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Short communication

A new vesicular compartment in *Encephalitozoon cuniculi*

Kaya Ghosh [a,b,1], Edward Nieves [c], Patrick Keeling [d], Ann Cali [b], Louis M. Weiss [a,e,*]

[a] *Department of Pathology, Division of Tropical Medicine and Parasitology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA*
[b] *Department of Biological Science, 195 University Avenue, Boyden Hall, Rutgers University, Newark, NJ 07102, USA*
[c] *Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA*
[d] *Botany Department, University of British Columbia, 6270 University Blvd., Vancouver BC, V6T 1Z4, Canada*
[e] *Department of Medicine, Division of Infectious Diseases, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

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Abstract

The microsporidia are emerging human and veterinary pathogens known to infect every tissue type and organ system. Their infectious spore possesses a number of peculiar organelles, including the diagnostic polar tube. In a proteomics-driven effort to find novel components of this organelle in the human-pathogenic species *Encephalitozoon cuniculi*, we unexpectedly discovered a protein which localizes to punctate structures consistent with the appearance of relic mitochondria, or mitosomes. However, this novel protein did not colocalize with ferredoxin, a mitochondrial iron–sulfur cluster protein which shows a similar localization pattern by light microscopy. The distribution pattern of this protein thus suggests either a novel vesicular compartment that is similar to mitosomes in size and distribution, the presence of subdomains or branching architecture within mitosomes, or heterogeneity in the protein composition of *E. cuniculi* mitosomes.

Keywords: Microsporidia; Mitosome; Mitochondria; Proteomics; Ferredoxin; Polar vesicles

1. Introduction

The microsporidia are obligate intracellular parasites that cause a wide variety of clinical syndromes in humans, especially in the context of immune suppression such as AIDS, chemotherapy, and organ transplantation [1]. The most common manifestation is diarrhea, which may progress to life-threatening wasting in the setting of HIV-infection. The uniting feature of the phylum is the internal polar tube, a biologically unique structure which lies coiled inside the spore until the moment of germination, when it everts and serves as a conduit for the parasite cytoplasm into the host cell [2,3]. Diagnosis of these pathogens is challenging and treatment options are limited, due partially to the fact that the basic biology of these organisms is not well understood. As recently as the 1990s, it was hypothesized that because they lack typical mitochondria, the microsporidia along with diplomonads (e.g., *Giardia*), parabasalia (e.g., *Trichomonas*), and archaemoebae (e.g., *Entamoeba*) represented primitive eukaryotes, similar to the type of cell that according to endosymbiotic theory might have accepted the first mitochondrial symbiont (discussed in [4]). While phylogenetic evidence at the time seemed to also support the notion of primitivity, this Archezoan hypothesis has since been completely discredited, and over the past fifteen years, molecular and genetic evidence has converged to suggest a close relationship between Microsporidia and Fungi. Mitochondrion-derived heat shock proteins were first reported in 1997 [5], suggesting that microsporidia secondarily lack mitochondria. In fact, the presence of a mitochondrial remnant, or "mitosome", has now been demonstrated in several microsporidia [6–8]. As a result of these and other

* Corresponding author. Albert Einstein College of Medicine, 1300 Morris Park Avenue, Room 504 Forchheimer, Bronx, New York 10461, USA. Tel.: +1 718 4302142; fax: +1 718 4308543.
E-mail address: Louis.weiss@einstein.yu.edu (L.M. Weiss).
[1] Present address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461 USA.

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molecular phylogenetic observations (see [9]) the microsporidia were accorded phylum status in a recently constructed phylogeny of the fungi by a diverse assemblage of sixty-some fungal taxonomists [10]. Thus it is now widely appreciated that the microsporidia are a highly evolved lineage exhibiting extreme reduction at both the genomic and cellular level.

Like the mitosomes of the parasitic protists *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*, and unlike the ATP- and hydrogen-producing parabasalian hydrogenosome (see [4]), the mitosome of many microsporidians (e.g., *Encephalitozoon*) appears to be of limited metabolic consequence to the cell, but has a more clearly supported function in iron–sulfur cluster assembly [11]. Immunolocalization experiments by Goldberg et al. [12] demonstrated that donors of iron and sulphur and the scaffold protein Isu1 colocalize with the mitosomal marker Hsp70, and in the same year Williams et al. showed that the iron–sulfur cluster protein ferredoxin also localizes to small punctate structures consistent with mitosomes [7]. In other microsporidians, however, the recent discovery of a mitosomal alternative oxidase suggests the mitosome is involved in providing a terminal electron acceptor in the species that possess this gene [13]. These exceptions notwithstanding, only a small number of mitosome proteins and functions have been described to date. In this report, we identify a novel protein located to a vesicular compartment with a mitosome-like distribution pattern that was unexpectedly discovered in a shotgun proteomic search for additional components of the polar tube.

2. Methods

2.1. Culture of microsporidia, spore fractionation, and proteomic analysis

Encephalitozoon cuniculi spores were harvested from culture supernatants of infected rabbit kidney cell line RK13 (American Type Cell Culture Collection, Manassas, VA), fractionated to enrich for polar tube proteins, and DTT-solubilized [14,15], as previously described but omitting the urea wash. The lysate was analysed by nanoLC ESI-MS/MS and detected peptides were mapped to hypothetical *E. cuniculi* ORFs also as previously described [14].

2.2. Immunolocalization of hypothetical protein *ECU09_0280* in situ

The ECU09_0280 ORF was cloned and expressed as a recombinant glutathione-*S*-transferase (GST)–tagged protein in a prokaryotic system and detected by Western blotting with a monoclonal anti-GST antibody as previously described [14]. Polyclonal antiserum to this protein was raised in murine hosts for protein localization *in situ* also as previously described. Briefly, a 1:50 dilution of ECU09_0280–GST antiserum was used to stain formaldehyde-fixed, bovine serum albumin-blocked *E. cuniculi*-infected RK13 host cell cultures followed by incubation with a 1:500 dilution of goat anti-mouse IgG conjugated to Alexa Fluor 488. Host and parasite nuclei were stained by 1 μg/mL 4,6-di-amidino-2-phenylindole (DAPI). Where indicated, cultures were co-stained by rabbit anti-ferredoxin polyclonal antibody (1:20) followed by a 1:500 dilution of goat anti-mouse IgG conjugated to ALexa Fluor 594. Stained slides were viewed with an epifluorescence-equipped microscope (Zeiss AxioVert 200M, Carl Zeiss, Goettingen, Germany). Images were captured with a Zeiss Axiocam monochrome digital camera (Carl Zeiss, Goettingen, Germany), and false color was assigned according to the known emission wavelength of each fluorophore. Deconvolved fluorescence images were obtained with an Olympus IX71 inverted microscope equipped with a Photometrics CoolSnap HQ2 CCD monochrome digital camera and softWorx 3.6.0 software.

2.3. Identification of homologs to ECU09_0280

Raw illumina reads from an ongoing *Encephalitozoon hellem* genome project were assembled using Velvet [16] and Consed [17] with parameters described previously [18]. Predicted ORFs on all preliminary scaffolds were searched for homologs of ECU10_1500 and ECU10_1070 using BLAST (Altschul et al., 1997), and the sequence quality and coverage of putative matches was confirmed manually. The *Encephalitozoon intestinalis* [18], *Enterocytozoon bieneusi* [19] and *Nosema ceranae* genomes [20] were also searched for

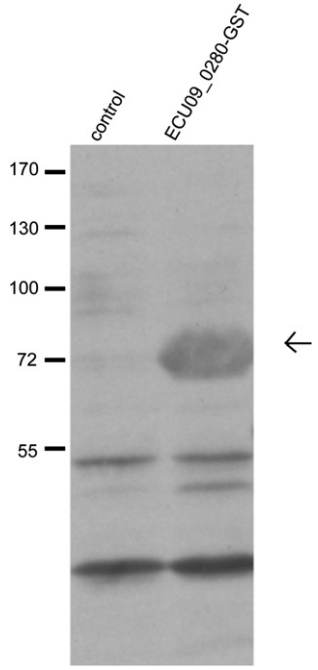

Fig. 1. Heterologous expression of *Enc. cuniculi* hypothetical gene ECU09_0280 in as a GST-fusion protein in *E. coli*. Right lane, expression of recombinant GST-tagged ECU10_1500 is clearly visible as an overexpressed band at approximately 72 kDa (arrow) which is recognized by anti-GST antibody. Left lane, negative control (i.e., bacteria transformed with the same expression vector encoding an unrelated parasite gene). The GST antibody also reacts with GST and adjacent vector-encoded tags at ~32 kDa and non-specific bands from bacterial proteins at ~50 kDa.

homologs of these proteins using BLAST [21]. Microsporidian genome data is available on MicrosporidiaDB (http://microsporidiadb.org/micro/).

3. Results

3.1. Proteomic detection of ECU09_0280 and generation of polyclonal antiserum

The peptide IKDGNAKEGTK was detected by nanoLC ESI-MS/MS (score 31, $p < 0.05$) and mapped to the ECU09_0280 open reading frame. The encoded 39-kDa amino acid sequence was expressed as a recombinant glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli* and detected by Western blot with anti-GST antibody (Fig. 1, arrow).

3.2. Immunolocalization of ECU09_0280

The gene product of ECU09_0280 appears to be localized to discrete small round punctuate structures which frequently appear in groups of two or three close to the nucleus (Fig. 2A, top and bottom panel insets) and sometimes four. The localization pattern of this protein is reminiscent of *E. cuniculi* ferredoxin, a mitochondrial iron–sulfur cluster protein that has been shown to localize to similar punctuate structures *in situ* [7,12]. Notably, ferredoxin-stained structures also occurred most often in groups of two or three around each parasite nucleus. It was noted by the authors that the appearance of these ferredoxin-stained structures was consistent with that of polar vesicles, membrane-bound structures occurring in several different microsporidia which have recently been

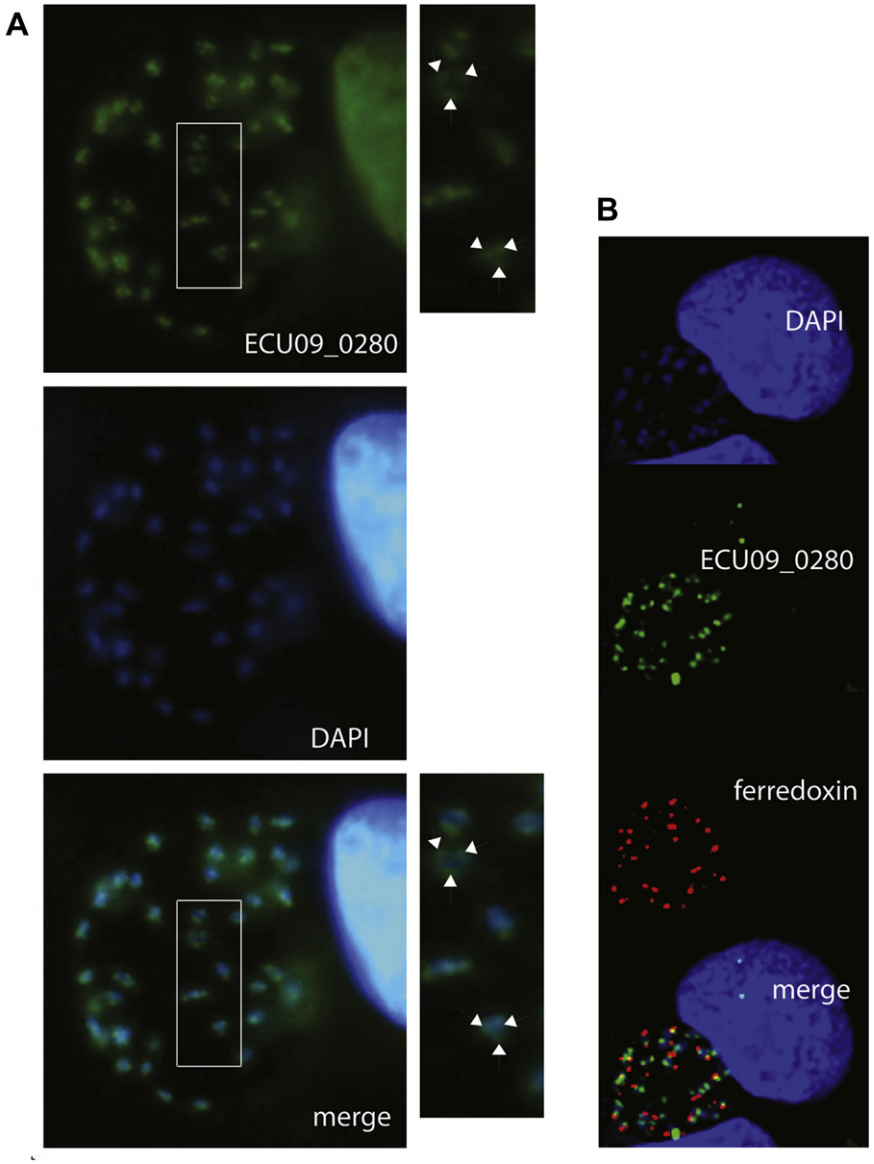

Fig. 2. Immunofluorescence localization of ECU09_0280. (A) This protein is localized to small, discrete, punctuate structures close to the nucleus (insets, top and bottom panels) which frequently appear in groups of two or three (arrows, top and bottom panels). Some antiserum cross reactivity occurs with the host cell nucleus (HN) but not with any other parasite structures. (B) Both ECU09_0280 (green) and ferredoxin (red) localize to structures consistent in appearance and location with mitosomes, but do not colocalize.

proposed on the basis of ultrastructural data to be mitosomes [22]. However, deconvolved images of *in situ* cultures co-stained for ferredoxin and ECU09_0280 (Fig. 2B) demonstrated that while both of these proteins localize to subcellular structures consistent with mitosomes within the parasite, they do not extensively colocalize.

3.3. Identification of homologs to ECU09_0280

ECU09_0280 has no putative conserved domains or significant similarity to characterized proteins or any other *E. cuniculi* proteins, but clear homologs were identified in the human-pathogenic microsporidia *E. hellem*, *E. intestinalis*, and *E. bieneusi*, as well as a potential homolog in the honey bee parasite *N. ceranae* (Fig. 3). No putative mitosome-targeting transit peptide was readily detected for any of these proteins (data not shown), but detection of such sequences in divergent clades such as the microsporidia is often challenging (e.g., see [6]).

4. Discussion

We have previously shown that proteins with biophysical properties similar to the polar tube proteins (PTPs) can co-purify with PTPs during the polar tube fractionation procedure we developed and can be demonstrated to localize to distinct, unrelated compartments within the cell such as the spore wall and the branching proteinaceous filamentous network within the parasitophorous vacuole [14]. Here we have localized a novel protein in *E. cuniculi*, which was isolated by a similar method, and demonstrated that it has a curious subcellular localization pattern similar to previous described mitosomal proteins such as ferredoxin. ECU09_0280 localizes to small punctate tructures near the parasite nucleus which frequently occur in groups of two or three, similar to the staining pattern observed with anti-ferredoxin antibody. Surprisingly, however, ferredoxin and ECU09_0280 did not colocalize, although they did stain markedly similar compartments of the same cell. It seems, then, that the ECU09_0280 protein distribution pattern represents either: (1) a novel vesicular compartment similar to mitosomes in size and distribution about the nucleus; (2) the presence of unique subdomains perhaps due to branching architecture within a mitosome; or (3) a functional or developmental heterogeneity of *E. cuniculi* mitosomes. Regarding the first possibility, it is important to note that the compartment so far shares only morphological similarity with the polar vesicle or mitosome, and only necessarily at the light-level; regarding the second, there is no precedent for sub-compartmentalization of mitosomes other than lumenal versus intermembrane, which would be challenging to resolve by light microscopy. However, it is possible that mitosomes, though comparatively tiny, may possess an as yet unappreciated branching structure as functional mitochondria often do. Electron microscopical investigations would shed light on these first two possibilities, however the currently available antibody does not recognize this protein on immuno-electron microscopy. The third hypothesis, that of mitosomal heterogeneity, is a formal possibility but challenging to prove.

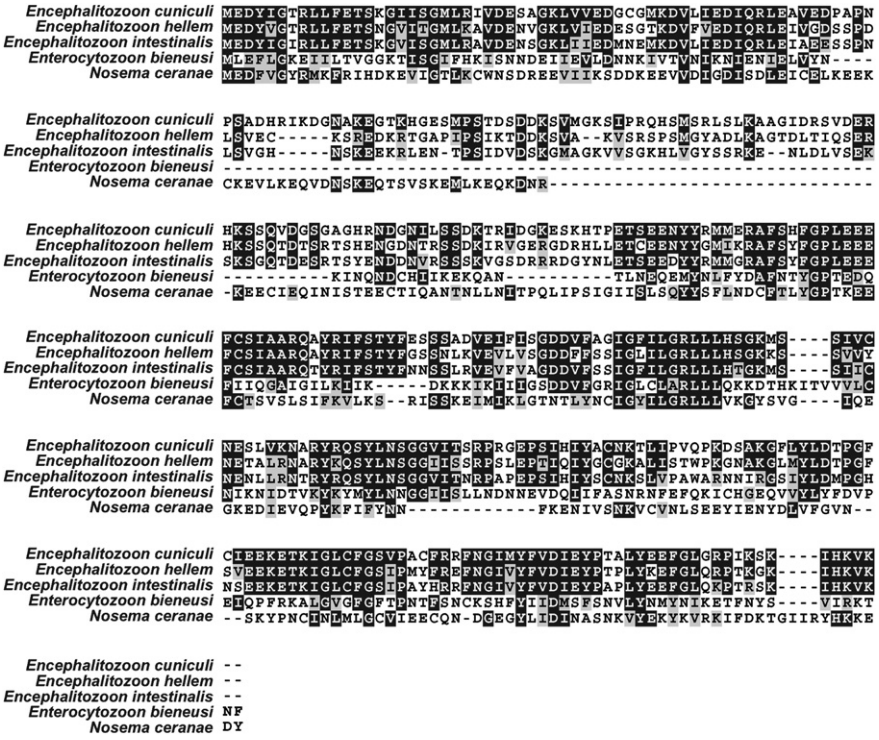

Fig. 3. BLASTP alignment of (a) ECU10_1500 and (b) ECU10_1070 and their presumptive homologs in *Enc. hellem*. Residues highlighted in black or gray indicate identity or similarity, respectively.

Attempts at colocalization with other mitosomal markers such as Hsp70 may prove useful. As little is known about the function of these diminutive relic organelles in the microsporidia, and ECU09_0280 displays no homology to proteins of known function or conserved functional domains, it is difficult to discriminate between these three possibilities, or to speculate on the function of this protein. However, its apparent conservation in the *Enterocytozoon/Encephalitozoon/Nosema* clade which contains many species of medical and agricultural importance suggests an interesting target for further study.

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References

[1] E.S. Didier, L.M. Weiss, Microsporidiosis: current status, Curr. Opin. Infect. Dis. 19 (2006) 485−492.
[2] M. Wittner, L.M. Weiss (Eds.), The Microsporidia and Microsporidiosis, ASM Press, Washington, D.C, 1999.
[3] A. Cali, L. Weiss, P. Takvorian, H. Tanowitz, M. Wittner, Ultrastructural identification of AIDS associated microsporidiosis, J. Eukaryot. Microbiol. 41 (1994) 24S.
[4] A.M. Shiflett, P.J. Johnson, Mitochondrion-related organelles in eukaryotic protists, Annu. Rev. Microbiol. 64 (2010) 409−429.
[5] A. Germot, H. Philippe, H. Le Guyader, Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*, Mol. Biochem. Parasitol. 87 (1997) 159−168.
[6] L. Burri, B.A. Williams, D. Bursac, T. Lithgow, P.J. Keeling, Microsporidian mitosomes retain elements of the general mitochondrial targeting system, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 15916−15920.
[7] B.A. Williams, A. Cali, P.M. Takvorian, P.J. Keeling, Distinct localization patterns of two putative mitochondrial proteins in the microsporidian Encephalitozoon cuniculi, J. Eukaryot. Microbiol. 55 (2008) 131−133.
[8] B.A. Williams, R.P. Hirt, J.M. Lucocq, T.M. Embley, A mitochondrial remnant in the microsporidian Trachipleistophora hominis, Nature 418 (2002) 865−869.
[9] S.C. Lee, N. Corradi, E.J. Byrnes 3rd, S. Torres-Martinez, F.S. Dietrich, P.J. Keeling, J. Heitman, Microsporidia evolved from ancestral sexual fungi, Curr. Biol. 18 (2008) 1675−1679.
[10] D.S. Hibbett, M. Binder, J.F. Bischoff, M. Blackwell, P.F. Cannon, O.E. Eriksson, S. Huhndorf, T. James, P.M. Kirk, R. Lucking, H. Thorsten Lumbsch, F. Lutzoni, P.B. Matheny, D.J. McLaughlin, M.J. Powell, S. Redhead, C.L. Schoch, J.W. Spatafora, J.A. Stalpers, R. Vilgalys, M.C. Aime, A. Aptroot, R. Bauer, D. Begerow, G.L. Benny, L.A. Castlebury, P.W. Crous, Y.C. Dai, W. Gams, D.M. Geiser, G.W. Griffith, C. Gueidan, D.L. Hawksworth, G. Hestmark, K. Hosaka, R.A. Humber, K.D. Hyde, J.E. Ironside, U. Koljalg, C.P. Kurtzman, K.H. Larsson, R. Lichtwardt, J. Longcore, J. Miadlikowska, A. Miller, J.M. Moncalvo, S. Mozley-Standridge, F. Oberwinkler, E. Parmasto, V. Reeb, J.D. Rogers, C. Roux, L. Ryvarden, J.P. Sampaio, A. Schussler, J. Sugiyama, R.G. Thorn, L. Tibell, W.A. Untereiner, C. Walker, Z. Wang, A. Weir, M. Weiss, M.M. White, K. Winka, Y.J. Yao, N. Zhang, A higher-level phylogenetic classification of the fungi, Mycol. Res. 111 (2007) 509−547.
[11] H. Lange, A. Kaut, G. Kispal, R. Lill, A mitochondrial ferredoxin is essential for biogenesis of cellular iron-sulfur proteins, Proc. Natl. Acad. Sci. U. S. A 97 (2000) 1050−1055.
[12] A.V. Goldberg, S. Molik, A.D. Tsaousis, K. Neumann, G. Kuhnke, F. Delbac, C.P. Vivares, R.P. Hirt, R. Lill, T.M. Embley, Localization and functionality of microsporidian iron−sulphur cluster assembly proteins, Nature 452 (2008) 624−628.
[13] B.A. Williams, C. Elliot, L. Burri, Y. Kido, K. Kita, A.L. Moore, P.J. Keeling, A broad distribution of the alternative oxidase in microsporidian parasites, PLoS Pathog. 6 (2k010): e1000761.
[14] K. Ghosh, E. Nieves, P. Keeling, J.F. Pombert, P.P. Henrich, A. Cali, L.M. Weiss, Branching network of proteinaceous filaments within the parasitophorous vacuole of *Encephalitozoon cuniculi* and *Encephalitozoon hellem*, Infect. Immun. 79 (2011) 1374−1385.
[15] E. Keohane, P.M. Takvorian, A. Cali, H.B. Tanowitz, M. Wittner, L.M. Weiss, The identification and characterization of a polar tube reactive monoclonal antibody, J. Eukaryot. Microbiol. 41 (1994) 48S.
[16] D.R. Zerbino, E. Birney, Velvet: algorithms for de novo short read assembly using de Bruijn graphs, Genome Res. 18 (2008) 821−829.
[17] D. Gordon, Viewing and editing assembled sequences using Consed, Curr. Protoc. Bioinformatics. (2003) (Chapter 11) Unit11.12.
[18] N. Corradi, J.F. Pombert, L. Farinelli, E.S. Didier, P.J. Keeling, The complete sequence of the smallest known nuclear genome from the microsporidian *Encephalitozoon intestinalis*, Nat. Commun. 1 (2010) 77. doi:10.1038/ncomms1082.
[19] D.E. Akiyoshi, H.G. Morrison, S. Lei, X. Feng, Q. Zhang, N. Corradi, H. Mayanja, J.K. Tumwine, P.J. Keeling, L.M. Weiss, S. Tzipori, Genomic survey of the non-cultivatable opportunistic human pathogen, *Enterocytozoon bieneusi*, PLoS Pathog. 5 (2009) e1000261.
[20] R.S. Cornman, Y.P. Chen, M.C. Schatz, C. Street, Y. Zhao, B. Desany, M. Egholm, S. Hutchison, J.S. Pettis, W.I. Lipkin, J.D. Evans, Genomic analyses of the microsporidian *Nosema ceranae*, an emergent pathogen of honey bees, PLoS Pathog. 5 (2009) e1000466.
[21] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389−3402.
[22] J. Vavra, "Polar vesicles" of microsporidia are mitochondrial remnants ("mitosomes")? Folia Parasitol. (Praha) 52 (2005) 193−195.