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Lineage-specific activities of a multipotent mitochondrion of trypanosomatid flagellates

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Summary

Trypanosomatids are a very diverse group composed of monoxenous and dixenous parasites belonging to the excavate class Kinetoplastea. Here we studied the respiration of five monoxenous species (*Blechomonas ayalai*, *Herpetomonas muscarum*, *H. samuelpessoai*, *Leptomonas pyrrhocoris* and *Sergeia podlipaevi*) introduced into culture, each representing a novel yet globally distributed and/or species-rich clade, and compare them with well-studied flagellates *Trypanosoma brucei*, *Phytomonas serpens*, *Crithidia fasciculata* and *Leishmania tarentolae*. Differences in structure and activities of respiratory chain complexes, respiration and other biochemical parameters recorded under laboratory conditions reveal their substantial diversity, likely a reflection of different host environments. Phylogenetic relationships of the analysed trypanosomatids do not correlate with their biochemical parameters, with the differences within clades by far exceeding those among clades. As the *S. podlipaevi* canonical respiratory chain complexes have very low activities, we believe that its mitochondrion is utilised for purposes other than oxidative phosphorylation. Hence, the single reticulated mitochondrion of diverse trypanosomatids seems to retain multipotency, with the capacity to activate its individual components based on the host environment.

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Introduction

Trypanosomatid flagellates constitute an obligatory parasitic group within the class Kinetoplastea, which belongs to the eukaryotic supergroup Excavata (Adl *et al.*, 2012). Due to their amenability for a wide range of methods of forward and reverse genetics, as well as an early knowledge of complete genomes of the three most important species (*Trypanosoma brucei*, *T. cruzi* and *Leishmania major* or TriTryps), they serve as model parasitic protists. Initial biochemical and molecular biology data were obtained from the fast and cheap growing non-pathogenic *L. tarentolae* and *Crithidia fasciculata* (Le Trant *et al.*, 1983; Priest and Hajduk, 1992; Benne, 1993; Speijer *et al.*, 1997). However, within the last decade these species have largely been abandoned in favour of *T. brucei*, which possesses an efficient RNAi machinery (LaCount *et al.*, 2000). Many other *Trypanosoma* and *Leishmania* spp. are being sequenced, yet they are less accessible to functional studies (Ackermann *et al.*, 2012; Akhoundi *et al.*, 2013; Roellig *et al.*, 2013; Rogers *et al.*, 2014), with the strength of the comparative analysis with the related *T. brucei* being extensively used. Most research is focused at the dixenous trypanosomatids, as they are the causative agents of serious diseases of humans and economically important vertebrates and plants.

Trypanosomatids represent one of the most diverse and widespread groups of parasitic protists and are capable of infecting plants, insects, as well as probably any vertebrate host. Only relatively recently, this anticipated diversity has been systematically mapped, and trypanosomatids isolated mostly from insects originating from different continents are becoming available for molecular studies. Phylogenetic analyses indicate a subdivision of the family Trypanosomatidae into about a dozen of major clades which, however, do not have a support in morphology (Maslov *et al.*, 2013). The flagellates studied so far by biochemical and molecular methods in any detail fall into just three clades represented by the genera *Trypanosoma* and *Phytomonas* and the subfamily Leishmaniinae. Virtually no information is available for the remaining clades,

although that is where most of the trypanosomatid diversity seems to be hidden.

To change this highly dixenous species-centred knowledge, we searched for trypanosomatids isolated from insects that fulfil the following criteria: (i) belong to a clade for which no or very little biochemical and molecular data is available, (ii) are easily cultivable and grow to high concentrations, and (iii) have been formally described based on their spliced leader (SL) and 18S rRNA sequences and morphology (Maslov *et al*., 2013; Votýpka *et al*., 2013; Yurchenko *et al*., 2014). Using these criteria, we selected *Blechomonas ayalai*, *Herpetomonas muscarum*, *H. samuelpessoai*, *Leptomonas pyrrhocoris* and *Sergeia podlipaevi*, which have been isolated from heteropteran, dipteran or siphonapteran hosts. These flagellates have been subjected to a panel of methods to allow their direct comparison with trypanosomatids characterised previously. We wondered whether phylogenetic position, lifestyles and/or different hosts would be reflected in their mitochondrial (mt) metabolism.

A text-book mitochondrion utilises Krebs cycle for two purposes. Firstly, the cycle serves as the metabolic crossroad of carbohydrate and amino acid metabolism via 2-oxoglutarate (connected to alanine, aspartate and glutamate metabolism), succinyl coenzyme A (connected to valine, leucine and isoleucine degradation), fumarate (connected to arginine, proline and tyrosine metabolism) or oxaloacetate (connected to alanine, aspartate and glutamate metabolism). Secondly, the cycle is a source of reduced co-factors to feed the mt respiratory chain oxidative phosphorylation (OXPHOS) (Scheffler, 2007). A reduced nicotinamid adenine dinucleotide (NADH) is re-oxidised at complex I (= NADH dehydrogenase), a multisubunit structure capable of pumping protons across the inner mt membrane. A flavin adenine dinucleotide (FAD) is regenerated at complex II (= succinate dehydrogenase), without the translocation of protons, while mt FAD-dependent glycerol-3-phosphate dehydrogenase (mtG3PDH) facing the mt intermembrane space constitutes part of the glycerol-3-phosphate:dihydroxyacetone phosphate (G3PDH: DHAP) shuttle, connecting respiration with glycolysis. Electrons from all three enzymes are channelled to ubiquinol, from where they flow to complex III (= cytochrome *c* reductase). To translocate protons across the inner membrane, this complex employs the so-called Q-cycle with electrons being further passed to soluble cytochrome *c* located in the intermembrane space. The reduced cytochrome *c* is then re-oxidised by complex IV (= cytochrome *c* oxidase), the last proton pump in the chain that sinks electrons to molecular oxygen, giving rise to water. The translocated protons are used for either transport of various molecules or ATP synthesis by complex V (= ATP synthase) (Scheffler, 2007).

Trypanosomatid flagellates studied previously display various deviations from the aforementioned classical situation (Verner *et al*., 2013). If used at all, their Krebs cycle serves as an entry point of the metabolism of amino acids under low glucose conditions, as is the case of *C. fasciculata*, *L. tarentolae* and *T. brucei* (Bringaud *et al*., 2006), whereas *P. serpens* has been reported to completely lack this central metabolic wheel (Tielens and van Hellemond, 2009). Complex I is prone to be lost upon cultivation in glucose-rich medium as was documented in *L. tarentolae* (Sloof *et al*., 1994) and *C. fasciculata* (Speijer *et al*., 1997). Both procyclic and bloodstream stages of *T. brucei* possess complex I; however, its function in mt metabolism remains enigmatic (Verner *et al*., 2011; Surve *et al*., 2012; Duarte and Tomás, 2014). In *T. brucei*, NADH appears to be primarily oxidised by an alternative rotenone-insensitive NADH dehydrogenase (Fang and Beattie, 2003; Verner *et al*., 2013), whereas other NADH dehydrogenases are active in the laboratory strains of *C. fasciculata* and *L. tarentolae* (Verner *et al*., 2014). Complexes III and IV were completely lost by *P. serpens* (Nawathean and Maslov, 2000; Gonzáles-Halphen and Maslov, 2004), rendering the functionality of complex I essential for survival of this plant pathogen (Čermáková *et al*., 2007). To sink electrons, *P. serpens* employs an alternative oxidase, an enzyme unable to pump protons (van Hellemond *et al*., 1998; Chaudhuri *et al*., 2006). A homologue of this protein is also present in both life cycle stages of *T. brucei* [where it is called Trypanosome Alternative Oxidase (TAO)], giving rise to a branched electron transport chain (Bringaud *et al*., 2006; Tielens and van Hellemond, 2009).

Given this variability in mt respiratory chain set up, we decided to investigate its composition and activities in five monoxenous trypanosomatids belonging to novel and/or previously not studied phylogenetic clades. To characterise their mt metabolism and physiology, we have subjected them to a battery of tests, the aim of which was to assay for the presence, size and activities of individual respiratory complexes, measure the activity of mtG3PDH, oxygen consumption and sensitivity to inhibitors, as well as potential carbon source preferences, allowing us to integrate the obtained data into the framework of mt and cellular physiology. The emerging pattern is compatible with a multipotent mitochondrion, the metabolism of which has been tailored to different needs.

Results

Properties of respiratory complexes

Initially, we performed western blot analyses after standard denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A) and Blue Native (BN) gel electrophoreses (Fig. 1B), followed by in-gel

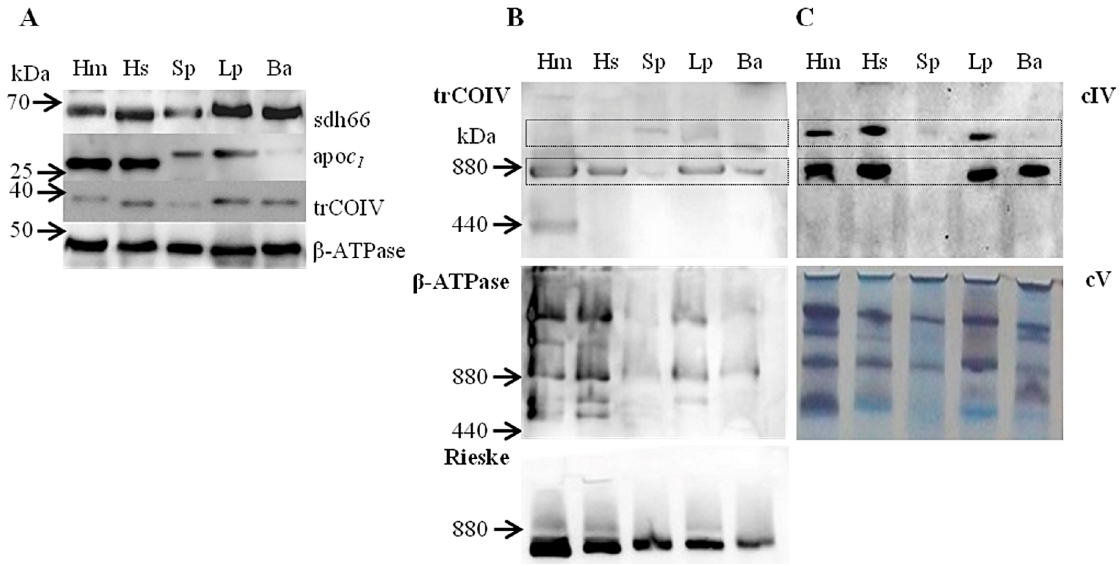

Fig. 1. Western blot analyses and histochemical activity staining.
A. Western blot analysis of the mitochondrion-enriched fractions upon a regular SDS-PAGE. Antibodies used correspond to sdh66 subunit of complex II (sdh66), apocytochrome c_1 of complex III (apoc_1), trCOIV subunit of complex IV (trCOIV) and β chain of complex V (β-ATPase).
B. Western blot analysis of the mitochondrial fractions upon Blue Native PAGE. Antibodies used correspond to Rieske Fe-S protein of complex III (Rieske), trCOIV subunit of complex IV (trCOIV) and β chain of complex V (β-ATPase).
C. Histochemically stained Blue Native PAGE for complexes IV (cIV, upper panel) and V activities (cV, lower panel). (B) and (C) panels are aligned so that the same region of the gel is visible for complexes IV and V. Areas used for quantification are framed (Suppl. Table S1).
Hm = *Herpetomonas muscarum*; Hs = *H. samuelpessoai*; Sp = *Sergeia podlipaevi*; Lp = *Leptomonas pyrrhocoris*; Ba = *Blechomonas ayalai*.

staining of activities of complexes IV and V (Fig. 1C). Immunodetection upon SDS-PAGE was successfully performed with antibodies against complexes II (sdh66), III (apo c_1), IV (trCOIV) and V (β-ATPase) (Fig. 1A), whereas analyses under native conditions were successful only with the last two antibodies. Consequently, the native western blot analysis was extended by the inclusion of an antibody against the Rieske subunit of complex III (Fig. 1B).

In all studied species SDS-PAGE confirmed the presence of all examined subunits, although with different signal intensity. Next, the results were compared with the in-gel activity staining of complexes IV and V (Fig. 1C). *B. ayalai* showed weak signals for both subunits of complex III and a single strong band in complex IV. *S. podlipaevi* gave an almost undetectable in-gel activity staining signal of complex IV accompanied by a very weak signal of trCOIV in both denatured and native gels. Surprisingly, in *S. podlipaevi* and *B. ayalai*, there was a constant discrepancy between the invariably strong β-ATPase signal in SDS-PAGE and weak corresponding signals in native gels (Fig. 1).

Combining BN-PAGE with either western blot analysis or in-gel activity staining gave us information not only about the presence, assembly and the putative formation of artificial in-gel aggregates of protein complexes but also about the activities of different forms. Although complex III is uniformly detected with an apparent molecular weight slightly below 880 kDa (Fig. 1B), complexes IV and V are usually represented by two and multiple bands respectively (Fig. 1B and C). A reliable in-gel activity staining of complex III has yet to be developed (Verner *et al.*, 2014), and thus we were unable to correlate the immunoanalysis of this complex with its activity. However, based on the presence of a single prominent band in western blot analysis upon BN gel electrophoresis, we concluded that this was the active form. Except for *S. podlipaevi* (Fig. 1C), complex IV forms an active band with a molecular weight slightly lower than 880 kDa (Fig. 1C; Suppl. Table S1A). In *H. muscarum*, *H. samuelpessoai* and *L. pyrrhocoris*, a second strong activity band was observed in the area of higher molecular weights, whereas only a very low activity appeared in the same area for *S. podlipaevi*. However, the immunodetection signal was very weak in all samples (Fig. 1B). Moreover, a weak 440 kDa signal, which may correspond to the monomer of complex IV, was detected in *H. muscarum* (Fig. 1B). This suggests that the oligomeric forms are not equally active (Fig. 1B and C; Suppl. Table S1A and B). The *S. podlipaevi* signal was the weakest one detected by both techniques.

Next, to resolve subunit composition of the detected complexes, we performed a 2D BN/SDS-PAGE. Coomassie staining upon the 2nd dimension showed that respiratory complexes of *L. pyrrhocoris* (Fig. 2), *H. samuelpessoai*, *S. podlipaevi* and *B. ayalai* (data not

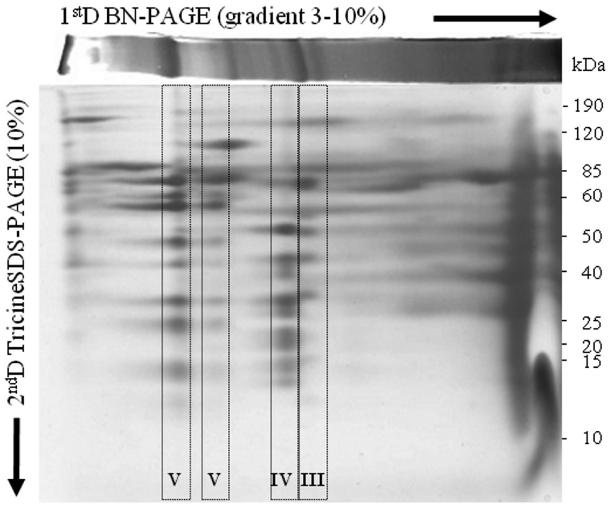

Fig. 2. Native versus denaturing 2D analysis of mitochondria-enriched fractions. Respiratory complexes in the mitochondrion-enriched fraction from *Leptomonas pyrrhocoris* resolved in a 2D gel (see *Materials and methods*) and stained with Coomassie blue. Molecular weight markers are shown to the right.

shown) are resolved in the same order as in other trypanosomatids studied so far (Verner *et al*., 2014). *H. muscarum* is the only trypanosomatid with complex IV migrating at a lower molecular weight (~ 440 kDa) than complex III (data not shown), which is represented by a single band in the 1st dimension, and forms a discrete column of subunits in an area slightly lower than 880 kDa. 2D gel analysis resolves two distinct columns of complex V, which are not equivalent in terms of the quantity of protein (Fig. 2).

Activities of respiratory enzymes

The above-mentioned methods provided information about the presence and activities of the respective enzymes. Next, we measured the specific OXPHOS enzymatic activities (mtG3PDH and complexes II–V) by a spectrophotometric approach to provide quantitative data (Table 1). Both mtG3PDH and complex II shift electrons to the respiratory chain by passing them to ubiquinone, connecting OXPHOS with glycolysis and Krebs cycle respectively. The highest mtG3PDH activity was measured in the *S. podlipaevi* lysate, which has virtually no complex II activity (Table 1). *H. samuelpessoai* has the same activity two times lower and complex II activity three times higher than the corresponding activities in *B. ayalai*, which are both relatively high. The last two flagellates, *L. pyrrhocoris* and *H. muscarum*, showed no or extremely low mtG3PDH activity, although their complex II activities were quite high (Table 1).

Complex III participates in the generation of mt membrane potential using electron flow from ubiquinone to cytochrome *c*, its activity being seemingly inversely related to mtG3PDH. Activity of this complex is low in *S. podlipaevi* and *B. ayalai*, which happen to have the highest mtG3PDH activity (Table 1). Given that complex III shuffles electrons to complex IV, which then sinks them to oxygen, one may predict a correlation between their activities. This holds true for *H. muscarum*, *H. samuelpessoai* and *L. pyrrhocoris*, which have high complex III activity. However, such correlation seems to be lost in *B. ayalai*, which has very low complex IV activity, and in *S. podlipaevi*, complex III of which shows very limited activity. Finally, the activity of complex V was determined via its capacity to hydrolyse ATP and varied substantially among studied species. Quite unexpected is its low activity in *H. muscarum*, which has high activities of the other respiratory complexes (Table 1).

Phylogenetic and clustering analyses of OXPHOS activities

We wondered whether the measured OXPHOS activities correlate with the phylogenetic positions of studied flagellates. For that purpose, the newly obtained data were combined with those available for the well-studied trypanosomatids (Verner *et al*., 2014), allowing a comparison of the hierarchical clustering. Phylogenetic topologies were

Table 1. Specific activities of OXPHOS.

Cell line	G3PDH [mU × mg^{-1}]	II [U × mg^{-1}]	III [mU × mg^{-1}]	IV [mU × mg^{-1}]	V [U × mg^{-1}]
H. muscarum	3.4 ± 2.6	92.0 ± 19.2	1142.6 ± 137.9	7.7 ± 0.8	302.1 ± 51.3
H. samuelpessoai	16.2 ± 3.4	230.0 ± 13.3	1518.1 ± 502.8	3.5 ± 0.5	1503.9 ± 264.2
S. podlipaevi	70.8 ± 14.7	2.2 ± 2.2	60.0 ± 24.6	3.1 ± 0.8	463.1 ± 53.7
L. pyrrhocoris	0.0	55.3 ± 10.0	1353.0 ± 261.8	2.7 ± 0.9	1078.6 ± 186.7
B. ayalai	31.8 ± 4.8	84.0 ± 9.8	595.8 ± 57.1	0.4 ± 0.2	424.5 ± 57.0

Average values ± standard deviation in units (U) × mg^{-1} for respiratory complexes II and V and in mU mg^{-1} for glycerol-3-phosphate dehydrogenase (G3PDH), complexes III and IV are shown. The values are calculated at least from three measurements. The U of appropriate activity is defined as an amount of enzyme required for conversion of: (i) 1 μmol of 2,6-dichlorophenolindophenol per min for G3PDH, (ii) 1 nmol of 2,6-dichlorophenolindophenol per min for complex II; (iii) 1 μmol of cytochrome *c* for complexes III and IV; (iv) 1 nmol of ATP (releasing of 1 nmol of free phosphate) per min for complex V.

inferred upon the inclusion of 18S rRNA and gGAPDH sequences from the studied flagellates into comprehensive alignments of these genes (Fig. 3). Although both examined *Herpetomonas* species belong to the well-known *Herpetomonas* clade, *S. podlipaevi* is a sole cultivable member of the recently established genus *Sergeia*, *B. ayalai* belongs to the newly erected and species-rich genus *Blechomonas*, and *L. pyrrhocoris* is a cosmopolitan representative of the subfamily Leishmaniinae (Fig. 3).

For visualisation, the measured quantitative data have been converted into a heat map. The resulting pattern differs from the phylogenetic relationships (Fig. 4A). Following analysis used the Ward method of hierarchical clustering (Fig. 4B), in addition to the single and complete linkages. None of the clustering methods gives a topology that corresponds with the phylogenetic reconstruction (Fig. 4C; Suppl. Fig. S2). In all analyses, the well-studied *T. brucei*, *C. fasciculata* and *L. tarentolae* clustered together, giving rise to a 'cold island'. This cluster contained *B. ayalai* and *H. muscarum* (Ward method), *S. podlipaevi* alone (single linkage) or *B. ayalai* and *S. podlipaevi* (complete linkage) (Fig. 4A and B; Suppl. Fig. S1).

There is a clear lack of correlation between the phylogenetic position of the analysed species and respective clustering analysis, based on the measured features of the respiratory chain (Fig. 4C). Indeed, biochemical differences within phylogenetic clades by far exceed those among clades, a situation evidenced by the scattered positions of the related species in hierarchical topologies. Both *Herpetomonas* species (*H. muscarum* and *H. samuelpessoai*) as well as representatives of the subfamily Leishmaniinae (*L. pyrrhocoris*, *C. fasciculata* and *L. tarentolae*) apparently differ more within these monophyletic groups than in-between them. Furthermore, monoxenous [M] or dixenous [D] life strategies do not correlate with the enzymatic activities. The best grouping is obtained using single linkage; however, the monoxenous *C. fasciculata* inevitably flaws the picture (Suppl. Fig. S1A).

Oxygen consumption, stimulation by different carbon sources and correlation analysis

To put the *in vitro* data into a physiological context, oxygen consumption by intact cells was measured. The highest and lowest rates of basal oxygen consumption were measured in *H. muscarum* and *S. podlipaevi* respectively (Fig. 5A). To assay whether the studied species contain active TAO, the basal respiration was inhibited using antimycin A, potassium cyanide (KCN) or salicylhydroxamic acid (SHAM), which are specific inhibitors of complexes III, IV and TAO respectively. All species were tested in a wide range of inhibitor concentrations (Suppl. Fig. S4). KCN inhibited 70–90% of oxygen consumption in *H. muscarum*, *H. samuelpessoai*, *L. pyrrhocoris and B. ayalai*, indicating that they rely mainly on the cytochrome *c*-containing pathway, on which *S. podlipaevi* is much less dependent. From all examined species, only *H. muscarum* is fully sensitive to antimycin A. An interesting situation was observed following the inhibition with SHAM, which does not have a strong effect on respiration of any analysed trypanosomatid except *L. pyrrhocoris* (Fig. 5B).

Next, we investigated whether variation in the oxygen consumption is mirrored by mt membrane potential that is created by proton-pumping complexes of the respiratory chain. However, standard measurements with tetramethylrhodamin ethylester (TMRE) did not result in any signal (data not shown), a situation so far encountered only in *C. fasciculata* (Verner *et al*., 2014). Hence, we focused on probing what pathway feeds the respiration.

We used the well-described metabolic map of *T. brucei* (Bringaud *et al*., 2006) as a starting point and measured respiration of the flagellates incubated in phosphate buffered saline (PBS) alone or in PBS supplemented either with 10 mM glucose (PBSG) or a mixture of 15 mM L-proline, L-threonine and L-glutamine (PBSA). In a pilot test, we estimated starvation time required for the cells to fully consume their inner reserves. *P. serpens* and *T. brucei*, used as controls, remained motile for up to 3 h in PBS and PBSG, and their survival was not compromised in PBSA (data not shown). To exclude the possibility that motility or survival are influenced by different osmotic pressures of these media, we demonstrate that in PBS supplemented with 45 mM sorbitol, and in PBSG with additional 35 mM sorbitol, neither motility nor survival are enhanced. Hence, we subsequently incubated all flagellates in the respiration buffers (PBS, PBSG or PBSA) at 27°C for 4 h prior to the measurement.

Most glucose-stimulated respiration was observed in *H. muscarum* and *H. samuelpessoai* with a ~3.3-fold increase in oxygen consumption (Fig. 6). Interestingly, the addition of amino acids showed a different tendency in these two species, as the respiration was markedly stimulated only in *H. samuelpessoai* (4.7×). In *L. pyrrhocoris*, the stimulatory effect of amino acids was also higher than that of glucose. Moreover, respiration of *B. ayalai* and *S. podlipaevi* remained virtually unaffected by either amino acids or glucose (Fig. 6).

Finally, we performed a statistical evaluation of collected data using Pearson's correlation coefficient (Table 2). We identified negative correlations between the activity of mtG3PDH and other measured characteristics ranging from weak (complex V, $r = -0.3$) to very strong (complex III, $r = -0.9$). All other characteristics were positively correlated with the exceptions of respiration being in mild negative correlation with complex V activity ($r = -0.6$) and amino acids ($r = -0.4$). The correlation analysis revealed an overall negative relationship between mtG3PDH and the remaining mt characteristics. The

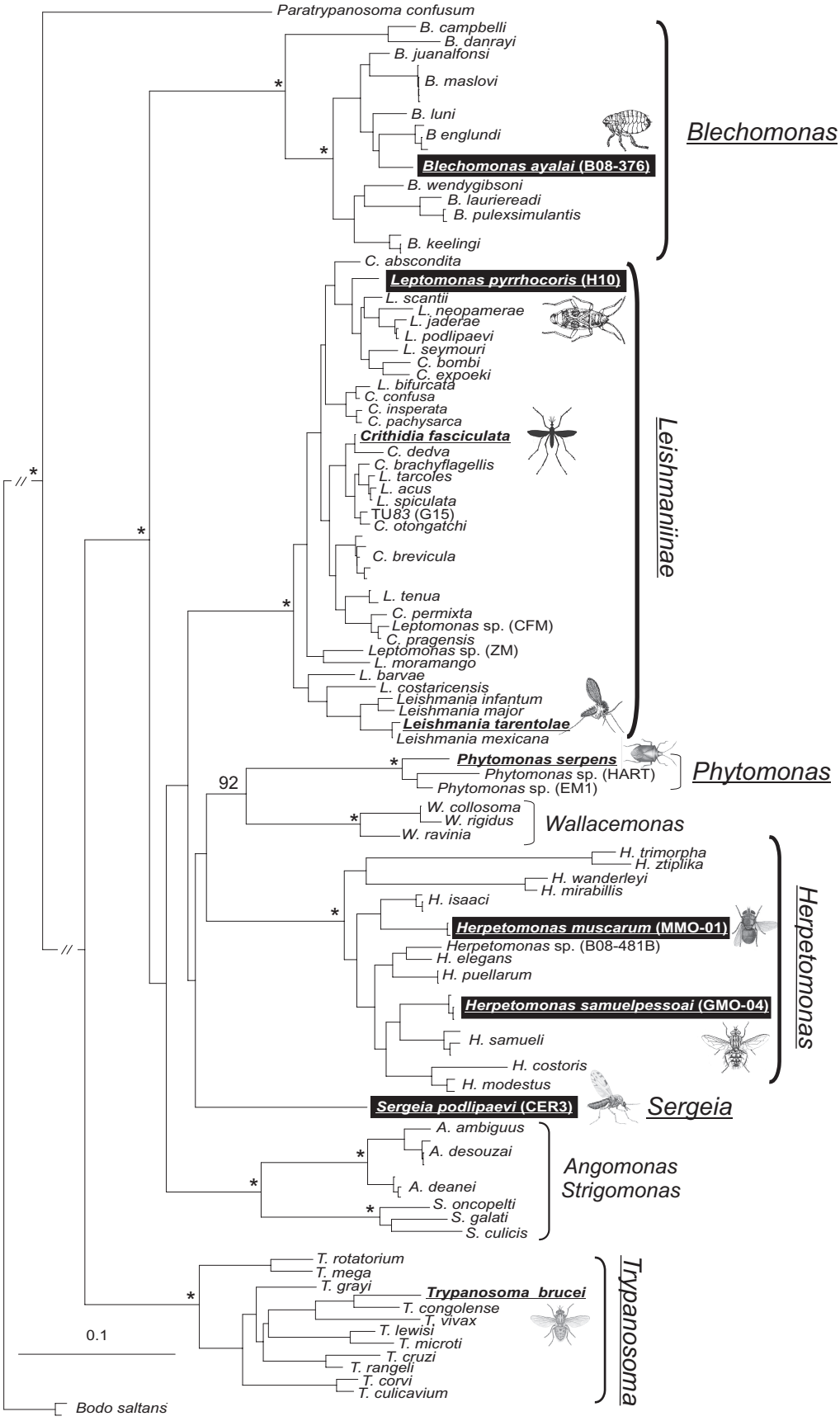

Fig. 3. Phylogenetic relationships of trypanosomatids. Maximum likelihood tree based on a concatenated alignment of glycosomal glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA genes. Underlined genera (subfamily) contain analysed trypanosomatid members; five species analysed in this study are highlighted; four species studied elsewhere are in bold and underlined. Maximum bootstrap supports are marked with asterisks; schematic icons depict the insect host.

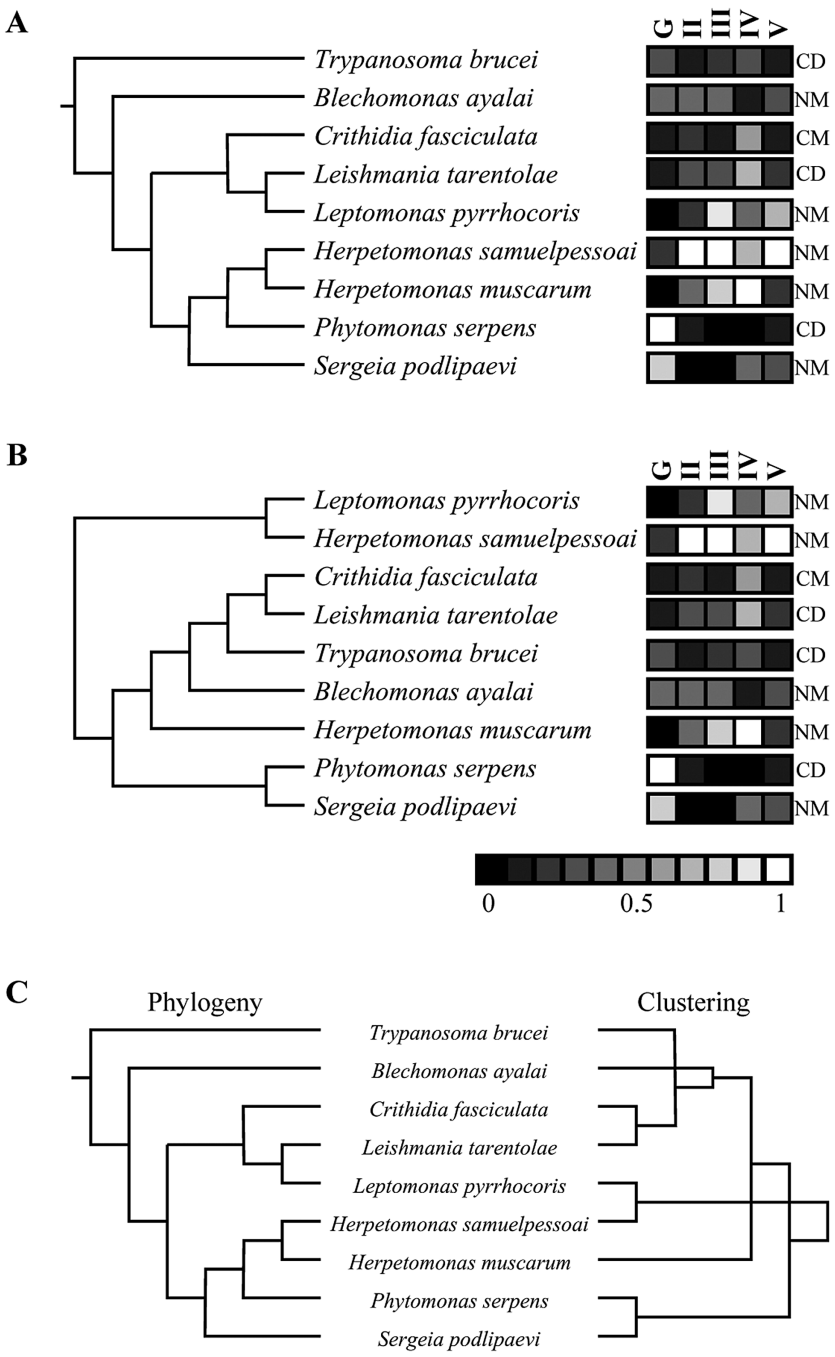

Fig. 4. Comparison of clustering analysis of enzymatic activities with phylogeny. Values from Table 1 were supplemented with the respective values from Verner *et al.* (2014), each characteristic was standardised towards the highest value and visualised as a heat map using MS Excel. Rows represent (from left to right) G3PDH (G) and respiratory complexes II, III, IV and V. CD – classical dixenous species (*T. brucei*, *P. serpens*, *L. tarentolae*); CM – classical monoxenous species (*C. fasciculata*); NM – new monoxenous species (*B. ayalai*, *H. muscarum*, *H. samuelpessoai*, *L. pyrrhocoris*, *S. podlipaevi*).
A. Heat map arrangement corresponding to the phylogenetic position of analysed species.
B. Heat map arrangement corresponding to hierarchical clustering analysis using the Ward method for distance calculation.
C. Mapping of the Ward method clustering analysis onto phylogeny.

inverse dependence between glycolytic generation of ATP and mt metabolism is illustrated by the strong inverse correlation with complex III, a proton pump directly influencing number of electrons to be passed to the second pump, complex IV. The correlation between activity of complex III and glucose-induced respiration implies that electrons originating from glucose oxidation pass through complex III, suggesting a lack of capacity of other putative terminal oxidases to cope with the electron flow. Lastly, the correlation between the amino acid-stimulated respiration and complex V fits perfectly with the bulk of ATP being generated in the mitochondrion, rather than by glycolysis or other pathways (Table 2).

Discussion

Due to the dearth of suitable morphological characters, our current view of trypanosomatid diversity has been shaped by the 18S rRNA-, SL RNA- and gGAPDH-based phylogenies. Moreover, several novel and sometimes

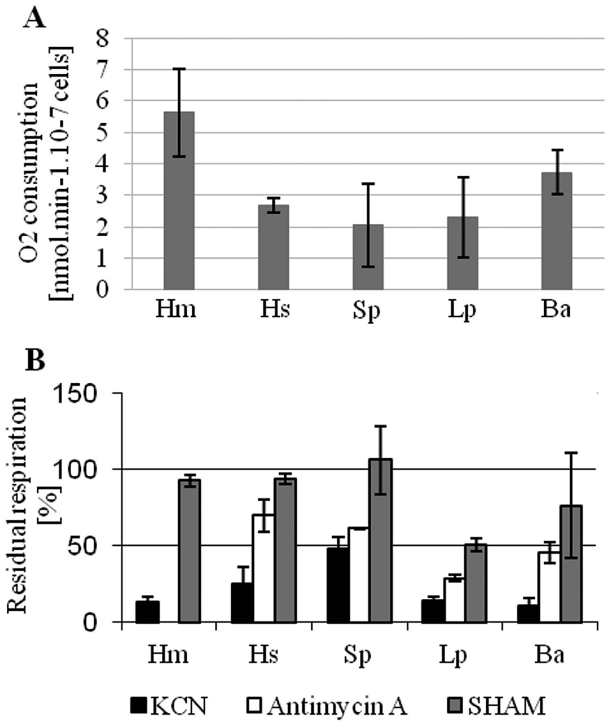

Fig. 5. Comparative analysis of respiration.
A. Basal oxygen consumption in cultivation media. Average values ± standard deviation for at least three measurements are shown.
B. Sensitivity of respiration to inhibitors of cytochrome *c*-containing (KCN and antimycin A) and alternative pathways (SHAM). Average values ± standard deviation for three to 10 measurements are shown. Hm = *H. muscarum*; Hs = *H. samuelpessoai*; Sp = *S. podlipaevi*; Lp = *L. pyrrhocoris*; Ba = *B. ayalai*.

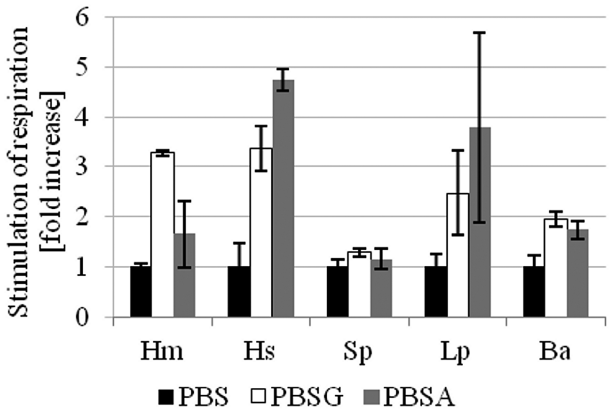

Fig. 6. Stimulation of respiration by various carbon sources. Cells were washed and resuspended in PBS or in PBS containing glucose (PBSG) or mixture of aminoacids (PBSA). Oxygen consumption was measured after 4 h and standardised towards respiration in PBS. Average values ± standard deviation for triplicates are shown, absolute values of respiration in PBS is shown in brackets following species name. Hm = *H. muscarum* ($4.1 \times 10^{-11} \pm 2.7 \times 10^{-12}$ µmol $O_2 \times min^{-1} \times cell^{-1}$); Hs = *H. samuelpessoai* ($2.8 \times 10^{-11} \pm 1.3 \times 10^{-11}$ µmol $O_2 \times min^{-1} \times cell^{-1}$); Sp = *S. podlipaevi* ($9.5 \times 10^{-12} \pm 1.5 \times 10^{-12}$ µmol $O_2 \times min^{-1} \times cell^{-1}$); Lp = *L. pyrrhocoris* ($1.9 \times 10^{-11} \pm 4.9 \times 10^{-12}$ µmol $O_2 \times min^{-1} \times cell^{-1}$); Ba = *B. ayalai* ($1.7 \times 10^{-11} \pm 3.6 \times 10^{-12}$ µmol $O_2 \times min^{-1} \times cell^{-1}$).

very species-rich clades are represented only by environmental sequences, with no cultivable strain available (Maslov *et al.*, 2013). Still, our systematic approach has resulted in the introduction into culture of five species that represent clades that to this time were observed only in insect hosts. For the chosen five seemingly monoxenous trypanosomatids, very little or no information is available. Measuring basal respiration and sensitivity to inhibitors of canonical and alternative enzymes of respiratory complexes, as well as following their mtG3PDH activity and the composition of, and electron flow through, respiratory complexes allowed basic characterisation of mt metabolism of the investigated species. The collected data were used for comparative analysis, into which an additional four well-studied trypanosomatids have been included (Suppl. Table S2).

Initially, we would like to stress that the data may represent only a section of mitochondrial metabolic capacity due to the fact that all the characterisations were performed under laboratory conditions. Although we believe we did not encounter dramatic deviations of any enzymatic activities from the levels occurring in natural environment, we cannot exclude this possibility as a direct comparison of *in vitro* and *in vivo* activities is not amenable for any of the described taxa at the moment. The dramatic change as a described loss of complex I in long-term cultivated isolates of *L. tarentolae* and *C. fasciculata* (Sloof *et al.*, 1994; Speijer *et al.*, 1997) most likely did not take place, although given these species were introduced into laboratory cultures might have biased the observed results. With this notion in mind the observed data are put into context.

Table 2. Correlation of measured mitochondrial features.

	G	II	III	IV	V	Resp	Glu
II	–0.46						
III	–0.90	0.72					
IV	–0.39	0.39	0.46				
V	–0.35	0.69	0.68	0.08			
Resp	–0.43	0.09	0.16	0.51	–0.56		
Glu	–0.82	0.78	0.90	0.73	0.45	0.48	
AA	–0.56	0.74	0.83	0.15	0.97	–0.38	0.60

Pearson's correlation coefficient was calculated based on average values of following mitochondrial features: G = activity of glycerol-3-phosphate dehydrogenase; II = activity of complex II; III = activity of complex III; IV = activity of complex IV; V = activity of complex V; Resp – overall basal respiration; Glu – glucose-stimulated respiration; AA – amino acid-stimulated respiration. Common correlation division: weak (0.1–0.3), medium (0.4–0.6), strong (0.7–0.8) and very strong (0.9–1.0; underlined). *P*-values were not calculated given the nature of the input data.

The activity of mtG3PDH is in a negative correlation with all examined activities of respiratory complexes. In glycosomes of bloodstream form *T. brucei*, G3PDH catalyses NADH-dependent conversion of dihydroxyacetone phosphate to glycerol-3-phosphate connected with a mt FAD-dependent isoenzyme working in an opposite direction. In this vein, the *T. brucei* bloodstream stage is devoid of the cytochrome *c*-containing respiration, with mtG3PDH directly linked to TAO (Clarkson *et al*., 1989). The succinate-producing branch in its glycosomes, previously thought to be absent (Michels *et al*., 2006), is not sufficient to provide enough oxidised co-factors, hence the activation of the G3PDH: DHAP shuttle. The rate of mtG3PDH reflects the high rate of glycolysis, and the negative correlation with respiratory enzymes suggests that the more the cell utilises glycolysis as a source of ATP, the less active mitochondrion it possesses in terms of classical respiration. In mt energy production, a high complex II activity reflects the employment of at least part(s) of Krebs cycle in trypanosomes. This is further documented by the situation in *P. serpens* and bloodstream *T. brucei*, where the high rate of mtG3PDH indicates that the connection between Krebs cycle and respiration is not operational (Clarkson *et al*., 1989; Sanchez-Moreno *et al*., 1992; Verner *et al*., 2014).

The association between complex II and mtG3PDH is not reflected by any other OXPHOS enzymes. A relatively high activity of complex III does not necessarily imply similarly high activity of complex IV. Due to large differences in the activities of complexes III and IV, we postulate that they do not readily respond to changes in their partner's activity. This conclusion is supported by our previous observation in which RNAi silencing of one complex had no effect on the activity of the following one and *vice versa* (Horváth *et al*., 2005). Similarly, there is no obvious correlation between the activities of oxidative phosphorylation enzymes and overall respiration.

Complex III forms a single band in all flagellates studied so far (this study; Speijer *et al*., 1997; Horváth *et al*., 2000; Horváth *et al*., 2005; Verner *et al*., 2014), although the pattern of complex IV is becoming increasingly convoluted. With the exception of *S. podlipaevi* and *B. ayalai*, several high molecular weight bands are detectable with antibodies against the complex IV subunit (Fig. 1B). However, their in-gel activity (Fig. 1C) does not always correspond to the intensity of detected signals, suggesting that not all agglomerates or oligomers are equally active. Combined, the results are compatible with the view that the dimer represents the active complex IV with the small forms being inactive, which is in agreement with data available for complex IV in beef heart mitochondria (Antonini *et al*., 1987). Unique to trypanosomatids is the invariably slower migration of complex IV as compared with complex III.

The presence of TAO in *T. brucei* and *P. serpens* (Chaudhuri *et al*., 1998; 2006; van Hellemond *et al*., 1998; Verner *et al*., 2014) and its likely absence in some monoxenous flagellates (this study; Suppl. Fig. S3) supports the evolutionary scenario postulating the presence of terminal alternative oxidase in the common eukaryotic ancestor and its preservation only in few lineages (McDonald *et al*., 2009). A similar scenario is observed within microsporidia where some lineages possess an alternative oxidase, whereas others have lost it (Williams *et al*., 2010).

The high and low complex IV and mtG3PDH activities of *H. muscarum*, respectively, indicate the presence of classical aerobic mitochondrion with the Krebs cycle as probably the main source of electrons for the electron transport chain. As a member of the same flies-parasitising genus, *H. samuelpessoai* differs from *H. muscarum* by higher specific activities of studied respiratory complexes, except for complex IV. Overall low sensitivity to SHAM is a common feature for all studied genera with the exception of *L. pyrrhocoris*, where the inhibition of alternative oxidase resembles the inhibition observed in *P. serpens*. The partition coefficients of KCN and SHAM are very similar (LogP = –0.78 and 0.7, respectively), and thus we expect them to cross the membrane under the same conditions. Therefore, the insensitivity to SHAM is most likely not caused by membrane impermeability for this inhibitor.

In vitro biochemical features and the 18S rRNA/gGAPDH-based phylogenies of species examined in this work do not correspond, as was documented by hierarchical clustering analysis. Moreover, we were unable to identify any key biochemical factor correlating with either phylogeny or life style, which leaves us with the conclusion that the obligatory parasitic trypanosomatids are multipotent in terms of respiratory chain complexes, and they seem to have retained all the essential elements of the mt metabolism over the course of evolution. Realisation of this potential is dictated by the availability of nutrients from the food of their final and/or intermediate host(s), and similar flexible or highly variable circumstances.

In summary, phylogenetic relationships of the studied trypanosomatids representing novel and potentially very widespread clades, do not correlate with the level of their enzymatic activities or mt physiology. Whether the unexpected variability of metabolic parameters is a consequence of diverse life style of these omnipresent parasites and/or the driver of their extreme success remains to be established. As parasites of perhaps up to 10% of all insect specimen inhabiting this planet (Maslov *et al*., 2013), trypanosomatids need a multipotent mitochondrion attached to a flexible metabolism. In any case, extending molecular and biochemical studies outside the well-known genera *Trypanosoma*, *Leishmania*, *Phytomonas* and *Crithidia*, as attempted for the first time herein, will be a fertile ground for the mt research and also for our

understanding of the emergence of the medically important dixenous species from their relatively innocuous monoxenous kins.

Experimental procedure

Organisms used and growth conditions

The following five species of monoxenous trypanosomatids were studied: (i) *H. muscarum*, strain MMO-01, was isolated from the hindgut part of the brachycerid fly male *Chrysomya putori* (Calliphoridae; Diptera) captured in 2010 in Ambatolampy, Madagascar (Týč *et al.*, 2013). The globally distributed *H. muscarum* is a type species of the genus *Herpetomonas*, one of the most species-rich clades of monoxenous trypanosomatids parasitising predominantly brachyceran flies. It displays the classical morphology of the genus *Herpetomonas* – promastigotes with a narrow flagellar pocket and pre-nuclear kinetoplast are elongated and needle-shaped, whereas a long and narrow flagellar pocket and post-nuclear localisation of the kinetoplast are features characteristic for the less frequent opisthomastigotes. (ii) Another globally distributed species of the same genus, *Herpetomonas samuelpessoai*, strain GMO-04, was isolated from the hindgut part of the brachycerid fly male of the subfamily Sarcophaginae (Diptera) collected in 2009 in Kokrobite, Ghana (Týč *et al.*, 2013). Promastigotes of this species are smaller, wider and shorter than those of *H. muscarum* and opisthomastigotes are rare in culture. (iii) *Sergeia podlipaevi*, strain CER3, was isolated from the Malpighian tubes and the abdominal midgut part of the biting midge female *Culicoides festivipennis* (Ceratopogonidae; Diptera) captured in 2000 in Mikulov, Czech Republic (Svobodová *et al.*, 2007). This is the type species of *Sergeia*, a genus that accommodates only two species found in Europe and America. Morphologically uniform cells are represented by elongated and needle-like promastigotes with the tendency to form rosette-like structures in culture. Another representative of an extremely species-rich clade is (iv) *Leptomonas pyrrhocoris*, strain H10, isolated from the abdominal midgut part of the fire bug female *Pyrrhocoris apterus* (Pyrrhocoridae; Heteroptera) and collected in 2008 in Prague, Czech Republic (Votýpka *et al.*, 2012). In culture, *L. pyrrhocoris* is invariably present in the form of highly heterogeneous promastigotes, varying from oval to long slender cells. The last monoxenous species studied herein is (v) *Blechomonas ayalai*, strain B08-376, isolated from the flea *Ctenophthalmus agyrtes* (Ctenophthalmidae; Siphonaptera) captured in a rodent nest in 2008 in Příbram, Czech Republic (Votýpka *et al.*, 2013). *B. ayalai* is a type species of the recently erected genus *Blechomonas* that represent a species-rich clade of global distribution (J.V. and J.L., unpubl. data). For *B. ayalai* a high morphological heterogeneity is characteristic, ranging from oval-shaped choanomastigotes to elongated promastigotes.

For long-term maintenance, these trypanosomatids were kept at 23°C on blood agar plates with an overlay. For scaled-up growth, the temperature was raised to 27°C. *H. samuelpessoai*, *L. pyrrhocoris* and *B. ayalai* were kept in liquid brain-heart infusion (BHI) medium (Fluka) supplemented with 10 mg ml^{-1} hemin (Sigma). *S. podlipaevi* and *H. muscarum* were cultivated in M199 + RPMI medium (1:1) (Fluka), supplemented with 10% heat-inactivated FBS (Fluka), 10 mg ml^{-1} hemin and sterile urine (1:50), as this species grows very slowly in BHI medium.

Phylogenetic and clustering analyses

Phylogenetic analyses were performed as described earlier (Votýpka *et al.*, 2012; Týč *et al.*, 2013). Briefly, the concatenated 18S rRNA and gGAPDH sequences were aligned via Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/), and the resulting alignments were edited manually using BioEdit 7.0.9.0 to remove the fast-evolving regions of 18S rRNA preventing unambiguous alignment. Final concatenated alignment included 3155 characters and the accession numbers retrieved from GenBank and used in phylogenetic reconstructions are available upon request from authors. Phylogenetic analyses were performed with MrBayes 3.2.1 program (Bayesian criteria: rates for six different types of substitution, proportion of invariant sites and shape parameter of the gamma correction for the rate heterogeneity with four discrete categories were allowed to vary; the covarion model was used to allow the rate heterogeneity along the tree; the Markov chain Monte Carlo was run for five million generations). Clustering analysis of the enzymatic activities was performed using free on-line application (http://www.wessa.net/rwasp_hierarchicalclustering.wasp).

Detection of mitochondrial respiratory complexes

Mitochondria-enriched fraction was isolated as described elsewhere for *T. brucei* (Horváth *et al.*, 2005), and protein content was measured by Bradford method. For BN-PAGE, mt proteins were lysed by 2% dodecylmaltoside followed by 1 h incubation on ice. Upon spinning, 15 µl of lysate was mixed with 1.5 µl Coomassie dye buffer (5% Coomassie Brilliant Blue G-250, 500 mM aminocaproic acid) and loaded on 2–12% gradient BN-PAGE as described previously (Horváth *et al.*, 2005). After electrophoresis, the gel slices were immediately used for downstream experiments or stored at –20°C.

Two-dimensional gel analysis

Analysis of respiratory complexes of purified mitochondria was performed by 3–10% 2D BN/Tricine-SDS-PAGE as

described previously (Horváth *et al*., 2005). A slice of the gel obtained from the first BN dimension was incubated at 37°C in denaturing buffer (1 M Tricine; 1% SDS, 1% 2-mercapthoethanol, pH 8.45) for 45 min. The incubation was followed by resolution of complexes in 10% Tricine-SDS-PAGE. The resulting gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 10% acetic acid and 30% methanol.

Immunodetection

Immunodetection was performed as described elsewhere (Verner *et al*., 2014). Briefly, protein complexes from BN-PAGE or SDS-PAGE were transferred to nitrocellulose membranes by wet blotting overnight using anchored 20 mA current. Membranes were probed with antibodies raised against: (i) the sdh66 subunit of succinate dehydrogenase from *T. brucei* (1:1000) (Kořený *et al*., 2012); (ii) apocytochrome c_1 (apo c_1) or Rieske protein of complex III from *T. brucei* (1:1000) (Horváth *et al*., 2005); (iii) the trCOIV subunit of complex IV from *L. tarentolae* (1:1000) (Maslov *et al*., 2002), and (iv) β chain of complex V from *T. brucei* (1:1000) (kindly provided by A. Zíková). Appropriate secondary antibodies (1:2000; Sigma) coupled to horseradish peroxidase were visualised using an ECL kit according to the manufacturer's protocol (Pierce). For specification of band intensities, Manual Rectangle Selection of regions of interest was used (Kodak Molecular Imaging).

In-gel activity staining and spectrophotometric assays

The staining was performed as described recently (Verner *et al*., 2014). For the detection of appropriate enzyme activities, BN gel slices were incubated overnight in 50 mM sodium phosphate, pH 7.2; 0.5 mg ml^{-1} 3,3′-diaminobenzidine; 1 mg ml^{-1} cytochrome *c* for complex IV and in 8.5 mM Tris-HCl, pH 7.0; 67 mM glycine; 14 mM magnesium sulphate; 0.2% lead nitrate; 8 mM adenosine 5-triphosphate for complex V. Band intensities were evaluated as described above.

Complex II activity was measured in 5 μg of mt lysate in SDH-A buffer (25 mM potassium phosphate, pH 7.2; 5 mM magnesium chloride; 20 mM succinate disodium salt; 0.014 mg ml^{-1} dichlorophenolindophenol; 65 μM ubiquinone Q_2) at 600 nm for 5 min. Activities of complexes III, IV and V were measured as described elsewhere (Horváth *et al*., 2005; Schnaufer *et al*., 2005) and FAD-dependent mtG3PDH was measured following Škodová *et al*. (2013).

Oxygen consumption and survival experiment

Oxygen consumption was determined with a Clark-type electrode (1302 Microcathode Oxygen Electrode; Strathkelvin). Basal oxygen uptake was recorded in 0.5 ml of logarithmically growing cell culture at 27°C. Cyanide (KCN), antimycin A and salicylhydroxamic acid (SHAM) were added in 4 min intervals to final concentrations of 0.1 mM, 1.2 μg ml^{-1} and 0.09 mM respectively. For survival experiments, approximately 2×10^7 cells ml^{-1} were washed twice in PBS (Fluka) and resuspended in 1 ml of PBS alone or PBS supplemented with 10 mM glucose (PBSG) or combination of L-threonine, L-proline and L-glutamine (15 mM each; PBSA). Respiration was measured in cells upon their incubation in appropriate buffer for 4 h at 27°C. Collected data were processed and analysed using either Microsoft Excel 2007 or GraphPad Prism 5.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.