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Kinetoplastids biology, from the group phylogeny and evolution into the secrets of the mitochondrion of one representative: *Trypanosoma brucei* – the model organism in which new roles of the evolutionary conserved genes can be explored

Ph.D. Thesis

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Annotation

This thesis is composed of two topics, for which trypanosomatids and evolution are common denominators. First part deals with phylogenetic relationships among monoxenous trypanosomatids, with emphasis on flagellates parasitizing dipteran hosts, analyzed mainly from biogeographical and evolutionary perspectives. Second part focuses on the trypanosomatid *Trypanosoma brucei*, causative agent of severe diseases, which serves as a model organism for functional studies of evolutionary conserved mitochondrial proteins, in particular those involved in replication, maintenance and expression of the mitochondrial genome, also termed the kinetoplast. This thesis identified the mtHsp70/mtHsp40 chaperone machinery as an essential component of replication and maintenance of the kinetoplast, and also identified numerous conditions under which mtHsp70 has a tendency to aggregate. Moreover, several conserved proteins, previously identified to be part of the mitochondrial ribosome, were shown to be important for translation of the mitochondrial transcripts.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 28. 3. 2015

RNDr. Jiří Týč

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List of papers and author's contribution

The thesis is based on the following papers and manuscripts (listed chronologically):

Paper I:
Týč J., Votýpka J., Klepetková H., Šuláková H., Jirků M. & Lukeš J. Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): Frequent cosmopolitism and moderate host specificity *Mol. Phyl. Evol.* 69(1), 255-264 (2013). doi: 10.1016/j.ympev.2013.05.024. IF = 4.018

JT was responsible for collecting samples of trypanosomatids from heteropteran and dipteran hosts in Bulgaria and Turkey and established axenic cultures. JT also isolated DNA from host samples and cultures, sequenced, analyzed and evaluated the data, and wrote most of the manuscript.

Paper II:
Lukeš J., Skalický T., Týč J., Votýpka J. & Yurchenko V. Evolution of parasitism in kinetoplastid flagellates (Review). *Mol. Biochem. Parasitol.* 195(2):115-122. (2014) doi: 10.1016/j.molbiopara.2014.05.007. IF = 2.243

JT contributed to writing of the manuscript.

Paper III:
Verner Z., Basu S., Benz C., Dixit S., Dobáková E., Faktorová D., Hashimi H., Horáková E., Huang Z., Paris Z., Pena-Diaz P., Ridlon L., Týč J., Wildridge D., Zíková A. & Lukeš J. Malleable mitochondrion of *Trypanosoma brucei* (Review). *Int. Rev. Cell. Mol. Biol.* 315*:73-151* (2015) doi: 10.1016/bs.ircmb.2014.11.001. IF = 4.52

JT participated in writing, and was the author of the chapters 2 and 2.1.

Paper IV:
Týč J., Klingbeil M.M. & Lukeš J. Mitochondrial heat-shock protein machinery Hsp70/Hsp40 is indispensable for proper mitochondrial DNA maintenance and replication. *mBio* 6(1):02425-14. (2015) doi:10.1128/mBio.02425-14. IF = 6.875

JT participated in designing the experiments, carried them out, processed and evaluated the data, wrote most of the manuscript.

Manuscript I:
Týč J., Novotná L., Maslov. D & Lukeš J. RSM22, mtYsxC and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei* (Manuscript in preparation)

JT carried out most of the experiments and data processing, participated in experimental design, data evaluation and wrote most of the manuscript.

Manuscript II:
Týč J., Haindrich A.C., Skalický T., Basu S., Flegontov P., Flegontova O. & Lukeš J. Aggregation of the Hsp70 chaperone in the mitochondrion of *Trypanosoma brucei* (Manuscript in preparation)

Together with A. Haindrich, JT carried out most experiments and data processing, participated in experimental design, data evaluation and wrote most of the manuscript.

Julius Lukeš, the corresponding author of all listed papers, approves the contribution of Jiří Týč in these papers as described above.

Prof. RNDr. Julius Lukeš CSc.

Contents

1.1 Trypanosomatids

Trypanosomatid flagellates are omnipresent and exceptionally successful obligatory parasites of various groups of eukaryotes, ranging from plants and invertebrates to vertebrates including human. Trypanosomatids also serve as valuable model organisms in studies of diversity, host specificity as well as distribution of parasites. Representative species, such as *Trypanosoma brucei*, *Trypanosoma cruzi, Leishmania tarentolae* and *Crithidia fasciculata* are used to study adaptations to parasitism as well as highly derived eukaryotic features at the cellular and molecular levels.

1.1.2 Phylogeny of the group

Trypanosomatids is a group with obligatory parasitic life style that constitutes a branch within the class Kinetoplastea, consisting of free-living, commensalic and parasitic flagellates (Lukeš et al., 2014; Moreira et al., 2004; Simpson et al., 2004a). Kinetoplastea obtained their name after the kinetoplast, a mass of mitochondrial DNA and proteins located in the periflagellar region of the mitochondrion (Lukeš et al., 2014). Along with Diplonemea, Euglenida and Symbiontida, Kinetoplastea forms the group Discicristata, whose representatives share discoidal mitochondrial cristae as their common character. In the evolutionary tree of all extant eukaryotes, these flagellates fall within the excavate kingdom Euglenozoa (Adl et al., 2012).

Not surprisingly, most studied representatives are those with medical, veterinary and economic impact. Best known is the genus *Trypanosoma*, the members of which are responsible for human Chagas disease in Americas and sleeping sickness in Africa. They also include causative agents of nagana, surra and dourine in cattle, horses, water buffalos, camels and other economically important animals. Species from the related genus *Leishmania* are responsible for leishmaniases, a wide array of clinically variable diseases affecting millions of people in the subtropical and tropical regions (Bañuls et al., 2007). Finally, flagellates ranked into the genus *Phytomonas* parasitize crops and other plants, such as oil and coconut palms (Camargo, 1999). These important pathogens are so-called dixenous (= two host) parasites, while the majority of trypanosomatid diversity lies within the monoxenous (= single host) parasites (Lukeš et al., 2014; Maslov et al., 2013).

Until present, hundreds of TUs (Typing units = molecular species) of trypanosomatids have been identified (Maslov et al., 2013) which can be divided into several major clades (Fig. 1) (Lukeš et al., 2014; Maslov et al., 2013). Dixenous *Trypanosoma* species appear at the base of the Trypanosomatidae clade, while the dixenous *Leishmania* and *Phytomonas* species branch off from within the monoxenous groups. Recently, one of the most evolutionary important trypanosomatids, *Paratrypanosoma confusum*, was described (Flegontov et al., 2013). It is the only flagellate that is basal even to trypanosomes, and is therefore positioned between all obligatory parasitic trypanosomatids and the free-living *Bodo saltans*. This isolate might be instrumental in resolving the long-lasting debate about whether the ancestors of trypanosomatids were originally parasites of aquatic vertebrates and only later acquired insect vectors (Minchin 1908), or whether they first colonized the insect hosts and only later gained the ability to be transmitted to vertebrates (Leger 1904). Due to accumulated molecular evidence, the latter view is now favored by most scientists.

Recent studies (Teixeira et al., 2011; Týč et al., 2013; Votýpka et al., 2012; Votýpka et al., 2010; Votýpka et al., 2013) revealed the existence of many new TUs and corrected taxonomy so far mostly based on rather misleading morphological characters. While the diversity of trypanosomatids is steadily growing, no new clades lately emerged, the *Blechomonas* group being the only exception (Votýpka et al., 2013). Hence, it seems that although most trypanosomatid species of insects remain yet to be identified, the overall phylogeny of the group is already well mapped, with no big expansions expected.

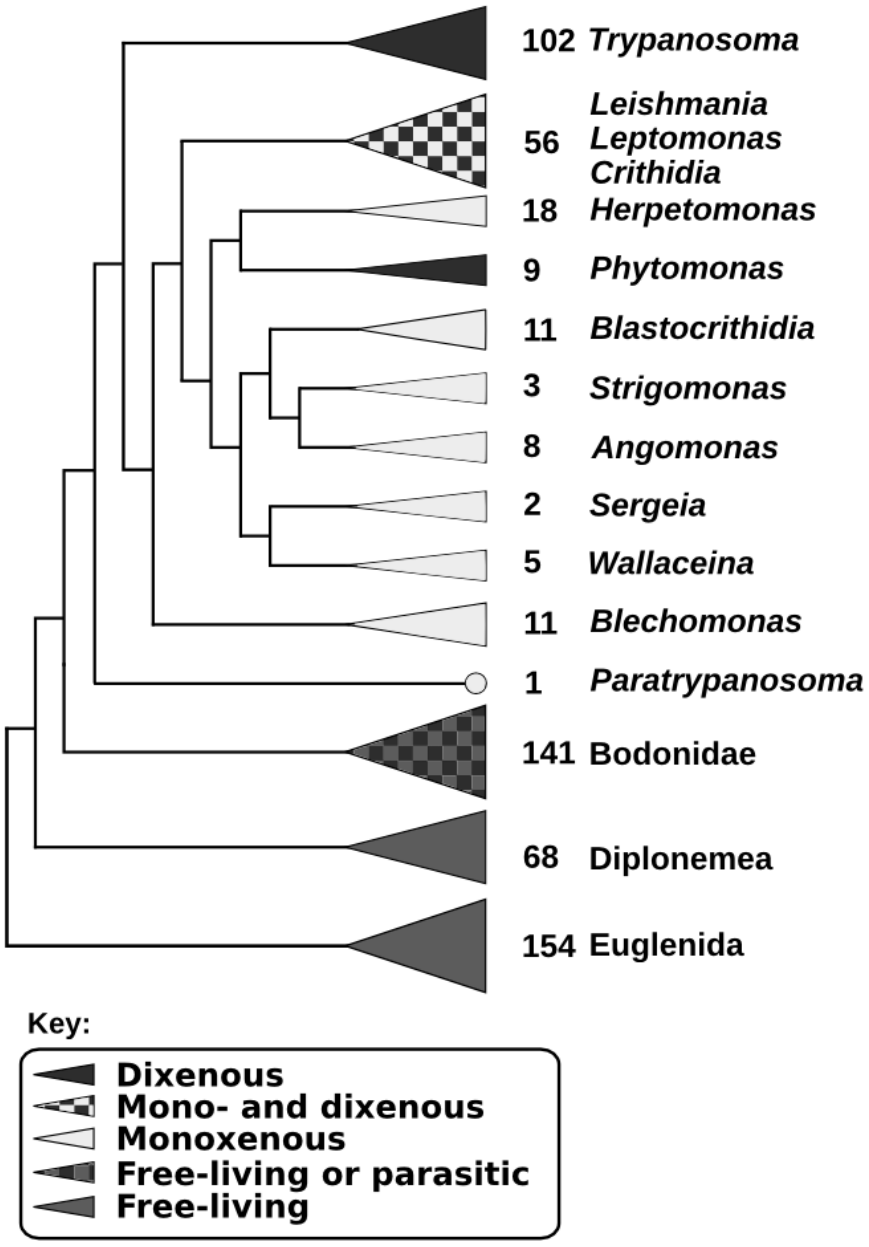

Figure 1: Phylogenetical tree of trypanosomatids based on SSU rRNA sequence. Numbers represents individual species for which sequence is available. Adopted from Lukeš et al., 2014.

1.2. Why to study trypanosomatids and what can they tell us?

Genomes of three dixenous trypanosomatids (*T. brucei, T.cruzi* and *L. major*) have been sequenced already a decade ago (Berriman et al., 2005; El-Sayed et al., 2005; Ivens et al., 2005), and with other strains and species are available in Tritrypdb.org (Aslett et al., 2010). Recently, two nuclear genomes of *Phytomonas* species were also sequenced (Porcel et al., 2014), and the number of fully sequenced *Leishmania* and *Trypanosoma* species is bound to grow. The TriTryp database is a valuable source for the community where researchers can look for interesting target genes for subsequent functional analyses. Some targets are obvious or already known, however, the function of the majority of protein-coding genes remains hypothetical (Aslett et al., 2010).

One of the essential questions is to identify genes responsible for the highly successful parasite's strategy allowing it to prosper within the vertebrate and especially human hosts. For this purpose, a comparative molecular and biochemical analysis between monoxenous and dixenous trypanosomatids can bring new important insight. Initial data, so far on a biochemical level, are now available (Škodová-Sveráková et al., 2014). Moreover, at least two draft genomes are publicly available, namely that of *Crithidia fasciculata* (http://tritrypdb.org) and *Leptomonas seymouri* (https://www.sanger.ac.uk/resources/downloads/protozoa/leptomonas-seymouri.html), and several more genomes of (likely) varying quality of assembly and annotation are on the way. Indeed, comparing the whole genomes and transcriptomes of monoxenous and dixenous trypanosomatids shall reveal differences in gene content and differential expression and therefore help to identify genes or gene families responsible for the ability to invade the vertebrate host and deceive its immune system. Identified target genes will be afterwards amenable to a thorough investigation.

This all means that with the sequencing costs progressively dropping, insect trypanosomatids sampled from around the world will be very useful for comparative studies and for understanding the evolution and diversity of these extremely successful and abundant parasites.

1.3. *Trypanosoma brucei*

T. brucei is a deadly human parasite from the phylum Trypanosomatidae. Two different strains *T. b. gambiense* and *T. b. rhodensiense* cause sleeping sickness or HAT (human African trypanosomiasis) in humans, while *T. b. brucei* is a causative agent of cattle disease nagana (Barrett et al., 2003). The two strains of *T. brucei* causing HAT differ in geographic distribution and symptoms but are morphologically indistinguishable (Gibson, 1986). The West African sleeping sickness, caused by *T. b. gambiense* is responsible for up to 98% of infections and it develops for up to 3 years without major symptoms. The East African sleeping sickness caused by *T. b. rhodesiense* develops faster and its symptoms become obvious within a few weeks. If untreated, the infections are mostly lethal, especially after the parasite crosses the blood brain barrier. *T. b. brucei* does not affect humans, as it is susceptible to trypanosome lytic factor in the human blood. All subspecies are transmitted via the bite of a blood-sucking tsetse fly (*Glossina* spp.) that populates African woodland and savannah zones. As a result, human populations in remote rural areas are predominantly affected. The effort to control the disease already brought results and in 2012 there were

only 10,000 newly reported infections. The total number is probably higher, as many cases remain unreported or undiagnosed, however, it is a significant drop in comparison to approximately 40,000 annual reports that occurred in sub-Saharan Africa before 1998 (WHO report, Geneva 2012). *T. brucei* is confined to Africa only due to the restricted area of its tsetse fly vector. Only flagellates that are able to find other means of their transmission are able to escape from Africa and spread worldwide. Such trypanosomes indeed exist and are known as *T. b. equiperum* and *T. b. evansi* (Lai et al., 2008). Their life strategies are related to the changes in kDNA and are described below.

1.3.1 Life cycle of *T. brucei*

The life cycle of *T. brucei* is quite complex, consisting of several developmental stages (Fig 2). Midgut of the tsetse fly is colonized by proliferating procyclic stage expressing procyclin as its protective surface coat. This stage subsequently develops into the epimastigote stage, which migrates into the salivary gland. The development in tsetse fly is terminated by the metacyclic stage, which does not proliferate and, being covered in masking coat of variable surface glycoprotein (VSG), is already pre-adapted for survival in the mammalian host. VSG serves to escape the mammalian immune system via a process called antigenic variation, via which the surface glycoproteins periodically changes (Pays et al., 2004). The transmission occurs during the tsetse fly's feeding.

In the bloodstream of the mammalian host, long slender dividing population and short-stumpy non-dividing forms can be distinguished. Short-stumpy form is the transitional stage, which does not proliferate and is preadapted to be ingested by tsetse fly where the life cycle is completed (Matthews, 1999) (Fig 2). Only the proliferating procyclic stage (PS) and the long slender bloodstream stage (BS) can be easily cultivated in the liquid media under laboratory conditions. For this reason these two stages are by far predominantly studied.

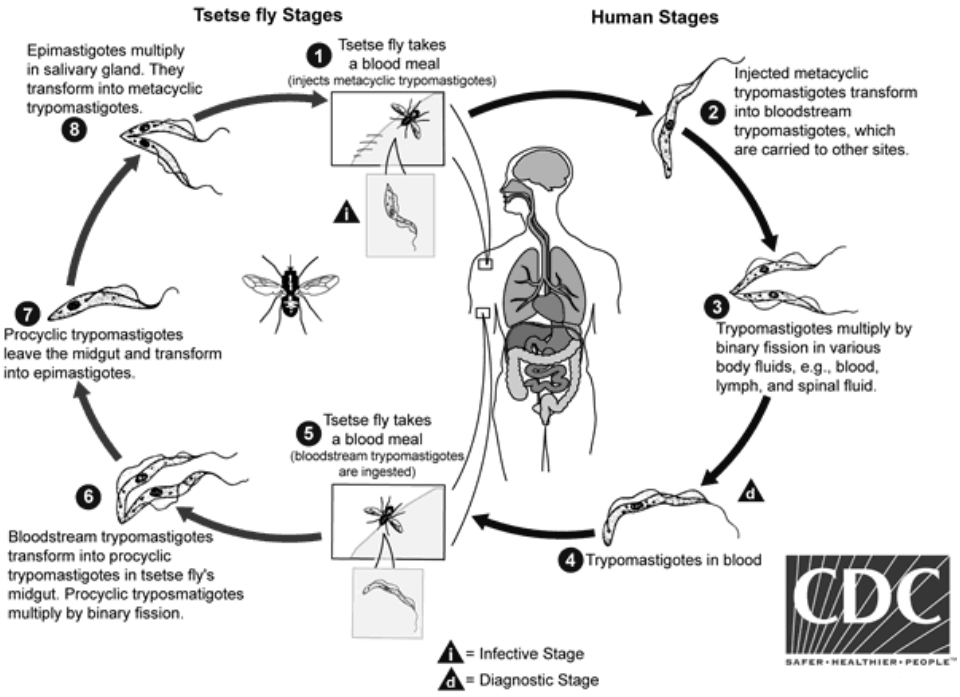

Figure 2: Life cycle of *Trypanosoma brucei*. Adopted from CDC.

1.3.2 Cell organization and peculiarities of *T. brucei*

T. brucei is a typical single eukaryotic cell with nucleus and organelles such as the Golgi apparatus, endoplasmic reticulum, mitochondrion and flagellum. In addition, together with other trypanosomatids, *T. brucei* features many distinct oddities for which these ancestral flagellates are intensely studied (Lukes et al., 2005; Montagnes et al., 2012). These includes polycistronic transcription (Muhich and Boothroyd, 1988), subsequent massive *trans*-splicing of mRNAs, variable surface glycoprotein coat (Pays et al., 2004) and specific cytoskeletal corset, composed of spiraled interlinked subpelicullar microtubules located just beneath the plasma membrane (Hemphill et al., 1991). Other unique feature of kinetoplastid flagellates is the paraflagellar rod, a highly organized protein structure running alongside the flagellum (Maga and LeBowitz, 1999; Vickerman, 1962). In *T. brucei* the flagellum is connected with the cell via an undulating membrane, which contributes to the characteristic movement of the parasite and enhances motility in the viscous medium such as blood (E.A., 2003). *T. brucei* has a number of other oddities, such as a specialized compartment for energy metabolism – the glycosome (Michels et al., 2006). This organelle, likely derived from the peroxisomes, contains enzymes of glycolysis and purine salvage pathway (Parsons et al., 2001). Finally, the single mitochondrion of *T. brucei*, with its extraordinarily complex mitochondrial DNA and editing of organelar RNA, deserves to be described in more details (see below).

1.3.3 *T. brucei* as a model organism

Protists can be used as informative model organisms to answer fundamental biological questions (Montagnes et al., 2012). From the trypanosomatid group the most prominent model organism is *T. brucei*, both because of its medical and economic importance, but also due to its amenability to a number of molecular biology approaches. Other kinetoplastid parasites such as *Leishmania* and *Phytomonas*, are also very important, yet most molecular tools for them are lacking.

T. brucei as a model benefits from the fact that its life cycle stages are extracellular and relatively easy to cultivate. Its whole genome has been sequenced and is available online together with *T. cruzi*, *Leishmania major* and other trypanosomatids (Aslett et al., 2010). Genome availability allows comparative studies as well as larger proteomic and transcriptomic studies (Butter et al., 2013; Kolev et al., 2010). Examples of commonly used strains in the laboratory for RNAi studies and ectopic copy expression are 29-13 strain of PS and the strain Lister 427 (cell line 90-12) of BS. Both were genetically modified to express T7 polymerase and tetracycline (TET) repressor allowing tight regulation (Wang et al., 2000; Wirtz et al., 1999).

Functional studies in the *T. brucei* model can also benefit from following methodological approaches. Most impact probably has RNA interference (RNAi), via which target mRNA is specifically down-regulated upon induced expression of double-stranded RNA (Motyka and Englund, 2004; Wang et al., 2000), leading to the ablation of target protein. Knock out of the gene by homologous recombination is also feasible in *T. brucei* (Gaud et al., 1997). This approach, however, is not easy and straightforward as RNAi. *T. brucei* is a diploid organism, so multiple rounds of transfection and multiple markers are

needed. Moreover, if the target gene is essential for cell survival, regulatable ectopic allele has to be introduced before any of the alleles is disrupted.

Important genetic manipulation include protein tagging techniques, with different tags being used for precise localization of the protein of interest, such as YFP, PTP, HA, V5, etc. (Huang et al., 2014; Týč et al., 2015; Týč et al., 2010b; Šubrtová et al., 2015). Even dynamic localization of the protein during cell cycle can be explored (Concepción-Acevedo et al., 2012). For cellular localization mostly immunoflorescence microscopy is used, but transmission electron microscopy can also be utilized for high resolution (Kovářová et al., 2014). Tagged proteins also serve for purification and identification of protein binding partners (Zíková et al., 2008), with the TAP and PTP tags being designed specifically for this purpose (Günzl and Schimanski, 2009).

T. brucei is particularly useful model for studying the function of proteins that significantly differ from its human host or are unique to the parasite. These proteins therefore can serve as potential drug targets, because their inhibition will not affect the vertebrate host (Ammerman et al., 2012; Týč et al., 2010b; Šubrtová et al., 2015).

Another group of proteins, for the studies of which *T. brucei* is well suited are the evolutionary conserved ones. As a member of the supergroup Excavata, *T. brucei* is evolutionary very distant from other model eukaryotes such as yeast, mice and human cells (members of the supergroup Opisthokonta) or plants (supergroup Archaeplastida). Thus, *T. brucei* gives us an opportunity to study highly conserved and therefore very important genes and pathways from unique perspective(s) (Basu et al., 2014; Týč et al., 2010a; Týč et al., 2015). For example, trypanosomatid oddities such as the single mitochondrion and single mitochondrial (mt) DNA that replicates only once per cell cycle can be used to address issues such as the involvement of mtHsp70/mtHsp40 chaperones in the replication of mtDNA from an angle that is not possible in other eukaryotes (Týč et al., 2015).

1.4. Mitochondrion of *T. brucei*

Mitochondrion is a double membrane organelle derived from a symbiotic α-proteobacterium, which hosts several key metabolic processes (Gray, 2012). The best known role of mitochondria is the production of ATP through the Krebs cycle and electron transport chain in the inner membrane. Other indispensable functions are synthesis of heme and steroids and the assembly of Fe-S clusters, just to mention a few (Nunnari and Suomalainen, 2012).

T. brucei contains a single large tubular mitochondrion, the activity of which depends on the life cycle stage. In the procyclic stage (PS), the mitochondrion is fully morphologically developed and metabolically active. Its numerous functions (oxidative phosphorylation, RNA editing, ATP production and others) are essential for the parasite's survival. Even though glucose is utilized if present, amino acids, especially L-proline, are the major energy source of this stage (Bringaud et al., 2006). Bloodstream stage (BS) of *T. brucei* swims in glucose-rich blood and for ATP production relies mostly on glycolysis. Its mitochondrion is reduced in size and the energy production function is silenced. Moreover, standard respiratory chain is absent and the respiration is carried out solely via trypanosome alternative oxidase (TAO) (Chaudhuri et al., 2006). The organelle is however still essential for parasite's survival, due to functions related to its Fe-S cluster assembly, kDNA replication and maintenance, transcription, RNA editing and translation (Cristodero et al., 2010). Even in absence of a

functional respiratory chain, the BS still needs complex V, functioning as an ATPase. This complex contains a single subunit that is encoded in the kDNA. Because of this single protein all the above-listed machineries have to be functional (Hashimi et al., 2010). ATP synthase actually reverses its function and hydrolyzes ATP, in the process pumping protons into the inter membrane space in order to maintain the membrane potential on the mitochondrial double membrane (Brown et al., 2006; Schnaufer et al., 2005). Loss of membrane potential is lethal as it is needed for proper functioning of numerous mitochondrial processes, such as protein import across the double membrane (Schleyer et al., 1982).

1.4.1 Kinetoplast DNA – structure and replication

One of the most remarkable features of the kinetoplastid mitochondrion is its extraordinary mitochondrial nucleoid, which even gave name to the whole group. *T. brucei* not only has one mitochondrion per cell, but also its mtDNA termed the kinetoplast (or kDNA) is located at a distinct periflagellar region of the organelle. Due its size, it is well visible even under the light microscope. As a matter of fact, it was the first extra-nuclear DNA ever observed (Steinert et al., 1958).

Kinetoplast DNA (kDNA) consist of two DNA entities - minicircles and maxicircles, which are catenated into a single complex network (Liu et al., 2005) (Fig. 3). Maxicircles are homologous to the mtDNA in other eukaryotes and carry protein-coding genes (Lukes et al., 2005). In *T. brucei* there are several dozens of identical copies per organelle, with their size being around 23 kb. Minicircles are smaller, approximately 1 kb in length, and each kDNA contains heterogeneous and highly diverse population of several thousands of them (Ntambi and Englund, 1985). Maxicircles encode two rRNAs and protein-coding genes, the products of which are mainly incorporated into the respiratory chain, while minicircles carry guide RNAs (gRNAs) essential for RNA editing (Clement et al., 2004). Transfer RNA genes are missing, and all tRNAs have to be imported from the cytosol (Alfonzo and Söll, 2009). Mitochondrial genes are transcribed polycistronically (Read et al., 1992) and extensive processing and editing is required during their maturation, which includes polyadenylation of mRNAs (Bhat et al., 1992) and polyuridylation of rRNAs and gRNAs (Adler et al., 1991; Stuart et al., 2005).

Kinetoplast DNA replicates once per cell cycle, predating nuclear DNA replication and cell division, which is quite unusual among eukaryotes (Englund, 1978; Woodward and Gull, 1990). Cell division starts by the division of the basal body (Robinson and Gull, 1991), which is an organizing center of cell duplication, and is physically connected to the kDNA via the tripartite attachment complex (TAC) (Gluenz et al., 2011). TAC controls and ensures the kDNA replication, separation and proper positioning within the mitochondrion (Gluenz et al., 2011). Replication of kDNA itself, during which the network doubles in size and then splits, is a highly complicated process which has to ensure that each minicircle and maxicircle is replicated and subsequently delivered into each daughter cell (Liu et al., 2005).

Replication of maxicircles occurs within the kDNA network via a theta structure intermediate (Carpenter and Englund, 1995). This replication occurs inside the kDNA disc, and given the fact that maxicircles are much less numerous than the minicircles, we know little about their replication. So far, only helicase Pif2, primase Pri1, and polymerases Pol1C and Pol1D were shown to play a role in the maxicircle replication (Hines and Ray, 2011; Jensen and Englund, 2012; Liu et al., 2009), with Pif2 helicase being the only known enzyme

that affects exclusively maxicircles. Maxicircles are probably the major component of the nabelschnur structure that connects the two daughter kDNA networks right until their final separation (Gluenz et al., 2011).

Minicircles are on the other hand easier to study and therefore their replication is much better understood. Each minicircle has to be released into kinetoflagellar zone (KFZ) before its replication (Drew and Englund, 2001). Pif1 helicase, Pri2 primase and Pol1B polymerase, along with other proteins, are then involved in their replication, which also occurs via the theta structure (Bruhn et al., 2010; Hines and Ray, 2010; Liu et al., 2010; Ryan and Englund, 1989). The sister minicircles that have been replicated then migrate to antipodal sites at the opposite sides of the kinetoplast, where they are being reattached to the growing disc (Melendy et al., 1988). Although most of the gaps and nicks are sealed during the process, some of them are kept in order to distinguish newly replicated and reattached minicircles from those that did not yet underwent the process (Englund, 1979). Only after the whole replication is finished, all the nicks and gaps are sealed and sister kDNA networks segregates.

This highly complex process obviously requires a sophisticated replication machinery. It has been estimated that total number of proteins involved in kDNA maintenance, replication and its regulation can easily reach 150 with just about 30 of them described so far (Jensen and Englund, 2012). The simplest explanation is that more complicated structure would require more proteins. Moreover, the kDNA replication must be highly reliable and faithful, as there is only one kDNA per cell. The increase in number of proteins involved in the replication and maintenance machinery is due to the involvement of several kinetoplastid-specific proteins and also by multiplication of the universal ones.

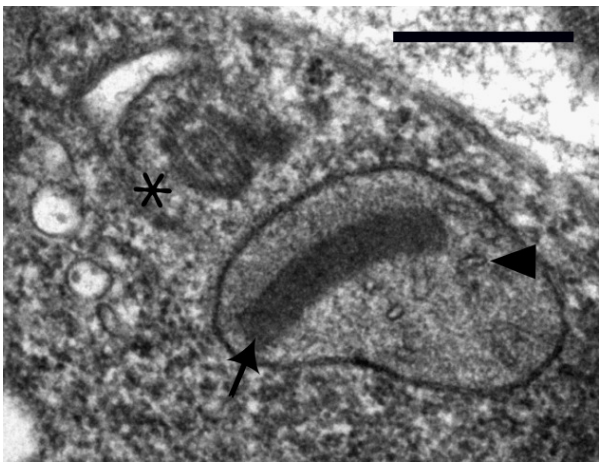

Figure 3: Electron micrograph of *T. brucei* kinetoplast DNA. Arrow points at kDNA, asterisk marks basal body and arrowhead points at cristae in the mitochondrion. Scale bar represents 500 nm.

Indeed, so far several topoisomerases (Bakshi and Shapiro, 2004; Lindsay et al., 2008; Melendy et al., 1988; Scocca and Shapiro, 2008; Wang et al., 2000), ligases (Sinha et al., 2004; Sinha et al., 2006), primases (Hines and Ray, 2010, 2011); six helicases (Liu et al., 2009) and seven DNA polymerases have been identified (Klingbeil et al., 2002; Rajão et al., 2009; Saxowsky et al., 2003). These numbers are highly unusual for a mitochondrion, where usually an opposite situation can be documented, with reduced complexity of the replication

process taking place (Kasiviswanathan et al., 2012). UMSBP (Universal minicircle sequence binding protein) (Tzfati et al., 1992), p38 (Liu et al., 2006), p93 (Li et al., 2007), and hypothetical protein (Tb927.2.6100) (Beck et al., 2013) are examples of the kinetoplastid-specific proteins. UMSBP and p38 are involved in binding to minicircle origin of replication, while the exact function of the latter two proteins is still uncertain.

1.4.2 Life without the kinetoplast

The kDNA and the editing of its transcripts are essential for the viability of all cell cycle stages of *T. brucei* (Ammerman et al., 2013; Jensen and Englund, 2012) and therefore are extensively studied as promising drug target against this deadly parasite. Surprisingly there are some flagellates that manage to live with damaged and nonfunctional kDNA, or even in total absence of it (Schnaufer et al., 2002). They have been compared, for good reasons, to the petite mutants of *S. cerevisiae* (Lai et al., 2008).

As described above, the PS mitochondrion is fully developed and metabolically active, while in the BS the energy is mostly obtained via glycolysis, with the mitochondrion being highly reduced. Still, kDNA and RNA editing remain essential, as the ATP synthase is still needed (Brown et al., 2006; Schnaufer et al., 2005). The BS also needs to keep its kDNA intact in order to complete the life cycle and return back to tsetse fly. However, it is easy to imagine that some part of the kDNA that are not necessary for the BS can be damaged. Flagellates carrying such kDNA are termed diskinetoplastic (Dk) if they lost part of the kDNA, or akinetoplastic (Ak) if the loss of kDNA is complete (Lai et al., 2008). Such a trypanosome will never be able to complete the standard life cycle and proceed with its development in the insect vector.

However, as one door closes, another opens – such a trypanosome no longer depends on its insect vector and therefore is no longer bound to Africa. This is the case of *T. b. equiperdum* and *T. b. evansi*, causative agents of dourine and surra, widespread diseases of horses, camels and buffalos (Desquesnes et al., 2013). For over 100 years, they were considered separate species, but these trypanosomes are genetically virtually identical to *T. brucei* and the difference lays mostly in the kDNA content (Carnes et al., 2015). *T. b. equiperdum* still retains part of the maxicircle kDNA, while *T. b. evansi* is completely devoid of maxicircles. Regarding kDNA minicircles – in both *T. brucei* subspecies, a gradual loss of minicircle classes and their homogenization have been documented (Lai et al., 2008). Interestingly, despite severely damaged or even completely missing kDNA, the cells retain import of all replication and editing proteins from the nucleus that are completely useless in the absence of organellar nucleic acids (Lai et al., 2008). The Dk and Ak cells also have to maintain their membrane potential. Recent data show that this is achieved by single point mutation of F_1F_0-ATPase subunit γ that is able to compensate for the loss of the single kDNA-encoded subunit (Dean et al., 2013). This event does not seem to be rare and it was suggested that there is a continuous flow of new individuals out of Africa (Lai et al., 2008; Lun et al., 2010; Schnaufer et al., 2002). On the other hand, this process does not seem to happen that easy, as so far there is only one successful attempt to create the Ak cells in the laboratory (Stuart, 1971).

1.5. Protein synthesis in the mitochondrion

Even though most of the genes of the endosymbiont have been relocated into the nucleus, in most aerobes there are still genes present in the organellar genome. Although the exact number of genes present in the mitochondrial genome varies depending on the organism, usually it encodes genes for rRNAs, tRNAs and several very hydrophobic proteins involved in the electron transport chain such as NADH dehydrogenase, cytochrome bc_1, cytochrome *c* oxidase and F_1F_0 ATPase (Chacińska and Boguta, 2000). Because of these protein-coding genes, the mitochondrion has retained complex replication, transcription and translation machineries. If these processes are compromised, the whole function of mitochondrion collapses. Mutations in the mitochondrial genome and the proteins involved in its maintenance have been connected to several human diseases (Calvo and Mootha, 2010; Copeland, 2008; El-Hattab and Scaglia, 2013; Goffart et al., 2009; Scharfe et al., 2009; Tyynismaa et al., 2005).

In case of *T. brucei*, mitochondrial ribosomes translate 18 mRNAs (Verner et al., 2015). Protein synthesis in its single mitochondrion has several unique features: i/ most mRNAs undergo extensive editing before translation (Stuart et al., 2005); ii/ all tRNAs have to be imported from the cytosol (Alfonzo and Söll, 2009); iii/ its 9S and 12S rRNAs are the smallest known (de la Cruz et al., 1985); iv/ mitochondrial protein synthesis is resistant to chloramphenicol (Horváth et al., 2002); v/ finally, an additional 45S particle (45S SSU*) resembling ribosome, that contains only the 9S rRNA, is part of the translation machinery (Maslov et al., 2007; Ridlon et al., 2013). After a lot of effort it was demonstrated that the edited mRNAs are indeed translatable, and the de novo synthesized proteins can be followed in two dimensional gels, departing from the main diagonal (Horváth et al., 2002; Ridlon et al., 2013; Týč et al., 2010a).

1.5.1 RNA editing

As already mentioned, many mitochondrial-encoded mRNAs undergo RNA editing during their maturation, to be rendered translatable. This phenomenon, now known to be widespread among eukaryotes, was actually discovered in *T. brucei* (Benne et al., 1986).

RNA editing is an extensive remodeling of the RNA sequence, in trypanosomes occurring in the form of multiple uridine insertions and deletions (Stuart et al., 2005). The process starts at the 3’ end of a given pre-mRNA and continues to its 5’ end (Maslov and Simpson, 1992), and uses gRNAs that serve as templates for specifying the exact positions of uridine insertions and deletions (Lukes et al., 2005). During this process frame shift changes occurs, start and stop codons are introduced in some cases and alternative editing was shown as a way to produce multiple proteins from a single transcript (Ochsenreiter et al., 2008). Dozens but perhaps hundreds of dedicated enzymes are required for the process, such as uridine-specific exonuclease, terminal uridylyltransferase, RNA ligase, RNA-binding proteins etc. The whole pathway has been extensively studied, but we still do not know some of its critical components (Ammerman et al., 2013; Aphasizhev et al., 2004; Benne, 1994; Hashimi et al., 2008; Kafková et al., 2012; Simpson et al., 2004b; Stuart et al., 2005).

1.5.2 Mitochondrial ribosomes

Ribosomes are high molecular weight complexes that carry out protein synthesis following the mRNA template. They always consist of two subunits – large subunit (LSU) and small subunit (SSU) both made up from rRNAs and proteins. In fact, enzymatically they are ribozymes. Proteins play still essential but likely only structural and supporting functions for the ribosomal stability, as they are mainly located on the surface of both subunits (Mears et al., 2006).

Mitochondrial ribosomes are derived from prokaryotic ribosomes of the bacterial ancestor of the organelle, and they indeed share more features with the bacterial ribosomes than with the cytosolic eukaryotic ones (O'Brien, 2002). Sedimentation coefficient of a typical prokaryotic ribosome is 70S with separate subunits 50S (LSU) and 30S (SSU). The small subunit contains 16S rRNA, and the large subunits contain 23S and 5S rRNAs (Schmeing and Ramakrishnan, 2009).

The sedimentation coefficient of mitochondrial ribosomes varies from the smallest one of 50S in *Leishmania* (Maslov et al., 2006) up to 78S in plants (Leaver and Harmey, 1972). In many organisms, the reduction of mitochondrial ribosomes took place, as f.e. mammalian mitochondria contain ribosomes sedimenting at 55S, with the rRNAs also reduced. Mitoribosomes are actually physically larger than the bacterial ones due to higher protein content, which is thought to compensate for the loss of rRNA (O'Brien, 2002).

Trypanosomatid mitochondrial ribosomes are the smallest known among the eukaryotes (50S total with LSU sedimenting at 40S and SSU at 30S) and with the shortest rRNAs ever described (12S and 9S, respectively) (Maslov et al., 2006). During the rRNA shrinkage, even the active site responsible for chloramphenicol sensitivity was lost in trypanosomatids resulting in their resistance to this drug (Eperon et al., 1983). Although rRNAs are not very well conserved in the primary sequence, it seems that secondary structures are more important and therefore kept (de la Cruz et al., 1985). Despite extremely reduced rRNAs, the total size of mitochondrial ribosomes of trypanosomatids is not as affected as in other mitochondrial ribosomes. It was found that they are also protein rich, look porous and many unique proteins have been recruited to the trypanosomatid mitochondrial ribosome, which is in correlation with possible need to compensate for very small rRNA (Aphasizheva et al., 2011; Maslov et al., 2007; Zíková et al., 2008).

Mitochondrial translation machinery of trypanosomatids features an additional unique complex named 45S SSU-related complex (45S SSU*). It can be detected by electron microscopy (Maslov et al., 2007; Sharma et al., 2009) and was shown to be essential for mitochondrial translation and survival of the cell (Ridlon et al., 2013). The whole complex is putatively composed of two subunits. One of the subunits corresponds to the SSU, while the second one is novel (Maslov et al., 2007) and contains a variety of proteins so far not associated with the ribosome function (Aphasizheva et al., 2011; Zíková et al., 2008). Interesting is the involvement of proteins containing the pentatricopeptide or tetratricopeptide repeat domains. These domains are able to bind specifically various RNAs and they are frequently found in proteins involved in RNA binding, splicing and editing (Aphasizheva et al., 2011). Indeed, proteins carrying these domains were found in trypanosomatid mitochondrial ribosome, RNA editing complexes and polyadenylation machinery (Aphasizheva et al., 2011). Function of 45S SSU* complex is still unknown but it may play a role in the mitoribosome by discriminating mature mRNAs from the pre-edited ones.

1.6. Chaperones

Chaperones or heat-shock proteins (HSPs) is a group of proteins that help the cell to maintain its inner environment and general homeostasis by facilitating folding and stabilizing other proteins. They were described as up-regulated after a heat shock (Schlesinger, 1990), but any other cellular stress such as oxidative stress condition would induce their expression too, because stress conditions inevitably lead to conformational damage of cellular proteins (Feder and Hofmann, 1999). Under normal conditions, chaperones are essential for folding, assembly, secretion, intracellular localization, and degradation of proteins (Young et al., 2004). All these functions of HSPs are so fundamental for the survival of the cell that chaperones belong to the most conserved genes among all domains of life and can be easily found in all available genomes (Boorstein et al., 1994).

Traditionally, HSPs are classified into families based on their sequence and molecular weight (Lindquist and Craig, 1988). During diversification of organisms, there were many multiplication events of these genes and specialization of some of them regarding the function as well as localization (Boorstein et al., 1994). They were all found in the genomes of trypanosomatids, some of them are even present in unexpectedly high numbers (Folgueira and Requena, 2007; Louw et al., 2010). This might be due to the complex life cycle, as there are big temperature differences and other (host immunity, etc.) stresses induced by switches between different stages, which have to be reflected in parasite's biology and its adaptations (Maresca and Carratù, 1992).

1.6.1 Hsp70

Is one of the most widely known chaperones present in all domains of life and belongs to the most conserved proteins (Boorstein et al., 1994). In principle it performs all the chaperone functions described above from protein folding, preventing aggregation, translocations across the membranes to protein degradation. In short, it does the quality control of proteins in the cell (Mayer and Bukau, 2005). We can distinguish two types of Hsp70 genes: first ones have inducible expression as an answer to the stress conditions, while the second ones are constitutively expressed. There are also specialized paralogs localizing to cytoplasm, mitochondrion and endoplasmic reticulum (Gupta et al., 1994).

Hsp70 consist of three domains, namely the N-terminal ATPase domain, the linker and the C-terminal substrate-binding domain (Louw et al., 2010). This chaperone is an ATPase and oscillates in cycle between the ATP-bound state with low affinity for hydrophobic peptide segments, and the ADP-bound state with high affinity for substrates (Szabo et al., 1994). Hsp70 associates with the hydrophobic parts of the misfolded proteins, thus preventing their aggregation or unwanted interactions with other proteins.

Hsp70 protein does not act alone. It needs co-chaperons and other partners to function properly. The shift between ATP and ADP bound state is facilitated by nucleotide exchange factors inevitable for all the processes where Hsp70 is involved. Its primary function is to release the ADP from mtHsp70 and therefore support its continuous cycling (Hartl and Hayer-Hartl, 2002). Interestingly, convergent evolution led to the emergence of two unrelated proteins (Mayer and Bukau, 2005). GrpE can be found in prokaryotes, and its relative Mge1 (mitochondrial GrpE) is present in the mitochondrion (Miao et al., 1997). While the same role is in the cytosol of the eukaryotic cell fulfilled by the Bag proteins

(Young et al., 2004). Contrary to the ubiquitous nucleotide exchange factors, there are the J proteins, which contain the so-called J and Zn-finger domains. They belong to a group of interacting partners with mtHsp70 that stimulate the ATPase activity (McCarty et al., 1995). They are more diverse and provide the specificity of the given reaction (Fan et al., 2003). In eukaryotes they are called mtHsp40 chaperones while in *E. coli* they are known as the DnaJ proteins (Fan et al., 2003; Gur et al., 2005).

1.6.2 Mitochondrial chaperones: mtHsp70, mtHsp40, Mge1 and Hep1

Mitochondrial Hsp70 (mtHsp70) is the organellar version of the cytosolic Hsp70. Mitochondrion usually contains a single type of a constitutively expressed mtHsp70 protein (Folgueira and Requena, 2007), which takes part in many essential processes in the organelle, such as the folding of newly synthesized proteins, as well as the folding of damaged and aggregated ones (Folgueira and Requena, 2007). It is also implicated in the degradation of denatured and unstable proteins (Voos and Röttgers, 2002). Moreover, the mitochondrial version of Hsp70 gained new functions such as the one in Fe-S cluster biogenesis (Dutkiewicz et al., 2003; Lill and Mühlenhoff, 2008), mtDNA replication and maintenance (Týč et al., 2015) and protein import across the organellar double membrane (Liu et al., 2003; Voos and Röttgers, 2002). Interestingly mtHsp70 is more closely related to its bacterial homolog DnaK than to its cytosolic counterparts (Gupta et al., 1994).

Among the organellar co-chaperones of mtHsp70 belongs the nucleotide exchange Mge1 needed for all functions of the chaperone (Miao et al., 1997; Schmidt et al., 2001; Slutsky-Leiderman et al., 2007) and the J domain-containing proteins (mtHsp40s) specific for the given functions. Examples of mtHsp40 proteins are Mdj1 protein necessary for protein folding (Voos and Röttgers, 2002), Jac1 in Fe-S cluster biogenesis(Lill and Mühlenhoff, 2008) and Pam16 and Pam18 involved in protein transport (Dudek et al., 2013).

Few years ago new protein was identified and connected to the mtHsp70 and named Hep1. This abbreviation stands for Hsp70 escort protein, which was later found to affect protein transport and is known to contain the Zn-finger domain. The most important finding was that mtHsp70, which is helping others proteins to fold properly, is prone to aggregation itself (Sichting et al., 2005) and Hep1 is the co-chaperone that serves in order to prevent such mtHsp70 self-aggregation. The structure of Hep1 is already known (Momose et al., 2007), and the binding to mtHsp70 has been described in details. Hep1 interacts with the linker part of the mtHsp70 connecting the ATPase and protein binding domains (Blamowska et al., 2010).

Chapter 2. Objective of the research

- Investigation into the diversity, host specificity and biogeography of trypanosomatids parasitizing Dipteran hosts.
- Investigation into the functions of conserved genes within the mitochondrion of *T. brucei*
- Investigation and description of mitochondrial chaperones mtHsp70/mtHsp40 and their putative association with mitochondrial DNA
- Analysis of the mtHsp70 tendency to aggregate
- Functional analysis of selected mito-ribosomal proteins

Chapter 3. Summary of results and discussion

Highlights of the core findings of the presented thesis:

(i) Dipteran hosts host their own specific clades of trypanosomatid parasites. 24 new TUs (typing units or molecular species) were discovered in brachyceran flies. Many TUs are truly cosmopolitan including the *Angomonas* and *Strigomonas* clades previously known only from South America. Our results also show that multiple infections of trypanosomatid parasites are in dipteran hosts more common than in hemipteran bugs.

(ii) The mtHsp70/mtHsp40 machinery is indispensable for proper replication of mitochondrial (= kinetoplast; k) DNA of *T. brucei*, which is lost in the absence of these enzymes. The observed phenotype corresponds to those triggered by depletion of proteins involved in early stages of kDNA replication.

(iii) MtHsp70 aggregates under various conditions with temperature being the most important factor. MtHsp70 also forms aggregations even in the absence of its co-chaperones Hep1, Mge1 and mtHsp40.

(iv) Three proteins (PNKD-like, mtYsxC and RSM22) containing conserved domains and were associated with mitochondrial ribosome are needed for the *de novo* protein synthesis in the mitochondrion of *T. brucei*. RSM22 was shown also to be important for the structural integrity of *T. brucei* mt ribosome.

3.1 Monoxenous trypanosomatids parasitizing flies

First project of this thesis intended to extend our knowledge about the monoxenous trypanosomatids, with the focus on brachyceran flies, which were collected on several continents (Týč et al., 2013). Although shown to be highly prevalent in dipterans (Podlipaev 1990), trypanosomatids were studied only occasionally in these hosts, as compared to extensive studies of these flagellates in the hemipteran bugs. Until this study, only few notes on trypanosomatids were available from dipterans, almost invariably focusing on morphological features only (Borghesan et al., 2013; Teixeira et al., 2011; Wilfert et al., 2011).

We chose the Brachyceran group of flies for our study, as due to their feeding strategy that does not include predatory behavior, they should contain only their own specific parasites. It was shown previously that insect predators such as the reduviid bugs have the highest diversity of parasites, which is likely caused by unspecific trypanosomatid infections obtained from their prey (Votýpka et al., 2012; Westenberger et al., 2004).

Samples were obtained from the following countries of four different continents: Bulgaria, Czech Republic, Ecuador, Ghana, Kenya, Madagascar, Mongolia, Papua New Guinea and Turkey. Such extensive sampling allowed us to address phylogenetic relationships, host specificity and geographic distribution of isolated flagellates from an almost global perspective. Spliced leader (SL) RNA gene repeats and small subunit (SSU) rRNA genes were used for the analyses.

In total, 40 positive fly specimens were included in the study, from which 36 different TUs of trypanosomatid parasites were derived, with 24 being novel. Multiple infections were found in more than 30% hosts, which is an unprecedently high occurrence, especially as compared with similar studies of the heteropteran insects (Votýpka et al., 2012; Votýpka et al., 2010).

Importantly brachyceran parasites are also more host-specific on the genus level. Indeed, strains isolated from dipteran insects from different locations around the world are more closely related to each other than to trypanosomatids from heteropteran insects caught at the same locations (Maslov et al., 2013; Votýpka et al., 2012; Votýpka et al., 2010). This study shows that members of the genera *Herpetomonas* and *Angomonas* are primarily associated with dipteran hosts, while *Blastocrithidia* and the ‘‘jaculum’’ clade are primarily found in heteropteran hosts. The subfamily Leishmaniinae and the genus *Strigomonas* along with the ‘‘collosoma’’ clade accommodate parasites from both host groups. Our results are in favour of the scenario that postulates most parasites being rather specific for a group of phylogenetically related hosts than being generalists (Poulin and Keeney, 2008).

From the geographical point of view, it is relevant to point out that many TUs are widely distributed around the world, and several can be considered as true cosmopolitans, including members of the endosymbiont-carrying genera *Angomonas* and *Stringomonas*.

3.2 Conserved proteins in the mitochondrion of *T. brucei*

Second part of the thesis focuses on molecular biology of *T. brucei*, which is the causative agent of deadly human and animal sleeping sickness (Barrett et al., 2003). I decided to dissect the function of mitochondrial proteins that are highly conserved among eukaryotes, yet their function is unknown or only poorly known. *T. brucei* is a model organism which has a single mitochondrion, and thanks to its amenability to various methods of forward and reverse genetics, is particularly suitable for the studies of mitochondrial processes and proteins, as discussed below.

Most research on mitochondria was so far performed on relatively closely related eukaryotes, mostly belonging to the supergroup Ophistokonta, which include yeast and human. *T. brucei* is a member of the arguably ancestral supergroup Excavata (Cavalier-Smith, 2010), and its studies have a potential to provide new insight from the evolutionary point of view.

Using all publicly available information in the online databases (Aslett et al., 2010), we were able to identify several interesting genes of high conservation and widespread presence in the mitochondrion across eukaryotic supergroups. Next, we proceeded to use available molecular tools in order to knock respective gene down, and/or to introduce its modified (usually tagged) version back into the parasite. Convenience and efficiency of RNA interference allowed detailed investigations of phenotypes that occur after the ablation of targeted transcript.

3.2.1 Mitochondrial chaperones and mitochondrial DNA

In frame of the second project presented in this thesis we examined mitochondrial Hsp70 and its partners in *T. brucei*. Mitochondrial chaperones are multifunctional enzymes, essential for the organellar homeostasis. The multifunctional mtHsp70 plays a role in folding and quality control of proteins (Dutkiewicz et al., 2003; Voos and Röttgers, 2002), Fe-S cluster biogenesis (Dutkiewicz et al., 2003; Lill and Mühlenhoff, 2008) and also in protein import across the organellar double membrane (Liu et al., 2003; Voos and Röttgers, 2002). In *T. brucei* we focused at the so far overlooked function of the highly conserved mtHsp70/mtHsp40 machinery in the replication and maintenance of mitochondrial DNA. In humans, mutations in or loss of mtDNA are associated with mitochondrial dysfunction and a variety of neural and muscular diseases (Calvo and Mootha, 2010; El-Hattab and Scaglia, 2013). Therefore understanding the maintenance and replication of mtDNA is a very important question, which is still far from being answered.

The mitochondrial DNA of *T. brucei* known as kinetoplast DNA (kDNA) is an excellent model that can be used to address role of protein involved in mitochondrial replication as it replicates only once per cell cycle, kDNA has clearly defined structure and is easily observable via DAPI staining and fluorescence microscopy, as well as electron microscopy. The whole process of replication and segregation of kDNA is extensively studied and several specific methods had been developed to explore kDNA replication in detail (Jensen and Englund, 2012).

We were able to show that the chaperones mtHsp70, mtHsp40 and their co-factor Mge1 are all essential for survival of the flagellate upon RNAi induction and the proteins (PTP-tagged or in case of mtHsp70 visualized by specific monoclonal antibody) are equally

distributed throughout the whole mitochondrion (Týč et al., 2015). Mitochondrial localization is actually not surprising when other functions of the chaperones that are needed in the lumen of the organelle are considered. It was shown previously that mtDNA polymerase 1D changes its localization during the cell cycle (Concepción-Acevedo et al., 2012) and we wandered if proteins studied by us are subject to similar redistribution. It is easy to imagine that enzymes important for kDNA replication would be needed only during the S phase and therefore acquire a different localization during the cell cycle, yet the localization of the studied chaperones did not exhibit any detectable changes.

After the RNAi-mediated ablation of the target chaperones we observed gradual loss of kDNA, monitored both by DAPi staining and transmission electron microscopy. Southern blot analysis of total DNA was employed in order to show that the kDNA loss is indeed caused by the decrease in overall kDNA minicircle and maxicircle content, and not just by their redistribution or disintegration of the kDNA network. In fact, maxicircles were affected predominantly and almost completely lost. Moreover, Southern blot analysis proved that it is the kDNA replication, which is affected in the absence of these chaperones.

The obvious question that has to be answered before claiming that these multifunctional enzymes are really indispensable for kDNA maintenance and replication was whether their other functions are not causing the observed phenotype. Therefore, cells ablated for proteins involved selectively in Fe-S biogenesis or protein import were checked and we were able to provide evidence that their disruption does not lead to kDNA loss and hence, the phenotype obtained following the down-regulation of mtHsp70, mtHsp40 and Mge1 was specific and primary.

Our finding confirms hints in the literature suggesting that these chaperones play a role in the replication of mt DNA, as they were found in the mt nucleoids (Bogenhagen et al., 2008; Ciesielski et al., 2013; Effron et al., 1993; Engman et al., 1989; Nosek et al., 2006; Sakasegawa et al., 2003; Wang and Bogenhagen, 2006), and in some cases even affected the levels and functionality of the mt DNA (Duchniewicz et al., 1999; Hayashi et al., 2006). The fact that bacterial homologues of mt chaperones were indeed shown to play a role in the replication of chromosomal (Sakakibara, 1988), plasmid (Sozhamannan and Chattoraj, 1993) as well as the bacteriophages DNA in *Escherichia coli* (Hoffmann et al., 1992) further confirms conservancy and importance of this so far overlooked function of the mtHsp70/mtHso40 machinery.

In this study we provide unambiguous evidence that mtHsp70/mtHsp40 play an important role in the mt DNA replication and maintenance. Importantly, the observed phenotype is comparable to phenotypes of other enzymes that play a role in the initial stages of replication of the *T. brucei* kDNA, including primase Pri1 (Hines and Ray, 2010), DNA polymerases Pol1D and Pol1C (Chandler et al., 2008; Klingbeil et al., 2002) and the origin of replication binding protein p38 (Liu et al., 2006).

3.2.2 MtHsp70 tendency to aggregate

The mtHsp70 chaperone, which is known to help other proteins to fold properly, is itself prone to aggregation. About a decade ago, a protein that can prevent this aggregation Hep1 (Hsp70 escort protein) was identified (Sichting et al., 2005). It was found in parallel by several teams, which resulted in the existence of two additional alternative names: Zim15 (Burri et al., 2004) and Tim17 (Yamamoto et al., 2005). Hep1 proteins are found only in the

mitochondria and chloroplasts and so far were not identified in prokaryotes, suggesting that they evolved as an adaptation after the symbiosis (Kluth et al., 2012; Willmund et al., 2008). Aggregation of mtHsp70 has been hypothesized to be caused by new functions gained throughout its evolution within these organelles. It is believed that Hep1 is important for the *de novo* folding of mtHsp70 after its import into the organelle (Blamowska et al., 2012; Willmund et al., 2008).

The question whether the main function of Hep1 is to prevent the aggregation of mtHsp70 is still open, as it is possible that any protein able to bind to mtHsp70 can protect it from aggregation (Momose et al., 2007). Indeed, Mge1 was also shown to prevent mtHsp70 aggregation in the mitochondrion (Momose et al., 2007), but the situation seems to be different in the chloroplast, where Mge1 has no such effect (Willmund et al., 2008). Our study confirms the hypothesis by Momose et al. (2007) that any protein that binds to mtHsp70 can have stabilizing effect, as our preliminary data indicates that in the *T. brucei* mitochondrion mtHsp70 not only aggregates when Hep1 is missing, but does so also in the absence of its other mtHsp70 co-chaperones, Mge1 and mtHsp40. In fact we observed also some limited aggregation after the depletion of Tim17, which is, in collaboration with mtHsp70, involved in protein import. On the other hand, almost no aggregation was observed in enzymes involved in the Fe-S cluster assembly, namely Isd11 and IscU. Unexpectedly, some aggregation was detected in these knock-down cell lines under elevated temperatures indicating that the whole process is more complicated than originally thought.

Our preliminary results show that in *T. brucei*, mtHsp70 aggregates under various conditions with temperature being the most important factor, a situation highly reminiscent of that in yeast (Sanjuan Szklarz et al., 2005). In addition we observed that the Hep1 co-chaperone is able to provide protection of the *T. brucei* cells during a heat-shock, again replicating similar observation made in the case of Hep1 in yeasts (Sichting et al., 2005).

3.2.3 Putative mito-ribosomal proteins

As discussed previously, the loss or damage of the mt DNA is usually lethal for the cell, as its mitochondrion fails to express essential organellar proteins. The same outcome occurs if other processes such as transcription and translation of those proteins are compromised.

Mitochondrial ribosomes were subject to considerable changes during evolution, the most significant being the reduction of their rRNA component and the acquisition of additional proteins that are thought to compensate for the diminution of rRNA (Smits et al., 2007). Their sedimentation coefficient (S) varies between the lowest value of 50S in *Leishmania* (Maslov et al., 2006) and 78S in plants (Leaver and Harmey, 1972). Mammalian ribosomes have a mass similar to the bacterial ribosomes, yet they contain only about half of the rRNA and incorporate about twice as many proteins. Purification of all proteins from such a big complex as is the ribosome is not straightforward, and hence the exact composition of this structure is still not completely known. It has to be taken into consideration that the problems with purification of total ribosomal proteome may to some extent explain differences in ribosomal composition among the organisms examined (Desmond et al., 2011; O'Brien, 2003; Zíková et al., 2008).

Protein synthesis in the single mitochondrion of *T. brucei* has been extensively studied and assays addressing mt translation and ribosome stability are already available (Horváth et al., 2000; Maslov et al., 2007; Ridlon et al., 2013; Týč et al., 2010a; Zíková et al., 2008),

making this flagellate a suitable model for dissecting the function of three putative mito-ribosomal components.

In our survey, in frame of which we were looking for mt proteins with unknown function highly conserved between human and *T. brucei*, we came across three proteins that according to the high throughput TAP-tag (tandem affinity purification) analysis (unpublished data) have a connection to the mito-ribosomes. In accordance with the literature, we termed these proteins as RSM22, mtYsxC (mitochondrial YsxC) protein and PNKD-like protein. First two were also identified as possible parts of the *T. brucei* mito-ribosome by Zíková et al. (2008). Moreover, Aphasizheva et al. (2011) performed another set of experiment confirming this assignment for the first two proteins and added also a third one (PNKD-like) as another possible component of the mito-ribosome. According to these studies, mtYsxC is associated with the large ribosomal subunit (LSU), while the other two proteins are connected to small subunit (SSU) (Aphasizheva et al., 2011; Zíková et al., 2008).

The goal of this project is to investigate the association of these proteins with mt translation, and to confirm or disprove their putative ribosomal appurtenance. All three cell lines exhibit growth phenotype upon depletion of the respective protein by RNAi. However, the growth arrest is observable only in glucose-free media when the parasite is not able to use glycolysis and has to rely on ATP production in its mitochondrion. The cells, in which mtYsxC mRNA is targeted showed only a modest growth phenotype, which is likely due to poor RNAi knockdown.

Quantitative PCR approach allowed us to check the levels of mito-rRNAs in the three knock-downs mentioned above. We were able to show that, as would be expected for ribosomal proteins, mito-rRNA of the ribosomal subunit with which the target protein is associated, was down-regulated following RNAi induction. These results indicate that the stability and/or assembly of a given ribosomal subunit were compromised.

Perfromed experiments revealed that the *de novo* mt translation is severely affected long time before a growth phenotype is observable, indicating important function of the candidate proteins in this process. To proof that it is organellar translation that is primarily affected, we investigated activities predating translation, namely transcription and RNA editing. Using mt RNA polymerase as a positive control, we showed by qPCR that both transcription and editing remain unaffected in all three knock-down cell lines.

From the studied proteins, only the yeast homologue of RSM22 was previously shown to be part of the mito-ribosomal SSU and was shown to be essential for yeast survival (Saveanu et al., 2001). This protein is not present in prokaryotes and is thus considered novel subunit of eukaryotic ribosome. Our results in an unrelated eukaryote confirmed its connection to the ribosome, moreover we proved the RSM to be essential for the mt translation and ribosome integrity.

MtYsxC shares domain with YsxC protein of *Bacillus subtilis* and *Staphylococcus aureus* in which it was shown to be important for ribosome assembly (Cooper et al., 2009; Schaefer et al., 2006; Wicker-Planquart and Jault, 2015). It was only suggested that eukaryotes bears organellar targeted homologues of this protein (Leipe et al., 2002), therefore, to the best of our knowledge, results presented herein are the first to prove this hypothesis correct. The fact that all YsxC domain-containing GTPases are likely LSU associated further demonstrates functional conservation of these proteins. Importantly our finding of mtYsxC in the ribosome of eukaryotes, might be important to note as it was considered as potential drug target against bacterial pathogens such as *Staphylococcus aureus* (Cooper et al., 2009).

The last protein on our list is PNKD-like, which contains the lactamase B superfamily domain. As it was identified only in one study to be putative component of the ribosome (Aphasizheva et al., 2011) and it does not play structural role in the ribosome (our study). The protein can be either Trypanosomatid specific or peripherally localized. Among the closest human homologs with highest similarity score is PNKD protein and some of its isoforms. Interestingly, mutations in this gene have been associated with the movement disorder paroxysmal non-kinesigenic dyskinesia (PNKD) (Charlesworth et al., 2013). The mutations identified are located in N-terminal targeting sequence and have been connected to destabilization of the whole protein which is still without the assigned function (Ghezzi et al., 2009; Shen et al., 2011). The possible ribosomal function of PNKD protein might therefore explain the disease symptoms.

We provide evidence that all three candidate proteins are essential for protein translation in the mitochondrion of *T. brucei* and that the SSU is destabilized in the absence of RSM22. Yet direct evidence that the proteins in question are really part of the mt ribosome is still missing. To show this, we will use already prepared TAP- or PTP-tagged cell lines to purify their partner proteins. Mass spectrometry analysis will be used to identify whether the target proteins really interact with other components of the mt ribosome of *T. brucei*.

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Woodward, R., and K. Gull, 1990, Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of Trypanosoma brucei: J Cell Sci, v. 95 (Pt 1), p. 49-57.

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Chapter 4. List of Papers and Manuscripts

Paper I:
Týč J., Votýpka J., Klepetková H., Šuláková H., Jirků M. & Lukeš J. Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): Frequent cosmopolitism and moderate host specificity *Mol. Phyl. Evol.* 69(1), 255-64 (2013). doi: 10.1016/j.ympev.2013.05.024. IF = 4.018

Paper II:
Lukeš J., Skalický T., Týč J., Votýpka J. & Yurchenko V. Evolution of parasitism in kinetoplastid flagellates (Review). *Mol. Biochem. Parasitol.* 195(2):115-122. (2014) doi: 10.1016/j.molbiopara.2014.05.007. IF = 2.243

Paper III:
Verner Z., Basu S., Benz C., Dixit S., Dobáková E., Faktorová D., Hashimi H., Horáková E., Huang Z., Paris Z., Pena-Diaz P., Ridlon L., Týč J., Wildridge D., Zíková A. & Lukeš J. Malleable mitochondrion of *Trypanosoma brucei* (Review). *Int. Rev. Cell. Mol. Biol.* 315*:73-151* (2015) doi: 10.1016/bs.ircmb.2014.11.001. IF = 4.52

Paper IV:
Týč J., Klingbeil M.M. & Lukeš J. Mitochondrial heat-shock protein machinery Hsp70/Hsp40 is indispensable for proper mitochondrial DNA maintenance and replication. *mBio* 6(1):02425-14. (2015) doi:10.1128/mBio.02425-14. IF = 6.875

Manuscript I:
Týč J., Novotná L., Maslov. D & Lukeš J. RSM22, mtYsxC and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei* (Manuscript in preparation)

Manuscript II:
Týč J., Haindrich A.C., Skalický T., Basu S., Flegontov P., Flegontova O. & Lukeš J. Aggregation of the Hsp70 chaperone in the mitochondrion of *Trypanosoma brucei* (Manuscript in preparation)

Paper I

Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): Frequent cosmopolitism and moderate host specificity

Paper II

Evolution of parasitism in kinetoplastid flagellates

Paper III

Malleable mitochondrion of *Trypanosoma brucei*

Paper IV

Mitochondrial heat-shock protein machinery Hsp70/Hsp40 is indispensable for proper mitochondrial DNA maintenance and replication

Manuscript I

RSM22, mtYsxC and PNKD-like proteins are required for mitochondrial translation in *Trypanosoma brucei*

Manuscript II

Aggregation of the Hsp70 chaperone in the mitochondrion of *Trypanosoma brucei*

Appendix

Curriculum vitae

Jiří Týč

Personal details
Born: 24 April 1985, Příbram, Czech Republic
email: tyc.jiri@centrum.cz

Current position
Research assistant at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic

Education
2010 - until now PhD student at the Department of Molecular biology, Faculty of Science, University of South Bohemia in České Budějovice, Thesis*: Kinetoplastids biology, from the group phylogeny and evolution into the secrets of the mitochondrion of one representative: Trypanosoma brucei – the model organism in which new roles of the evolutionary conserved genes can be explored.*

2010 RNDr. Molecular biology, Department of Molecular biology, Faculty of Science, University of South Bohemia in České Budějovice, Thesis*:* YCF45 protein, usually associated with plastids, is targeted into the mitochondrion of *Trypanosoma brucei*

2008-2010 MSc. Molecular biology, Faculty of Science, University of South Bohemia in České Budějovice, graduated with honors, Thesis: Functional analysis of prohibitin in *Trypanosoma brucei*

2005-2008 BSc. Biology, Faculty of Science, University of South Bohemia in České Budějovice, graduated with honors, Thesis: Functional analysis of the YCF 45 gene in procyclic *Trypanosoma brucei*

Research interests
I am interested in molecular and evolutionary parasitology. Mostly, I have been working with *T. brucei* as a suitable model flagellate, but I am also involved in studies exploring the diversity and evolution of monoxenous trypanosomatids in insect.

Intership and stay abroad
Feb 7 – May 8, 2014, DNA Replication Laboratory of Prof. Michele Klingbeil, University of Massachusetts, Amherst, USA

Teaching
Lecturer at project EKOTECH in Advanced Methods of Molecular biology (reg.num. CZ.1.07/2.3.00/09.0200) (Biology Centre of the Academy of Sciences of the Czech Republic, v. v. i.)

Awards
2010 Dean Prize, Faculty of Science, University of South Bohemia, České Budějovice

2008 – 2009 Premium scholarship for outstanding study results, Faculty of Science, University of South Bohemia, České Budějovice

2007 – 2008 Premium Scholarship for outstanding study results, Faculty of Science, University of South Bohemia, České Budějovice

2007 Award for best poster at 11th Evolutionary Biology Meeting at Marseilles, France

Grants and fellowships

2014 EMBO Short-Term fellowship ASTF 539-2013 to cover the stay in Michele Klingbeil laboratory at Amherst, USA

2013 Holz-Conner travel grant to cover travel cost for ICOP meeting at Vancouver, Canada

2013 Principal investigator of project - Impact and importance of conserved hypothetical mitochondrial ribosome proteins on the structure and function of mitochondrial ribosome in *T. brucei*, No. GAJU 110/2013/P (from internal Grant agency of University of South Bohemia)

2012 Principal investigator of project - MtHsp70s connection with mtDNA and tendency to aggregate, No. GAJU 039/2012/P (from internal Grant agency of University of South Bohemia)

2007 Principal investigator of project - What is the function of the chloroplasts gene YCF 45 in *Trypanosoma brucei*?, No. SGA2007/004 (from internal Student grant agency of Faculty of Science, University of South Bohemia)

Publications

1. **Týč J.**, Klingbeil M.M. & Lukeš J. Mitochondrial heat-shock protein machinery Hsp70/Hsp40 is indispensable for proper mitochondrial DNA maintenance and replication. *mBio* 6(1):02425-14. (2015) doi:10.1128/mBio.02425-14
2. Verner Z., Basu S., Benz C., Dixit S., Dobáková E., Faktorová D., Hashimi H., Horáková E., Huang Z., Paris Z., Pena-Diaz P., Ridlon L., **Týč J.**, Wildridge D., Zíková A. & Lukeš J. The malleable mitochondrion of *Trypanosoma brucei* (Review). *Int. Rev. Cell. Mol. Biol.* **315*:73-151*** (2015) doi: 10.1016/bs.ircmb.2014.11.001
3. Lukeš J., Skalický T., **Týč J.**, Votýpka J. & Yurchenko V**.** Evolution of parasitism in kinetoplastid flagellates (Review). *Mol. Biochem. Parasitol.* 195(2):115-122. (2014) doi: 10.1016/j.molbiopara.2014.05.007
4. **Týč J.**, Votýpka J., Klepetková H., Šuláková H., Jirků M. & Lukeš J. Growing diversity of trypanosomatid parasites of flies (Diptera: Brachycera): Frequent cosmopolitism and moderate host specificity *Mol. Phyl. Evol.* 69(1), 255-264 (2013). doi: 10.1016/j.ympev.2013.05.024
5. **Týč J.**, Long S., Jirků M. & Lukeš J. YCF45 protein, usually associated with plastids, is targeted into the mitochondrion of *Trypanosoma brucei. Mol. Biochem. Parasitol.* 173, 43-47 (2010).doi: 10.1016/j.molbiopara.2010.05.002
6. **Týč J.**, Faktorová D., Kriegová E., Jirků M., Vávrová Z., Maslov D.A. & Lukeš J. Probing for primary functions of prohibitin in *Trypanosoma brucei*. *Int. J. Parasitol.* 40, 73-83 (2010). doi: 10.1016/j.ijpara.2009.07.008

Conferences

2013 Poster presentation, topic: Highly conserved putative mitochondrial ribosomal proteins in *Trypanosoma brucei,* ICOP meeting at Vancouver, Canada

2013 Poster presentation, topic: Mitochondrial chaperone and kDNA. Kinetoplastid Molecular Cell Biology Meeting at Woods Hole, USA

2013 Poster presentation, topic: Mitochondrial chaperone and kDNA. 43nd International Meeting of Czech Society for Protozoology at Nový Dvůr, Czech Republic

2012 Oral presentation, topic: Mitochondrial chaperone and mitochondrial DNA. European Meeting on Gene Expression in *T. brucei* at Würzburg, Germany

2012 Oral presentation, topic: Mitochondrial chaperone and mitochondrial DNA. 42nd International Meeting of Czech Society for Protozoology at Kouty, Czech Republic

2011 Oral presentation, topic (in Czech): Ssc1, Hep1, Mge1 - mitochondriální chaperon a jeho vlastní chaperony a kofaktory. 41st International Meeting of Czech Society for Protozoology at Benecko, Czech Republic

2010 Oral presentation, topic (in Czech): Prohibitin – mitochondriální protein spojovaný s apoptózou i rakovinou. Co ale ovlivňuje u jednobuněčné *Trypanosomy brucei*? 40th International Meeting of Czech Society for Protozoology at Ledeč nad Sázavou, Czech Republic

2007 Poster presentation, topic**:** Is gene YCF 45 a remnant of a plastid in *Trypanosoma brucei?* 11th Evolutionary Biology Meeting at Marseilles, France (Award for the best poster)

2007 Oral presentation, topic (in Czech): Je gen ycf45 pozůstatkem plastidu u *Trypanosoma brucei*? 37th International Meeting of Czech Society for Protozoology at Vranov nad Dyjí, Czech Republic

Others

Member of Czech Society for Protozoology

2010 - 2015 Reviewer of grant proposals for Student grant agency of the Faculty of Science (SGA)

2013 Main organiser of 43nd International Meeting of Czech Society for Protozoology