the Q-module, which contains Fe–S clusters, through which electrons are transferred to ubiquinone, forming the interface between two domains. The hydrophobic P-module (composed of the ND1, ND2, ND4 and ND5 multi-protein modules taking part in the proton pumping) is embedded in the inner mitochondrial membrane (Yagi and Matsuno-Yagi, 2000; Brandt, 2006, 2013; Berriers and Szanazov, 2009). The core of this enzyme consists of 14 essential subunits that are fairly conserved across different domains of life (Gabaldón et al., 2005). Mammalian complex I additionally contains up to 32 accessory subunits that are not directly associated with energy conservation (Carroll et al., 2006; Kmita and Zickermann, 2013). These proteins may be involved in the regulation of enzymatic activity, stability of the complex or auxiliary functions, for example, the fatty acid synthesis (Janssen et al., 2006; Pereira et al., 2013). Two of the most commonly used inhibitors of the mitochondrial complex I are rotenone (stabilizing the semiquinone intermediate within the complex) and capsaicin (antagonizing either formation or release of the quinol product) (Degli Esposti, 1998; Okun et al., 1999).

In addition to complex I, another NADH dehydrogenase, NDH2, has been discovered in the mitochondria of several organisms. It catalyses the transfer of electrons from NADH to ubiquinone without pumping protons out of the matrix (Matus-Ortega et al., 2013). In extreme cases (for example, in Saccharomyces cerevisiae), the complex I is completely missing and its function is taken by the alternative dehydrogenases (Overkamp et al., 2000). Trypanosomatids (class Kinetoplastea) is a group of obligate parasitic flagellates confined exclusively to insects (monoxenous species) or transmitted by insects or annelids to vertebrates or plants (Lukeš et al., 2018; Maslov et al., 2019). Functionality of the trypanosomatid complex I has long been debated. Bioinformatics analysis identified 29 orthologue genes of the prototypical eukaryotic subunits and further 34 genes encoding unique accessory proteins in genomes of dixenous Trypanosoma brucei, T. cruzi and Leishmania major (Opperdoes and Michels, 2008; Perez et al., 2014; Opperdoes et al., 2016). These genomic data suggest that trypanosomatid complex I is composed of over 60 subunits and its molecular mass is over 2 MDa, which is twice as large as its bovine or yeast counterpart (Abdrakhmanova et al., 2004; Carroll et al., 2006). The mitochondrial DNA of
Trypanosoma spp. encodes eight complex I subunits. ND1–ND5 are orthologues to the mitochondrial subunits (which participate in protons pumping and bind ubiquinone and rotenone), while ND7–ND9 are orthologues to the nuclear-encoded subunits NDUF2 (Fe–S cluster and binding site for ubiquinone), NDUF8 (two Fe–S clusters) and NDUF53 in humans. The genes for ND4L and ND6 had been assigned to neither the mitochondrial nor to the nuclear DNA (Opperdoes and Michels, 2008). However, it has been proposed that these proteins are encoded in the mitochondria by CR3 and CR4 genes (Duarte and Tomás, 2014). Recent data demonstrated that trypanosomatids possess all the proteins necessary for NADH regeneration by complex I: those involved in electron transfer, ubiquinone binding and reduction and proton pumping. Proteomic analysis confirmed the presence of both canonical and auxiliary subunits encoded in the nuclear genome of T. brucei. It was clearly shown that the complex I subunits are organized into the high molecular weight proteins in trypanosomal mitochondria (Panigrahi et al., 2008; Acestor et al., 2011). However, none of the proteomic studies published to date has been able to detect complex I subunits encoded by the mitochondrial genome of trypanosomatids.

The importance of the complex I in trypanosomatids has been disputed. Indeed, both dyskinetoplastic Trypanosoma evansi and T. equiperdum thrive without it (Schnafer et al., 2002). The natural T. cruzi mutants with deletions in ND4, ND5 and ND7 genes showed no alterations in mitochondrial bioenergetics compared to the wild type (Carranza et al., 2009). Long-term cultivated isolates of Leishmania tarentolae and Crithidia fasciculata have lost guide RNAs for editing of ND3, ND8 and ND9 genes and no complex I activity had been detected in them (Sloof et al., 1994; Thiemann et al., 1994). Complex I is also not essential in the studied stages of T. brucei. Ablation of NDUFV1 and NDUF57 in the procyctic and bloodstream forms did not produce any effect on the detected NADH dehydrogenase activity (Verner et al., 2011; Surve et al., 2012), which was also not sensitive to the rotenone (Verner et al., 2014). Of note, the presence of alternative NDH2 has been documented in T. brucei (Fang and Beattie, 2002; Verner et al., 2013) and Phytomonas serpens (Gonzalez-Halphen and Maslov, 2004; Čermáková et al., 2007). Its elimination in both procytic and bloodstream forms of T. brucei had only a modest effect on the viability of the tested cells (Verner et al., 2013; Surve et al., 2017).

The only trypanosomatid species with essential mitochondrial complex I known to date is P. serpens (Čermáková et al., 2007). However, it lacks respiratory chain complexes III and IV (Nawathean and Maslov, 2000). The size of the complex I in that species is about 2.2 MDa and its NADH dehydrogenase activity, as well as mitochondrial membrane potential are sensitive to rotenone (Moyes and Barrabin, 2004; Verner et al., 2014). It was demonstrated that the complex contains subunits NDUF56 and NDUF59 (Čermáková et al., 2007).

Here, we investigated NADH dehydrogenase activity in P. serpens and seven monoxenous trypanosomatids: Blechomonas ayalai (Votýpka et al., 2013), Herpetomonas tarakana (Yurchenko et al., 2016), Kinetomonas sorsogonicus (Votýpka et al., 2014), Leptomonas seymouri (Wallace, 1977), Noymphonas esmeraldas (Kostyogov et al., 2016), Sergioa podlipaevi (Svobodová et al., 2007) and Wallacemonas raviniae (Kostyogov et al., 2014). We provide evidence that functional complex I is present in two more trypanosomatids. In these molecular complexes, we detected not only a majority of the nuclear DNA-encoded proteins, but (for the first time) also several subunits from the mitochondrial DNA. In all of them we also spotted the mitochondrial DNA. In all of them we also spotted the mitochondrial DNA. (Blum and Simpson, 1990).

**Materials and methods**

**Cultivation of trypanosomatids**

*Phytomonas serpens* (strain 9T) was grown at 27°C in brain heart infusion (BHI) medium (Becton, Dickinson and Co, Sparks, USA) supplemented with 10 μg mL$^{-1}$ haemin (AppliChem, Darmstadt, Germany) (Lukeš et al., 2006). *Herpetomonas tara- kana* (strain OSR18) was cultivated at 27°C in the complete M199 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 2 μg mL$^{-1}$ haemin, 10% foetal bovine serum (FBS, Biosera, Kansas City, USA), 100 U mL$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin (Sigma-Aldrich), 2 μg mL$^{-1}$ biopterin (Sigma-Aldrich) and 25 μM HEPES (AppliChem). *Blechomonas ayalai* (strain BO8-376), *K. sorsogonicus* (strain MF08-01), *L. seymouri* (strain ATCC30220), *N. esmeraldas* (strain E262.01), *S. podlipaevi* (strain CER3) and *W. raviniae* (strain Mbr-04) were cultured at 23°C in BHI medium supplemented with 10 μg mL$^{-1}$ haemin, 10% FBS, 100 U mL$^{-1}$ penicillin, 100 μg mL$^{-1}$ streptomycin.

**Preparation of mitochondrial lysate**

The mitochondria-enriched fractions from 5 × 10⁸ cells were isolated by hypotonic lysis as described elsewhere (Horváth et al., 2005). Mitochondria were re-suspended in 0.5 M aminocaproic acid and 2% (w/v) dodecyl maltoside (both AppliChem). Lysis was performed for 1 h on ice and the lysates were centrifuged for 10 min at 20 000 × g at 4°C. The supernatants were recovered, and protein concentration was determined by the Bradford assay (Bradford, 1976).

In silico analyses

The genome of T. brucei [available from the TriTrypDB (Aslett et al., 2010)] was used as a template to search for genes of nucleus-encoded complex I subunits and NDH2 in other trypanosomatid genomes – B. ayalai (Opperdoes et al., 2016), L. seymouri (Kraeva et al., 2015), N. esmeraldas (manuscript in preparation) and W. raviniae (manuscript in preparation) – using BLAST v.2.6.0+ (Camacho et al., 2009).

**NADH dehydrogenase activity assay**

NADH dehydrogenase activity was measured in 1 mL NDH buffer (50 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 0.2 mM KCN), containing 20–30 μg proteins from the mitochondrial lysates and 5 μL of 20 mM NADH (AppliChem). After the addition of 2 μL 10 mM coenzyme Q$_{1}$ (Sigma-Aldrich), the change in absorbance at 340 nm was followed for 3 min (Čermáková et al., 2019). A unit of activity was defined as the amount of enzyme that catalyses the oxidation of 1 mmol NADH per min, assuming an extinction coefficient of 6.2 L mmol$^{-1}$ cm$^{-1}$ (Gonzalez-Halphen and Maslov, 2004). Solutions of the inhibitors were freshly prepared. Capsaicin (Sigma-Aldrich) was dissolved in ethanol, rotenone (Serva, Heidelberg, Germany) and DPI (diphenyl iodonium, Sigma-Aldrich) – in dimethylsulphoxide and methanol, respectively. Rotenone and DPI were added to the assay mixture immediately before the start of the reaction, capsaicin was pre-incubated for 3 min. Native electrophoresis and in-gel activity staining methods were adapted from Zerbetto et al. (1997) and Wittig et al. (2007) and performed as described previously (Verner et al., 2014).

**In-gel digestion and mass spectrometry analysis**

Procedure was performed as previously described (Shevchenko et al., 2006). Briefly, proteins were separated by native gradient
gel, bands of interest were cut into small pieces and incubated in 100 mM ammonium bicarbonate buffer. The samples were reduced in 10 mM DTT (30 min, 56°C) and dehydrated in acetonitrile. Alklylation reaction was performed in the presence of 15 mM iodoacetamide (20 min, room temperature, dark) and samples were dehydrated as described above. For protein digestion, 500 ng of the sequencing grade trypsin (Promega, Madison, USA) and 1 mM CaCl2 were added and the samples were incubated on ice for 30 min (if digestion was incomplete, the reaction was incubated overnight at 37°C). Digested peptides were eluted with acetonitrile and dried in SpeedVac (Thermo Fisher Scientific, Waltham, USA).

For liquid chromatography–mass spectroscopy (LC-MS) analysis, the set of a Nano-trap column (Acclaim PepMap100 C18, 75 μm × 20 mm) and Nano-separation column (Acclaim PepMap C18, 75 μm × 500 mm, both Dionex, Sunnyvale, USA/Thermo Fisher Scientific) attached to the UltiMate 3000 RSLCnano system (Dionex) was used. The peptides were separated for 120 min in a 3–43% gradient of buffer B with two mobile phases used: 0.1% formic acid (v/v) (buffer A) and 80% acetonitrile (v/v) with 0.1% formic acid (buffer B). Spectral data were collected by using the Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) operating in the data-dependent mode using the Top15 strategy for the selection of precursor ions for the HCD fragmentation (Michalski et al., 2012). Obtained datasets were processed by MaxQuant v.1.5.3.30 with built-in Andromeda search engine (Cox et al., 2011). The specific parameters for searching were: carbamidomethylation (C) as permanent modification and oxidation (M) and acetyl (protein N-terminus) as variable modifications. The search was performed against protein datasets of Phytomonas sp. (Hart1), T. brucei (TREU927), T. brucei (Lister 427), L. major (Friedlin) (TriTrypDB, downloaded 10.10.2020) and against a sequence database of U insertion/deletion editing in kinetoplastid mitochondria (Simpson et al., 1998).

Results

We have characterized NADH dehydrogenase activity in P. serpens and seven monoxenous trypanosomatids. We selected species from different clades of Trypanosomatidae (Lukš et al., 2018). These are the members of the subfamilies Leishmaniinae (Kostygov and Yurchenko, 2017) (L. seymouri and N. esmeraldas), Strigomonadinae (Votýpka et al., 2014) (K. sorsogonicus), Phytomonadinae (Yurchenko et al., 2016) (H. tarakana), Blechomonadinae (Votýpka et al., 2013) (B. ayalai), as well as two genera not formally classified into any subfamily – Sergetia (Svobodová et al., 2007) and Wallacemonas (Kostygov et al., 2014). These species differ not only in host specificity (Dicyoptera, Diptera, Heteroptera or Siphonaptera), but also geographical distribution and particulars of their life cycle. Novynomonas esmeralda and K. sorsogonicus harbour endosymbiotic bacteria, which have been acquired by host species independently in evolution (Kostygov et al., 2017; Silva et al., 2018), while L. seymouri and B. ayalai are heavily infected with dsRNA viruses (Grybchuk et al., 2018a, 2018b).

In silico analyses

We examined the presence of 19 core subunits of both the membrane and peripheral domains of the complex I, whose human orthologues were identified in T. brucei (Duarte and Tomás, 2014), and an alternative pathway enzyme, NDH2, in analysed species of trypanosomatids. The genomic data were available only for four species. The genomes of B. ayalai and L. seymouri are in TriTrypDB and two genomes were sequenced by us: N. esmeralda (32 Mbp; N50 197 811 bp; 1422 scaffolds) and W. ravinae (27 Mbp; N50 58 925; 1386 scaffolds) (both unpublished data). The correspondent sequences of T. brucei TREU927 from the TriTrypDB (Aslett et al., 2010) were used as queries to search the N. esmeralda and W. ravinae assemblies with TBLASTN (v.2.6.0) (Camacho et al., 2009) using a threshold of 10−50. The obtained hits were reciprocally BLASTed against the NCBI database. In all the cases, the genes of interest were located in syntenic genomic positions. All tested genes were detected in the genomes of all analysed trypanosomatids (Table 1 and Supplementary Table 1). Of note, multiple copies for genes encoding subunits NDUF8, NDUFB10 and NDUFa12 were documented in the genome of W. ravinae.

NADH dehydrogenase activity

Mitochondrial proteins of the studied strains were separated in 2–12% clear native gradient gel and NADH dehydrogenase activity was detected by in-gel staining (Fig. 1A). In the high molecular weight range, we detected NADH dehydrogenase activity in all species tested. However, the intensity and number of active bands differed significantly. We also noticed significant differences when comparing two different types of native electrophoresis – clear native (Fig. 1A) and blue native (Fig. 1C). NADH dehydrogenase activity in the low molecular weight range was observed for K. sorsogonicus and N. esmeralda. Its molecular weight around 130 kDa could correspond to the NDH2 dimer. For distinguishing different NADH dehydrogenase activities we performed in-gel staining in the presence of 100 μM DPI (Fig. 1B), which inhibits NDH2 and incomplete complex I (Čermáková et al., 2007). In the case of analysed trypanosomatids, DPI has inhibited most of the signals – strong bands remained visible only in the samples of P. serpens, N. esmeralda and S. podlipaevi. This suggests that DPI-resistant activity in N. esmeraldas and S. podlipaevi corresponds to the complex I, as was previously shown in P. serpens (Čermáková et al., 2007).

NADH dehydrogenase activity was spectrophotometrically measured in four trypanosomatid species (selected based on either the strongest intensity of in-gel staining signal or the presence of activity in low molecular weight range – N. esmeralda, S. podlipaevi, W. ravinae and K. sorsogonicus) in the absence or presence of specific inhibitors of the eukaryotic complex I (rotenone and capsaicin) and DPI. Our data demonstrated that contribution of the complex I and NDH2 is about equal in P. serpens (Table 2). Although the inhibitory effect of rotenone and capsaicin was comparable in this species, in all other trypanosomatids rotenone did not inhibit NADH dehydrogenase activity. In addition to P. serpens, capsaicin inhibited NADH dehydrogenase activity in N. esmeralda and S. podlipaevi. A comparable degree of inhibition by capsaicin and DPI in all three species implies the presence of both the functional complex I and the alternative NDH2. Kentomonas sorsogonicus and W. ravinae were not sensitive to capsaicin, while sensitive to DPI, which inhibited over 80% NADH dehydrogenase activity in the W. ravinae and blocked it completely in K. sorsogonicus (Table 2). These results correlate with DPI sensitivity of NADH dehydrogenase determined in the gel (Fig. 1B) and do not indicate the presence of a fully functional complex I in the tested life stage of both W. ravinae and K. sorsogonicus.

Protein composition of the NADH dehydrogenase complex

Four native gel’s strips in the high molecular weight range of P. serpens, N. esmeralda and S. podlipaevi and around 130 kDa of N. esmeralda (Fig. 1C) were subjected to LC-MS analysis (Supplementary Table 2). Most of the returned hits were hypothetical proteins, yet we were able to identify 29 nuclear-encoded subunits of the P. serpens complex I and 22 and 23 subunits of...
this complex in *N. esmeraldas* and *S. podlipaevi* datasets, respectively (Table 3). All the identified subunits were localized to the complex I modules: the N-module forming the peripheral arm, the Q-module binding the ubiquinone, and ND1, ND4 ND5 modules forming the membrane part of the complex I. The only part, from which no subunit has been identified, is the ND2 module. The acyl-carrier protein NDUFAB1 (that is not part of any module) was also detected (Fig. 2, Table 3). In addition to the subunits orthologues to those in other organisms, we also recognized some additional trypanosomatid-specific complex I subunits (Duarte and Tomás, 2014) and a few other proteins that are annotated in the TriTrypDB as NDAD dehydrogenase subunits without detailed specification. In addition to the complex I proteins, we also identified nine proteins encoded in mitochondrial DNA: ND8, ND7 and several proteins that are annotated in the TriTrypDB as NADH dehydrogenases without detailed specification. In addition to the complex I proteins, we also identified nine proteins encoded in mitochondrial DNA: ND8, ND7 and three subunits of the cytochrome c oxidase and ten subunits of the succinate dehydrogenase, NDH2 and two components (E2 and E3) of the 2-oxoglutarate dehydrogenase complex in *N. esmeraldas* (Supplementary Table 2).

### Table 1. *In silico* analysis of the selected complex I genes and alternative dehydrogenase NDH2 encoded by nuclear DNA

| Species            | Homo sapiens | Trypanosoma brucei | Blechromonas ayalai | Leptomonas seymouri | Novymonas esmeraldas | Wallacemonas raviniae |
|--------------------|--------------|--------------------|--------------------|--------------------|---------------------|----------------------|
| **Membrane domain**|              |                    |                    |                    |                     |                      |
| NDUF81             | Tb927.11.7390| Baya_011_0530       | Lsey_0055_0260     | +                  | +                   |                      |
| NDUF87             | Tb927.9.16660| Baya_016_0220       | Lsey_0192_0100     | +                  | +                   |                      |
| NDUF89             | Tb927.11.15810| Baya_019_0320       | Lsey_0010_0080     | +                  | +                   |                      |
| NDUF810            | Tb927.11.9930| Baya_039_0260       | Lsey_0091_0010     | +                  | +                   | (2)                  |
| NDUF811            | Tb927.4.440  | Baya_160_0080       | Lsey_0525_0020     | +                  | +                   |                      |
| NDUFAB1            | Tb927.3.860  | Baya_111_0040       | Lsey_0115_0040     | +                  | +                   |                      |
| NDUF55             | Tb927.3.5340| Baya_092_0110       | Lsey_0041_0050     | +                  | +                   |                      |
| NDUFA6             | Tb927.10.14860| Baya_244_0010       | Lsey_0011_0010     | +                  | +                   |                      |
| NDUFA8             | Tb927.10.12930| Baya_093_0130       | Lsey_0013_0050     | +                  | +                   | (3)                  |
| NDUFA9             | Tb927.10.13620| Baya_084_0070       | Lsey_0157_0100     | +                  | +                   |                      |
| **Peripheral domain**|              |                    |                    |                    |                     |                      |
| NDUFA13            | Tb927.11.8910| Baya_029_0060       | Lsey_0071_0190     | +                  | +                   |                      |
| NDUFA12            | Tb927.9.12680| Baya_004_0460       | Lsey_0186_0050     | +                  | +                   | (2)                  |
| NDUFA5             | Tb927.10.4130| Baya_191_0090       | Lsey_0112_0100     | +                  | +                   |                      |
| NDUFA2             | Tb927.11.16870| Baya_038_0390       | Lsey_0241_0060     | +                  | +                   |                      |
| NDUFS7             | Tb927.11.1320| Baya_018_0020       | Lsey_0065_0230     | +                  | +                   |                      |
| NDUFS6             | Tb927.6.4270| Baya_060_0270       | Lsey_0209_0010     | +                  | +                   |                      |
| NDUFS5             | Tb927.10.12540| Baya_080_0190       | Lsey_0113_0150     | +                  | +                   |                      |
| NDUFV2             | Tb927.7.6350| Baya_155_0060       | Lsey_0197_0040     | +                  | +                   |                      |
| NDUFV1             | Tb927.5.450  | Baya_008_1080       | Lsey_0248_0020     | +                  | +                   |                      |
| **NDH2**           |              | Tb927.10.9440       | Baya_062_0020      | Lsey_0004_0940     | +                  |                      |

All selected genes were detected in all analysed trypanosomatid genomes. The table lists either the names of genes in the TriTrypDB that was used for *T. brucei, B. ayalai* and *L. seymouri* or the ‘+’ sign indicating the presence in unannotated databases for *N. esmeraldas* and *W. raviniae*. All genes were found in one copy, except for a few genes of *W. raviniae*, for which a higher copy number is given in parentheses. Names of *H. sapiens* orthologues are also provided.

### Discussion

As was already mentioned above long-term cultivated trypanosomatids *L. tarentolae* and *C. fasciculata* have lost ability to edit some of complex I subunits and do not possess active form of this enzyme (Sloof *et al.*, 1994; Thiemann *et al.*, 1994). It was shown for *T. brucei* that complex I is not essential for its blood-stream life form (Surve *et al.*, 2012, 2017). Complex I contributes up to 20% of the electron flux of the respiratory chain in the pro-cyclic form of *T. brucei* but it is also not essential and does not pump protons across the inner mitochondrial membrane (Verner *et al.*, 2011). The main pathways of the electrons entry into the respiratory chain appear to be the complex II (Turrens, 1989; Denicola-Seoane *et al.*, 1992) and/or the alternative enzyme NDH2 (Verner *et al.*, 2013). Although some authors conclude that NDH2 is matrix-oriented (Surve *et al.*, 2017), our previous results strongly suggest that NDH2 is oriented into the intermembrane space and therefore cannot regenerate NAD+ in the matrix (Verner *et al.*, 2013). Within the mitochondria, mitochondrial NDAD-dependent fumarate reductase, which converts fumarate to succinate, utilized by complex II, may have this function (Coustou *et al.*, 2005). The only known exception to date was *P. serpens*, in which the complex I was demonstrated to be not...
Fig. 1. In-gel NADH dehydrogenase activity staining. (A, B) Clear native and (C) blue native gradient gel; 100 μg of mitochondrial proteins from Phytomonas serpens (PS), Blechomonas ayalai (BA), Herpetomonas tarakana (HT), Kentomonas sorsogonicus (KS), Leptomonas seymouri (LS), Novymonas esmeraldas (NE), Sergeia podlipaevi (SP) and Wallacemonas raviniae (WR) were applied to each lane. The NADH dehydrogenase activity was detected without (A, C) or with (B) 100 μM DPI. The slices with NADH dehydrogenase activity from blue native gel (C) subjected to MS analysis are marked by numbers 1–4. The positions of molecular weight markers (dimer of BSA and monomer, dimer and trimer of ferritin) are indicated.

Table 2. Specific NADH dehydrogenase activity with and without inhibitors

| Species                      | Specific activity (U mg⁻¹) | Inhibitor | Inhibition (%) |
|------------------------------|----------------------------|-----------|---------------|
| **Phytomonas serpens**       | 28 ± 11                    | Rotenone  | 30 ± 4        |
|                              |                            | Capsaicin | 42 ± 6        |
|                              |                            | DPI       | 37 ± 4        |
| **Kentomonas sorsogonicus**  | 39 ± 8                     | Rotenone  | 2 ± 3         |
|                              |                            | Capsaicin | 9 ± 8         |
|                              |                            | DPI       | 100 ± 0       |
| **N. esmeraldas**            | 20 ± 9                     | Rotenone  | 9 ± 7         |
|                              |                            | Capsaicin | 34 ± 12       |
|                              |                            | DPI       | 35 ± 8        |
| **Sergeia podlipaevi**       | 27 ± 10                    | Rotenone  | 6 ± 3         |
|                              |                            | Capsaicin | 27 ± 9        |
|                              |                            | DPI       | 20 ± 4        |
| **W. raviniae**              | 110 ± 24                   | Rotenone  | 7 ± 4         |
|                              |                            | Capsaicin | 8 ± 2         |
|                              |                            | DPI       | 81 ± 13       |

NADH dehydrogenase activity was measured in the mitochondrial lysates of P. serpens, K. sorsogonicus, N. esmeraldas, S. podlipaevi and W. raviniae in the absence or presence of 10 μM rotenone, 300 μM capsaicin and 100 μM DPI. Average values and s.e. of activities and their inhibition (in %) from at least three independent biological replicated (each measured in triplicates) are presented. One unit (U) of NADH dehydrogenase activity catalyses the oxidation of 1 nmol NADH per minute. Specific activity is calculated as U mg⁻¹ of mitochondrial proteins.
only fully functional, but also the only proton pump in the respiratory chain (Nawathean and Maslov, 2000; Gonzalez-Halphen and Maslov, 2004; Čermáková et al., 2007). Our *in silico* analysis confirmed the presence of the genes encoding the complex I subunits and the alternative dehydrogenase NDH2 in the genomes of all analysed species (*B. ayalai*, *L. seymouri*, *N. esmeraldas* and *W. raviniae*). Most genes are present only in one copy, with the exception of *W. raviniae*, where some subunits are encoded by several genes. However, the mere presence of the genes encoding the complex I subunits is not equal to the functional enzymatic activity. *Leishmania tarentolae* and *C. fasciculata*, for example, also possess all the complex I subunit genes in their genomes, and yet their enzymes are not active because the subunits encoded by mitochondrial DNA are not edited (Sloof et al., 1994; Thiemann et al., 1994). Procyclic form of *T. brucei* has essentially no direct contribution of complex I to the mitochondrial membrane potential (Verner et al., 2011).

Significant differences in NADH dehydrogenase activity within the examined trypanosomatids confirm the statement that complex I is the most controversial enzyme of these parasites (Opperdoes and Michels, 2008; Duarte and Tomás, 2014). Regardless of the strong intensity of some bands, most of them

| Subunits of mitochondrial complex I detected by mass spectrometry analysis |
|-----------------------------|-----------------------------|-----------------------------|
|                             | *P. serpens*               | *N. esmeraldas*             | *S. podlipaevi*             |
| **N-module**                | NDUFV1                     | NDUFV1                     | NDUFV1                     |
|                             | NDUFA12                    | NDUFA12                    | NDUFA12                    |
|                             | NDUFV2                     | NDUFV2                     | NDUFV2                     |
|                             | NDUFA2                     | NDUFA2                     | NDUFA2                     |
|                             | NDUF51                     | NDUF51                     | NDUF51                     |
|                             | NDUFA6                     | NDUFA6                     | NDUFA6                     |
| **Q-module**                | NDUF5                      | NDUF5                      | NDUF5                      |
|                             | NDUF7                      | NDUF7                      | NDUF7                      |
|                             | NDUFA9                     | NDUFA9                     | NDUFA9                     |
|                             | NDUF8 (ND8*)               | NDUF8 (ND8*)               | NDUF8 (ND8*)               |
| **ND1-module**              | NDUFA8                     | NDUFA8                     | NDUFA8                     |
|                             | NDUFA13                    | NDUFA13                    | NDUFA13                    |
|                             | ND1*                       | ND1*                       | ND1*                       |
| **ND4-module**              | NDUFB11                    | NDUFB11                    | NDUFB11                    |
|                             | NDUFB10                    | NDUFB10                    | NDUFB10                    |
|                             | NDUFB1                     | NDUFB1                     | NDUFB1                     |
| **NDS-module**              | NDUFB7                     | NDUFB7                     | NDUFB7                     |
|                             | NDUFB9                     | NDUFB9                     | NDUFB9                     |
| **Acyl carrier protein (ACP)** | NDUFAB1                    | NDUFAB1                    | NDUFAB1                    |
|                             | NDUTB2                     | NDUTB2                     | NDUTB2                     |
|                             | NDUTB3                     | NDUTB3                     | NDUTB3                     |
|                             | NDUTB5                     | NDUTB5                     | NDUTB5                     |
|                             | NDUTB10                    | NDUTB10                    | NDUTB10                    |
|                             | NDUTB11                    | NDUTB11                    | NDUTB11                    |
|                             | NDUTB12                    | NDUTB12                    | NDUTB12                    |
|                             | NDUTB15                    | NDUTB15                    | NDUTB15                    |
|                             | NDUTB17                    | NDUTB17                    | NDUTB17                    |
|                             | NDUTB25                    | NDUTB25                    | NDUTB25                    |
|                             | NDUTB26                    | NDUTB26                    | NDUTB26                    |
|                             | NDUTB31                    | NDUTB31                    | NDUTB31                    |
| **Unique trypanosomatids accessory subunits** | | | |
|                             | NDUTB2                     | NDUTB2                     | NDUTB2                     |
|                             | NDUTB3                     | NDUTB3                     | NDUTB3                     |
|                             | NDUTB5                     | NDUTB5                     | NDUTB5                     |
|                             | NDUTB10                    | NDUTB10                    | NDUTB10                    |
|                             | NDUTB11                    | NDUTB11                    | NDUTB11                    |
|                             | NDUTB12                    | NDUTB12                    | NDUTB12                    |
|                             | NDUTB15                    | NDUTB15                    | NDUTB15                    |
|                             | NDUTB17                    | NDUTB17                    | NDUTB17                    |
|                             | NDUTB25                    | NDUTB25                    | NDUTB25                    |
|                             | NDUTB26                    | NDUTB26                    | NDUTB26                    |
|                             | NDUTB31                    | NDUTB31                    | NDUTB31                    |
| **Others**                  | Tb927.10.5500               | Tb927.10.5500               | Tb927.10.5500               |
|                             | Tb927.11.7212               | Tb927.11.7212               | Tb927.11.7212               |
|                             | Tb927.11.15440              | Tb927.11.15440              | Tb927.11.15440              |
|                             | MURF2*                     | MURF2*                     | MURF2*                     |
| **Total**                   | 32                         | 24                         | 27                         |

Distribution of identified subunits to the modules of complex I is indicated in the left column. Designation of *H. sapiens* subunits in modules and ACP rows and *T. brucei* subunits in other rows were used. Subunits encoded by mitochondrial DNA are marked with * (ND1, ND7, ND8 and MURF2).
were sensitive to 100 μM DPI, similarly to the case of *T. brucei* (Verner et al., 2011, 2014). In addition to the expected resistance to DPI of the 2.2 MDa complex of *P. serpens* (Čermáková et al., 2007), we documented a similar phenomenon only in *N. esmeraldas* and *S. podlipaevi*. However, in contrast to *P. serpens*, these species have also the DPI-resistant activity in the range of about 1.3 MDa (Fig. 1B). It appears that this lower molecular weight complex is even more stable under conditions of native electrophoresis, as its activity was shown to be slightly stronger than that of the upper band under clear native conditions (Fig. 1A) and much stronger in the blue native gel (Fig. 1C). It also differs from the lower *P. serpens* bands (~600 kDa, DPI-sensitive) in our previous studies, which were suggested to be incomplete forms of the complex I (Čermáková et al., 2007; Verner et al., 2014).

It has been suggested that the 2-oxoglutarate dehydrogenase complex may be responsible for the detected NADH dehydrogenase activity in *T. brucei*, as up to four proteins of this enzyme were localized to the activity band (Panigrahi et al., 2008; Acestor et al., 2011). Our analysis revealed only one 2-oxoglutarate dehydrogenase subunit in *P. serpens*, two in *N. esmeraldas* and none in *S. podlipaevi* together with the complex I subunits. Therefore, we concluded that 2-oxoglutarate dehydrogenase does not contribute to the NADH dehydrogenase activity in the bands that we have analysed.

In this study, we detected a NADH dehydrogenase signal in the low molecular weight range (around 130 kDa) for the first time in trypanosomatids (*K. sorsogonicus* and *N. esmeraldas*). In the yeast *Yarrowia lypolitica*, the signal in the corresponding range comes from an alternative dehydrogenase (Čermáková et al., 2007). Our results confirm that this is also the case of *N. esmeraldas*, as we have detected the NDH2 protein in this area by LC-MS analysis. Interestingly, we have also identified NDH2 in the high molecular range along with the complex I subunits in this species. This could suggest that NDH2 functions in association with other proteins. Nevertheless, we revealed it with the complex I only in *N. esmeraldas*, but not in *P. serpens* or *S. podlipaevi*. We explain this discrepancy by either species-specific peculiarities, transient nature of this protein complex, or inconsistencies in databases used for downstream analysis. For example, we used proteome of the exact species *N. esmeraldas* for *Novymonas* but had to rely on data from *P. serpens* isolate Hart1 for the analysis of our model strain, 9T.

Spectrophotometric measurement of enzyme activities is more accurate and quantifiable than in-gel staining. Among the analysed trypanosomatids, sensitivity of the complex I activity to the low and high concentrations of rotenone has been previously documented only for *P. serpens* (Moysey and Barrabin, 2004; Čermáková et al., 2007) and *T. brucei* (Beattie and Howton, 1996; Fang et al., 2001), respectively. However, high concentrations of this inhibitor were shown to evoke non-specific effects (Hernandez and Turrens, 1998). It was later demonstrated that lower rotenone concentrations do not affect the NADH dehydrogenase activity of procyclic *T. brucei*, probably because the complex I is incomplete in this organism (Verner et al., 2011, 2014). In our experiments, rotenone inhibited the NADH dehydrogenase only in *P. serpens*. This can imply that none of the tested trypanosomatids have the *P. serpens*-like complex I. However, our experiments with capsaicin (which is another specific inhibitor of the complex I) led a different conclusion. The effect of capsaicin on NADH dehydrogenase activity in *P. serpens* was comparable to that of rotenone and inversely proportionally correlated with the effect of DPI in four other investigated species. Capsaicin was not effective in *K. sorsogonicus* and *W. ravinae*, while DPI inhibited their NADH dehydrogenase activity by 80% or more. The effects of DPI and capsaicin were similar in *N. esmeraldas*, *S. podlipaevi* and *P. serpens*. The resistance to rotenone in *N. esmeraldas* and *S. podlipaevi* may be explained by possible amino acid substitutions in NDUFS2, as has been described in other organisms, i.e. a substitution Tyr144Phe leads to 4× lower sensitivity to rotenone in *Y. lypolitica* (Tocilescu et al., 2010; Angerer et al., 2012). Taken together, our data strongly indicate the presence of a fully functional complex I in *N. esmeraldas* and *S. podlipaevi*.

MS analysis of the high molecular weight NADH dehydrogenase activity bands in *P. serpens* identified 32 subunits of the complex I (29 nuclear and 3 mitochondrial DNA-encoded) (Table 3). The total number of identified subunits is much closer to that of *Bos taurus* (45 subunits) (Carroll et al., 2006) or *Y. lypolitica* (42 subunits) (Abdrakhmanova et al., 2004) than to over 60 predicted subunits for trypanosomatids (Duarte and Tomás, 2014). Nevertheless, the complex I of *Y. lypolitica* migrates at about 880 kDa, which differs from the migration at over 2 MDa for *P. serpens* (Čermáková et al., 2007) and 1.3 MDa for *N. esmeraldas* and *S. podlipaevi* (this study). This can be explained by a higher number of the involved complex I subunits in trypanosomatids, or their significantly higher molecular weight. For example, the NDUF-A6 subunit in most eukaryotes is about 15 kDa, whereas its predicted size in trypanosomatids varies from 77 to 83 kDa (Čermáková et al., 2007).

There could be several reasons why we did not detect all the complex I proteins in our analysis: (i) we used protein databases...
of the related species; (ii) we could not identify unique subunits, similarly to the case of trCOIV subunit of the complex IV (Maslov et al., 2002; Perez et al., 2014) and (iii) some predicted proteins were too short (Duarte and Tomás, 2014) or hydrophobic. A smaller number of subunits identified in N. esmeraldas and S. podlipaevi samples reflects the lower molecular weight used for the MS analysis. This complex may be depleted of some weaker-bound subunits.

Importantly, we also detected several proteins of complex I encoded by mitochondrial DNA. This is the first experimental evidence for their existence in trypanosomatids. So far, only subunits of the complexes III, IV and V have been detected (Horváth et al., 2000a, 2000b, 2002; Acestor et al., 2011; Škodová-Sveráková et al., 2015a). We identified the ND8 subunit in three analysed species, ND1 in two and ND7 only in S. podlipaevi. We also detected the MURF2 – a mitochondrial protein of unknown function (Blum and Simpson, 1990). Its co-occurrence with other subunits of the complex I in all analysed species strongly suggests that it could be another subunit of this enzyme.

Comparison of bioenergetic metabolism in several trypanosomatid species suggests that these parasites have retained all the essential genes during evolution. Their expression depends on the specific living conditions – the availability of food and host–parasite relationships (Škodová-Sveráková et al., 2015b). Data obtained in this study indicate that the same rules apply to the complex I. Its loss is not only induced by the prolonged cultivation in vitro, but also may be influenced by natural conditions in different trypanosomatid species.

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References

Abdrakhmanova A, Zickermann V, Bostina M, Radermacher M, Schagger H, Kerscher S and Brandt U (2004) Subunit composition of mitochondrial complex I from the yeast Yarrowia lipolytica. Biochimica et Biophysica Acta 1658, 148–156.

Acecor N, Žiková A, Dalley RA, Anupama A, Panigrahi AK and Stuart KD (2011) Trypanosoma brucei mitochondrial respiratory composition: organisation and organization in procyclic form. Molecular and Cellular Proteomics 10, M110096908.

Angerer H, Nasiri HR, Niedergessav V, Kerscher S, Schwabhe H and Brandt U (2012) Tracing the tail of ubiquinone in mitochondrial complex I. Biochimica et Biophysica Acta 1817, 1776–1784.

Aslett M, Aurrecoechea C, Berriman M, Brestelli J, Brunk BP, Carrington M, Deplège DP, Fischer S, Gajria B, Gao X, Gardner MJ, Gingle A, Grant G, Harb OS, Heiges M, Hertz-Fowler C, Houston R, Innamorato F, Iodice J, Kissinger JC, Kraemer E, Li W, Logan FJ, Miller JA, Mitra S, Myler PJ, Nayak V, Pennington C, Phan I, Pinney DF, Ramasamy G, Rogers MB, Roos DS, Ross C, Sivam D, Smith DF, Srinivasasamoorthy G, Stoeckert CJ, Subramanian S, Thibodeau R, Tivey A, Treutman C, Velarde G and Wang H (2010) TriTryppDB: a functional genomic resource for the Trypanosomatidae. Nucleic Acids Research 38, D457–D462.

Beattie DS and Howton MM (1996) The presence of rotenone-sensitive NADH dehydrogenase in the long slender bloodstream and the procyclic forms of Trypanosoma brucei brucei. European Journal of Biochemistry 241, 888–894.

Berrisorf JM and Sazanov LA (2009) Structural basis for the mechanism of respiratory complex I. Journal of Biological Chemistry 284, 29773–29783.

Blum B and Simpson L (1990) Guide RNAs in kinetoplast mitochondria have a non-transcribed 3’ oligo(U) tail involved in recognition of the preedited region. Cell 62, 391–397.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Brandt U (2006) Energy converting NADH:quinone oxidoreductase (complex I). Annual Review of Biochemistry 75, 69–92.

Brandt U (2013) Inside view of a giant proton pump. Angewandte Chemie 52, 7358–7360.

Camacho C, Courniouris G, Avyagan V, Ma N, Papadopoulos J, Bealer K and Madden TL (2009) BLAST+: architecture and applications. BMC Bioinformatics 10, 421.

Carranza JC, Kowaltowski AJ, Mendonca MA, de Oliveira TC, Gadela FR and Zingales B (2009) Mitochondrial bioenergetics and redox state are unaltered in Trypanosoma cruzi isolates with compromised mitochondrial complex I subunit genes. Journal of Bioenergetics and Biomembranes 41, 299–308.

Carroll J, Fearnley IM, Skehel JM, Shannon RJ, Hirst J and Walker JE (2006) Bovine complex I is a complex of 45 different subunits. Journal of Biological Chemistry 281, 32724–32727.

Čermáková P, Verner Z, Man P, Lukej J and Horváth A (2007) Characterization of the NADH:ubiquinone oxidoreductase (complex I) in the trypanosomatid Phytomonas serpens (Kinetoplastida). FEBS Journal 274, 3150–3158.

Čermáková P, Kovalinka T, Ferenczoyvá K and Horváth A (2019) Coenzyme Q2 is a universal substrate for the measurement of respiratory chain enzyme activities in trypanosomatids. Parasite 26, 17.

Chomyn A, Mariiottini P, Cleter MW, Ragan CJ, Matsuno-Yagi A, Hafezi Y, Doililtte RF and Attardi G (1985) Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. Nature 314, 592–597.

Coustou V, Bестиore S, Rivière L, Biran M, Biteau N, Franconi JM, Boshart M, Balz G and Brandt U (2005) A mitochondrial NADH-dependent fumarate reductase involved in the production of succinate excreted by procyclic Trypanosoma brucei. Journal of Biological Chemistry 280, 16559–16570.

Cox J, Neuhauser N, Michalski A, Schtelerna RA, Olsen JV and Mann M (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. Journal of Proteome Research 10, 1794–1805.

Degli Esposti M (1998) Inhibitors of NADH-ubiquinone reductase: an overview. Biochimica et Biophysica Acta 1364, 222–235.

Denicola-Seano A, Rubbo H, Prodano E and Turrens JF (1992) Succinate-dependent metabolism in Trypanosoma cruzi epimastigotes. Molecular and Biochemical Parasitology 54, 43–50.

Duarte M and Tomás AM (2014) The mitochondrial complex I of trypanosomatids – an overview of current knowledge. Journal of Bioenergetics and Biomembranes 46, 299–311.

Fang J and Beattie DS (2002) Novel FMN-containing rotenone-insensitive NADH dehydrogenase from Trypanosoma brucei brucei: isolation and characterization. Biochemistry 41, 3065–3072.

Fang J, Wang Y and Beattie DS (2001) Isolation and characterization of complex I, rotenone-sensitive NADH: ubiquinone oxidoreductase, from the procyclic forms of Trypanosoma brucei. European Journal of Biochemistry 268, 3075–3082.

Gabaldon T, Rainey D and Huyven MA (2005) Tracing the evolution of a large protein complex in the eukaryotes, NADH:ubiquinone oxidoreductase (complex I). Journal of Molecular Biology 348, 857–870.

Gonzalez-Halphen D and Maslo D (2004) NADH-ubiquinone oxidoreductase activity in the kinetoplasts of the plant trypanosomatid Phytomonas serpens. Parasitology Research 92, 341–346.
Grba DN and Hirst J (2020) Mitochondrial complex I structure reveals ordered water molecules for catalysis and proton translocation. Nature Structural & Molecular Biology 27, 892–900.

Grychuk D, Akopyants NS, Kostygov AY, Konovalov A, Lye LF, Dobson DE, Zanger H, Fasel N, Butenko A, Frolov AO, Votýpka J, d’Avila-Ley CM, Kulich P, Moravcova J, Plevka P, Rogozin IB, Serva S, Lukes J, Busuioc SM and Yurchenko V (2018) Viral discovery and diversity in trypanosomatid protozoa with a focus on relatives of the human parasite Leishmania. Proceedings of the National Academy of Sciences of the United States of America 115, E506–E515.

Grychuk D, Kostygov AY, Macedo DH, Votýpka J, Lukes J and Yurchenko V (2018b) RNA viruses in Blechomonas (Trypanosomatidae) and evolution of Leishmaniaanivirus. MBio 9, e01932–e01918.

Hernandez FR and Turrens JF (1998) Rotenone at high concentrations inhibits NADH-fumarate reductase and the mitochondrial respiratory chain of Trypanosoma brucei and T. cruzi. Molecular and Biochemical Parasitology 93, 135–137.

Horváth A, Berry EA and Maslov DA (2000a) Translation of the edited mRNA for cytochrome b in trypanosome mitochondria. Science (New York, N.Y.) 287, 1639–1640.

Horváth A, Kiang TG and Maslov DA (2000b) Detection of the mitochondrial encoded cytochrome c oxidase subunit I in the trypanosomatid protozoan Leishmania tarentolae. Evidence for translation of unedited mRNAs in the kinetoplast. Journal of Biological Chemistry 275, 17160–17165.

Horváth A, Nebohacova M, Luke J and Maslov DA (2002) Unusual poly-peptide synthesis in the kinetoplast mitochondria from Leishmania tarentolae. Identification of individual de novo translation products. Journal of Biological Chemistry 277, 7222–7230.

Horváth A, Horáková E, Dunajčíková P, Verner Z, Pravdová E, Slápetová I, Cuninová I and Lukes J (2005) Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic Trypanosoma brucei. Molecular Microbiology 58, 116–130.

Janssen RJ, Nijtmans LG, van den Heuvel LP and Smeitink JA (2006) Mitochondrial complex I: structure, function and pathology. Journal of Inherited Metabolic Disease 29, 499–515.

Kampf D and Sazanov LA (2020) The coupling mechanism of mammalian respiratory complex I. Science (New York, N.Y.) 370, eabc2409.

Kmita K and Zickermann V (2013) Accessory subunits of mitochondrial complex I. Biochemical Society Transactions 41, 1272–1279.

Kostygov AY and Yurchenko V (2017) Revised classification of the subfamily Leishmaniinae (Trypanosomatidae). Folia Parasitologica 64, 620.

Kostygov AY, Grychuk-ieremenko A, Malysheva MN, Frolov AO and Yurchenko V (2014) Molecular revision of the genus Wallaceina. Protist 165, 594–604.

Kostygov A, Dobokova E, Grychuk-ieremenko A, Vahala D, Maslov DA, Votýpka J, Lukes J and Yurchenko V (2016) Novel trypanosomatid – bacterium associations: evolution of endosymbiosis in action. MBio 7, e01985–e01915.

Kostygov AY, Butenko A, Nenarokova A, Tashyreva D, Flegontov P, Luke J and Yurchenko V (2017) Genome of Ca. Pandoraea novymonadis, an endosymbiotic bacterium of the trypanosomatid Phytomonas serpens. Journal of Proteome Research 16, 2841–2853.

Kraeva N, Butenko A, Hlaváčová J, Kostygov A, Myšková J, Grychuk D, Leštinová T, Votýpka J, Volf P, Oppeders F, Flegontov P, Lukes J and Yurchenko V (2015) Leptomonas seymouri: adaptations to the xenogenous life cycle analyzed by genome sequencing, transcriptome profiling and co-infection with Leishmania donovani. PLoS Pathogens 11, e1005127.

Lukes J, Paris Z, Regmi S, Breitling R, Mureev S, Kushnir S, Pyatkov K, J, Butenko A, Hashimi H, Maslov DA, Votýpka J and Yurchenko V (2014) Translational initiation in Trypanosoma brucei: a molecule and a complex of unique oxidoreductase complex. Molecular and Cellular Proteomics 13, 534–545.

Pereira B, Videira A and Duarte M (2013) Novel insights into the role of Neurospora crassa NDUF2, an evolutionarily conserved mitochondrial complex I assembly factor. Molecular and Cellular Biology 33, 2623–2634.

Panigrahi AK, Ziková A, Dalley RA, Acestor N, Ogata Y, Anupama A, Myler PJ and Stuart KD (2008) Mitochondrial complexes in Trypanosoma brucei: a novel complex and a unique oxidoreductase complex. Molecular and Cellular Proteomics 7, 534–545.

Silvera D, Butenko A, Votýpka J, Lukes J and Yurchenko V and Alves JMP (2015a) Identification of the mitochondrial respiratory chain of the secondary endosymbiont Blechomonas phytomonads. Mitochondrion 19, 338–349.

Schnaufer A, Domingo GJ and Stuart K (2002) Natural and induced dyskine- netotropic trypanosomatids: how to live without mitochondrial DNA. International Journal for Parasitology 32, 1071–1084.

Shevchenko A, Tomas H, Havlis J, Olsen JV and Mann M (2006) In-gel digestion of endosymbiont proteins for mass spectrometric characterization of proteins and pro- teomes. Nature Protocols 1, 2856–2860.

Silva FM, Kostygov AY, Spodarova VV, Butenko A, Tossou R, Lukes J, Yurchenko V and Alves JMP (2018) The reduced genome of Candidatus Kinetoplastibacterium sorosongicus, the endosymbiont of Kentomonas sorosongicus (Trypanosomatidae): loss of the haem-synthesis pathway. Parasitology Research 117, 1285–1293. doi: 10.1007/s00436-019-06046-7.

Simpson I, Wang SH, Thiemann OH, Alfonzo JD, Maslov DA and Avila HA (1998) U-insertion/deletion edited sequence database. Nucleic Acids Research 26, 170–176.

Skovakova I, Horvath A and Maslov DA (2015a) Identification of the endosymbiont-encoded subunit 6 of FILO ATPase in Trypanosoma brucei. Molecular and Biochemical Parasitology 201, 135–138.

Skovakova I, Verner Z, Skalicky T, Votýpka J, Horváth A and Lukes J (2015b) Lineage-specific activities of a multipotent mitochondrion of trypanosomatid flagellates. Molecular Microbiology 96, 55–67.

Sloof P, Arts GJ, van der Burg J, van der Spek H and Benne R (1994) RNA editing in mitochondria of cultured trypanosomatids: transcriptable mRNAs for NADH-dehydrogenase subunits are missing. Journal of Bioenergetics and Biomembranes 26, 193–203.

Stroud DA, Surgenor EE, Formosa LE, Reljic B, Frazier AE, Dibley MG, Osellame LD, Stait T, Beilharz TH, Thorburn DR, Salim A and Ryan MT (2016) Accessory subunits are integral for assembly and function of human mitochondrial complex I. Nature 538, 123–126.
Surve S, Heestand M, Panicucci B, Schnaufer A and Parsons M (2012) Enigmatic presence of mitochondrial complex I in Trypanosoma brucei bloodstream forms. Eukaryotic Cell 11, 183–193.

Surve SV, Jensen BC, Heestand M, Mazet M, Smith TK, Bringaud F, Parsons M and Schnaufer A (2017) NADH dehydrogenase of Trypanosoma brucei is important for efficient acetate production in bloodstream forms. Molecular and Biochemical Parasitology 211, 57–61.

Svobodová M, Zdíková L, Čepička I, Obozník M, Lukej J and Votýpka J (2007) Sergei podlipaevi gen. nov., sp. nov. (Trypanosomatidae, Kinetoplastida), a parasite of biting midges (Ceratopogonidae, Diptera). International Journal of Systematic and Evolutionary Microbiology 57, 423–432.

Thiemann OH, Maslov DA and Simpson L (1994) Disruption of RNA editing in Leishmania tarentolae by the loss of minicircle-encoded guide RNA genes. EMBO Journal 13, 5689–5700.

Tocilesco MA, Fendel U, Zwicker K, Drose S, Kerscher S and Brandt U (2010) The role of a conserved tyrosine in the 49-kDa subunit of complex I for ubiquinone binding and reduction. Biochimica et Biophysica Acta 1797, 625–632.

Turrens JF (1989) The role of succinate in the respiratory chain of Trypanosoma brucei procyclic trypomastigotes. Biochemical Journal 259, 363–366.

Verner Z, Čermáková P, Škodová I, Kriegová E, Horváth A and Lukej J (2011) Complex I (NADH:ubiquinone oxidoreductase) is active in but non-essential for procyclic Trypanosoma brucei. Molecular and Biochemical Parasitology 175, 196–200.

Verner Z, Škodová I, Poláková S, Ďurisiova-Benkovicova V, Horvath A and Lukej J (2013) Alternative NADH dehydrogenase (NDH2): intermembrane-space-facing counterpart of mitochondrial complex I in the procyclic Trypanosoma brucei. Parasitology 140, 328–337.

Verner Z, Čermáková P, Škodová I, Kovácová B, Lukej J and Horváth A (2014) Comparative analysis of respiratory chain and oxidative phosphorylation in Leishmania tarentolae, Crithidia fasciculata, Phytomonas serpens and procyclic stage of Trypanosoma brucei. Molecular and Biochemical Parasitology 193, 55–65.

Votýpka J, Suková E, Kraeva N, Ishemgulova A, Duží I, Lukej J and Yurchenko V (2013) Diversity of trypanosomatids (Kinetoplastea: Trypanosomatidae) parasitizing flies (Insecta: Siphonaptera) and description of a new genus Blechomonas Gen. n. Protist 164, 763–781.

Votýpka J, Kostygov AJ, Kraeva N, Grybchuk-ieremenko A, Tesařová M, Grybchuk D, Lukej J and Yurchenko V (2014) Kentomonas Gen. n., a new genus of endosymbiont-containing trypanosomatids of Strigonomadinae subfam. n. Protist 165, 825–838.

Walker JE, Arizmendi JM, Dupuis A, Fearnley IM, Finel M, Medd SM, Pilkington SJ, Runswick MJ and Skehel JM (1992) Sequences of 20 sub-units of NADH:ubiquinone oxidoreductase from bovine heart mitochondria. Application of a novel strategy for sequencing proteins using the polymerase chain reaction. Journal of Molecular Biology 226, 1051–1072.

Wallace FG (1977) Leptomonas seymouri sp. n. from the cotton stainer Dystercus saturellus. The Journal of Protozoology 24, 483–484.

Wittig I, Karas M and Schagger H (2007) High resolution clear native electrophoresis for in-gel functional assays and fluorescence studies of membrane protein complexes. Molecular and Cellular Proteomics 6, 1215–1225.

Yagi T and Matsumo-Yagi A (2003) The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked. Biochemistry 42, 2266–2274.

Yurchenko V, Kostygov A, Havlová J, Grybchuk-Ieremenko A, Ševčíková T, Lukej J, Ševčík J and Votýpka J (2016) Diversity of trypanosomatids in cockroaches and the description of Herpetomonas tarakana sp. n. Journal of Eukaryotic Microbiology 63, 198–209.

Zerbetto E, Vergani I and Dabbeni-Sala F (1997) Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels. Electrophoresis 18, 2059–2064.