A new method of metabarcoding Microsporidia and their hosts reveals high levels of microsporidian infections in mosquitoes (Culicidae)

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Abstract

Microsporidia are ~~a large and diverse group of~~ obligate intracellular eukaryotic parasites that infect nearly all animal ~~phyla~~groups, including humans ~~and protists~~. The most common molecular methods for Microsporidia detection rely on species-targeting qPCR or end-point PCR using group-specific primers. However, these methods could be not specific enough or fail in case of mixed infections. We developed a method for parallel detection of both microsporidian infection and the host species. We designed new primer sets: one specific for the classical Microsporidia (targeting hypervariable V5 region of ssu rDNA), and a second one targeting a shortened fragment of the COI gene (standard metazoan DNA-barcode); both markers are well suited for a NGS approach. The analysis of ssu rDNA dataset representing 607 microsporidian species (120 genera) indicated that the V5 region enables identification of >98% species in the dataset (596/607). To test the method, we used microsporidians that infect mosquitoes in natural populations. Using mini-COI data, all field-collected mosquitoes were unambiguously assigned to seven species; among them almost 60% of specimens (127/212) were positive for at least 11 different microsporidian species, including a new microsporidian ssu rDNA sequence (Microsporidium sp. PL01). Phylogenetic analysis of Microsporidium sp. PL01 ssu rDNA showed that this species belongs to one of the two main clades in the Terresporidia. In addition, the level of microsporidian mixed infections was relatively high (9.4~~3~~%). The numbers of sequence reads for the OTUs suggest that the occurrence of *Nosema* spp. in co-infections could benefit them; however, this observation should be re-tested using more intensive host sampling. The proposed method for detection of Microsporidia can be applied to all types of DNA extracts, including medical and environmental samples.

~~Since they are studied mostly as zoonotic agents of human disease or veterinary parasites, the actual species diversity in this group probably remains undefined. Here we present a method for rapid and sensitive detection of microsporidia and their hosts based on~~

~~a metabarcoding approach. Our method comprises amplification and sequencing of the V5 region of ssu rDNA using the newly developed primer set specific for the classical Microsporidia. For the host species identification, we propose using a shorter fragment of the standard COI barcode; both markers are well suited for a next-generation sequencing approach. To test the method, we studied microsporidia that infect mosquitoes. The metabarcoding allows detecting of 100 spores per 1 mL which is comparable with conventional PCR-based methods. Applying this method to study microsporidian diversity in natural populations, we found the new species Microsporidium sp. nov. PL01 that dominated in all tested mosquito species. In total, more than half of field-collected mosquitoes analyzed in this study were positive for at least 11 different microsporidian species; also, the level of mixed infections was relatively high (9.43%). Our data suggest that the occurrence of some microsporidians in co-infections could benefit them; however, this observation should be re-tested using more intensive host sampling. A comparison of the metabarcoding and the conventional method for microsporidia detection showed that the metabarcoding is a 3x more sensitive method than standard amplicon sequencing confirmed 37% of the microsporidia-positive samples found by metabarcoding; moreover, Sanger sequencing failed to show mixed samples.~~

Keywords:

rDNA, DNA Barcoding, Molecular Diagnostics, Molecular Phylogeny, Coinfection, *Nosema* spp.

Introduction

Microsporidia are a large and diverse group of obligate intracellular eukaryotic parasites that infect nearly each animal phylum~~a~~ and certain protist species~~protists~~.

Molecular data suggest that Microsporidia and Rozellomycota form a monophyletic group, which is in a sister relation to the fungi (Corsaro et al., 2019; James et al., 2006, 2013; Karpov et al., 2014). The phylum Microsporidia consists of three evolutionary lineages: the so-called "classical Microsporidia" and two poorly studied groups, the Metchnikovellida and the Chytridiopsida. This classification results from structural and developmental characteristics (Vossbrinck, Debrunner-Vossbrinck, & Weiss, 2014); recent molecular phylogenetic analyses seem to support this classification, especially the monophyletic classical Microsporidia (Bass et al., 2018; Corsaro et al., 2019).

Classical Microsporidia are the largest group in the phylum, with more than 1,500 described species belonging to at least 200 genera (Vávra & Lukeš, 2013; Becnel, Takvorian, & Cali, 2014). Moreover, this group includes 17 species causing human disease; the most common belong to the *Enterocytozoon* and *Encephalitozoon* genera (Fayer & Santin-Duran, 2014; Franzen & Müller, 2001; Stentiford et al., 2016). In the immunosuppressed host, including humans, ~~they~~ Microsporidia can infect any organ system; ~~and~~ the most ~~common~~ reported~~s~~ ~~relate to~~ infections concern encephalitis, myositis, ocular infection and sinusitis (Sharma, Balne, & Das, 2014; Wang et al., 2018; Weiss, 2014; Weiss & Schwartz, 2015).

The real species diversity in this group probably is largely unknown, s~~S~~ince Microsporidia are studied mostly as zoonotic and/or waterborne agents of human disease or veterinary parasites~~, the real species diversity in this group probably is largely unknown~~. ~~This especially concerns the species that are parasites of invertebrates and protists.~~ Sometimes, Microsporidia are recorded incidentally during fine structure analysis of their hosts (Radek, Kariton, Dabert, & Alberti, 2015; Ribeiro & Passos, 2006). Many microsporidian species are known only based on unique DNA sequence coding for nuclear ribosomal RNA (henceforth: rDNA) (Vossbrinck, Andreadis, Vávra, & Becnel, 2004; Williams, Hamilton, Jones, & Bass, 2018; Krebes, Blank, Frankowski, & Bastrop, 2010).

Difficulties in the identification of microsporidians result mainly from their specific modifications ~~concerning~~ of both morphological and genomic characteristics. ~~– resulting from their strictly parasitic lifestyle.~~ The classical microsporidian cell is characterized by ~~the~~ mitochondria ~~that have been~~ significantly reduced to mitosomes (Vávra, 2005; Williams, Hirt, Lucocq, & Embley, 2002), the absence of Golgi apparatus (Beznoussenko et al., 2007; Vávra & Lukeš, 2013) and lack of peroxisomes or other simple organelles of this type (Fast, Law, Williams, & Keeling, 2003; Vávra & Lukeš, 2013). In addition, their ribosomes have a prokaryote-like structure with subunits of 50S and 30S (Ishihara & Hayashi, 1968). The microsporidian spores, the only developmental stage with the ability to survive outside the host cell, are equipped with a characteristic infection apparatus, called polar tube or polar filament, which ~~allows for the injection of~~injects infective sporoplasm into the host cell (Franzen, 2004). The length and bending angle of the polar filament along with life cycle observations are the main morphological diagnostic features for the microsporidian species ~~morphological~~ identification ~~of a species in this group~~ (Xu & Weiss, 2005). Increasingly, the description of ~~the~~ new species~~, next to life cycle and morphological characters,~~ is being supplemented by molecular data (Nishikori et al., 2018; Sokolova & Overstreet, 2018; Vávra, Fiala, Krylová, Petrusek, & Hyliš, 2019).

The standard ~~m~~Microsporidia detection methods ~~for the detection of Microsporidia~~ base on ultrastructural assessment of an infected material. Light microscopy-based methods mainly consist in the detection of a thick chitin wall of spores using different stains (Field, Hing, Milliken, & Marriott, 1993; Ignatius et al., 1997; Moura et al., 1997; Peterson, Spitsbergen, Feist, & Kent, 2011; van Gool et al., 1993). ~~However~~On the one hand, the staining for light microscopy rarely enables species identification. ~~, while~~ On the other, electron microscopy has low sensitivity because only a small amount of sample can be examined (Weber, Bryan, Schwartz, & Owen, 1994). To identify pathogenic microsporidia in clinical samples, antigen-based detection assays are used to recognize characteristic

pathogen-specific antigens, mostly located in the spore wall or the polar tube (del Aguila et al., 1998; Furuya et al., 2008; Luján et al., 1998; Singh, Sheoran, Zhang, Carville, & Tzipori, 2005; Zhang et al., 2005). Serological tests are rarely used because they can lead to false results (Hollister, Canning, & Willcox, 1991; Kučerová-Pospišilová & Ditrich, 1998; van Gool et al., 1997).

Nucleic acid-based detection methods are used for both diagnostic and environmental applications. The methods used in medical diagnostics are strictly targeted. Usually, they involve ~~the presence of human-pathogenic microsporidia is confirmed by~~ nested- or quantitive-PCR ~~methods~~ techniques ~~with the use of~~employing species-specific primers (Ghosh & Weiss, 2009; Ghosh, Schwartz, & Weiss, 2014; Ghoyounchi et al., 2019; Javanmard et al., 2018) and probes (Wolk, Schneider, Wengenack, Sloan, & Rosenblatt, 2002; Menotti et al., 2003; Huibers et al., 2018; Verweij, Ten Hove, Brienen, & van Lieshout, 2007; Wang, Orlandi, & Stenger, 2005).

~~Molecular methods for m~~Microsporidia detection in natural populations, on the other hand, base on end-point PCR amplification of ~~DNA fragments coding for ribosomal RNA~~ ~~(~~rDNA~~)~~ fragments and direct Sanger sequencing of the resulted amplicons. Most often, as a target for sequencing, researches amplify fragments of nuclear small subunit rRNA gene (henceforth: ssu rDNA) using ~~universal for~~ Microsporidia-specific (Weiss & Vossbrinck, 1998) or semi-~~universal~~ specific primer sets (e.g. Emsen et al., 2016; Grabner, 2017; Grabner et al., 2015; Quiles et al., 2019). ~~At present~~Presently, the primer pair V1F (Zhu et al., 1993) and 530R (Baker, Vossbrinck, Didier, Maddox, & Shadduck, 1995)~~, targeting about 400-bp covering hypervariable V1-V3 region of ssu rDNA,~~ seem~~ss~~ to be the most commonly used for the detection of microsporidian DNA. V1F/530R primer set targets a fragment of about 400-bp which covers hypervariable V1-V3 regions of ssu rDNA. This approach has proven its effectiveness in numerous microsporidian lineages (e.g. Bojko et al., 2015; Evans, Llanos, Kunin, & Evison, 2018; Simakova, Tokarev, & Issi, 2018; Sokolova, & Overstreet, 2018;

Wattier et al., 2007). However, in case of co-infection with different microsporidian species, such an approach based on direct amplicon sequencing using conventional method could fail due to ambiguous Sanger sequencing results or can give false results if one of the species dominates in the sample.

Recent advances in next-generation sequencing (NGS) have made great progress in studies concerning microbial diversity. The first attempt to use high throughput sequencing for microsporidian DNA detection was performed by Williams et al. (2018). They applied the standard V1F and 530R primers to check the diversity of Microsporidia in environmental samples. Using this primer set, they were able to uncover new microsporidian diversity; however, their raw data contained mostly non-target sequences (see Figure 2 in Williams et al. 2018). The high percentage of non-microsporidian sequences found in this study suggests that the standard V1F/530R primer set is not specific enough to amplify exclusively microsporidian DNA.

Our work aimed to develop a new molecular method for the rapid and sensitive microsporidia detection using a DNA marker better suited for NGS approach. Additionally, we present a new primer set to amplify the short DNA-barcode based on the mitochondrial cytochrome *c* oxidase subunit I (COI) gene for parallel identification of the microsporidian host species. As a model, we used microsporidia that infect mosquitoes (Culicidae). Microsporidia are common to mosquitoes: over 90 microsporidian species have been recorded worldwide from this host. In addition, some microsporidians parasitic in mosquitoes also infect ~~different~~ other species of insects, crustaceans, and vertebrates (Andreadis, 2007; Becnel, White, & Shapiro, 2005; Vossbrinck, Andreadis, Vávra, & Becnel, 2004). ~~Moreover, it has been suggested that microsporidia could influence the infection success of other intracellular pathogens infecting vector species of mosquito, including viruses and *Plasmodium* spp. (Duncan et al., 2012).~~

MATERIAL AND METHODS

Material

To determine sensitivity of the metabarcoding method we used three commercial lines of microsporidian spores: *Encephalitozoon cuniculi* (P103C @ 1x10/6), *E. hellem* (P103H @ 1x10/6), and *E. intestinalis* (P103I @ 1x10/6) from Waterborne Inc. New Orleans, LA. For each species, a series of ten-fold dilutions were prepared, ranging from 10 to 10,000 spores per mL. In addition, to prove that the new method is able to recover microsporidian species in co-infections, four samples consisting of a mixture of *E. cuniculi*, *E. hellem* and *E. intestinalis* spores ~~were~~ was prepared, ranging from 10:10:10 to 10:10:10,000 spores, respectively.

As positive controls, we used DNA isolates of *Anncaliia algerae* and *Vavraia culicis* from the Mosquito and Fly Research Unit, USDA-ARS Center for Medical, Agricultural and Veterinary Entomology (CMAVE), Gainesville, FL, USA and a DNA sample of *Enterocytozoon bieneusi* isolated in the Department of Biology and Medical Parasitology, Faculty of Medicine I, University of Medical Sciences, Poznan, Poland.

~~For~~ As microsporidia-negative control, we used colony mosquitoes believed to be free of microsporidia bred at the USDA-ARS-CMAVE. In total, we analyzed 200 mosquito individuals representing four species of 50 individuals each: *Aedes aegypti*, *Anopheles quadrimaculatus*, *Culex quinquefasciatus* and *Uranotaenia lowii*. ~~(50 individuals per one species).~~

Field-collected mosquito sample consisted of 212 adult females collected between July and August 2016 from periphery of mixed birch-oak and riparian forests, near the city of Poznan, Western Poland. Mosquitoes were ~~harvested~~ collected at night using human-landing catches~~by human landing catch~~. Captured mosquitoes were preserved in 80% ethanol at 4°C until DNA extraction.

DNA extraction

Before DNA extraction, mosquitoes were washed three times in 96% ethyl alcohol. Individual mosquitoes were placed in 1.5 ml Eppendorf tubes until DNA extraction, while ~~T~~the alcohol collected after third washing was filtered through the MF-Millipore Membrane Filter, 0.22 µm pore size (Merck KGaA, Germany). Then the filter was cut and placed in an Eppendorf tube containing 180 µl of ATL lysis buffer (Qiagen, Germany) and incubated with 0.2 mg of Proteinase K (Bio Basic Inc., Canada) for 48 h at 56°C. Later, 100 µl of the lysate was used for column-based DNA extraction using DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol for animal tissues.

Genomic DNA was isolated from spores and mosquitoes using a modified ammonium hydroxide method (Rijpkema & Bruinink, 1996). Two hundred µl of 0.7 M ammonium hydroxide (POCH S.A., Poland) was added to 100 µl of spore suspension or to one mosquito individual and homogenized for 30 seconds using the Pellet Cordless Motor Pellet (DWK Life Sciences, USA) with disposable micro pestles (Scientific Specialties Inc., USA). Samples were incubated for 20 min at 99°C with shaking and then the tubes were opened and further left under the same conditions to concentrate the lysate to about 100 µl volumes. Then, the samples were centrifuged for 5 min at 10,000 rpm, and the supernatant was collected. Before PCR amplification, DNA extracts from mosquitoes were normalized with sterile water to the concentration of about 10 ng/µl.

Standard molecular approach for microsporidia detection

~~To~~ We compared the performance of our new metabarcoding approach ~~and~~ with the standard PCR-based detection method. ~~s, w~~We amplified and sequenced the V1-V3 region of ssu rDNA using V1F (CACCAGGTTGATTCTGCCTGAC) and 530R

(CCGCGGCKGCTGGCAC) primers. PCRs were prepared in two technical replicates, each in a total volume of 10 µl containing Hot FIREPol DNA Polymerase (Solis BioDyne, Estonia), 0.25 µM of each primer and 1 µl of template DNA. Amplification program was as follows: 12 min at 95˚C, followed by 35 cycles of 15 s at 95˚C, 1 min at 60˚C and 1 min at 72˚C, with a final extension step at 72˚C for 10 min. DNA isolated from 100 spores of *E. intestinalis* was used as a positive control. After amplification, technical replications were pooled.

Standard COI-barcode (Hebert, Cywinska, Ball, & deWaard, 2003~~about 670 bp~~) was amplified using bcdF01 and bcdR04 (Dabert, Witalinski, Kazmierski, Olszanowski, & Dabert, 2010) primers. PCRs were prepared in a volume of 10 µl containing Hot FIREPol DNA Polymerase, 0.5 µM of each primer and 1 µl of template DNA. PCR program was as follows: 12 min at 95˚C, followed by 35 cycles of 15 s at 95˚C, 30 s at 50˚C and 1 min at 72˚C, with a final extension step at 72ºC for 5 min. DNA isolated from *A. aegypti* was used as a positive control.

Five µL of the PCR reaction was analyzed by electrophoresis on 1.5% agarose gel stained with GelRed (Biotium, USA) according to the manufacturer's instruction. Samples containing visible bands were purified with *E. coli* exonuclease I and FastAP Alkaline Phosphatase (Thermo Scientific, USA) and sequenced using BigDye v3.1 kit and ABI Prism 3130XL Genetic Analyzer (Applied Biosystems, USA), following manufacturer's instructions. Sequence chromatograms were checked for accuracy and, if necessary, manually edited in Geneious R11.1.5 (Biomatters Ltd.).

Designing new primers for microsporidia detection

New microsporidia-specific primer set was developed based on ssu rDNA sequence data published in GenBank. In total, 1,133 sequences representing 120 genera belonging to the classical Microsporidia were aligned using MAFFT and L-INS-i algorithm (Katoh, Misawa,

Kuma, & Miyata, 2002; Katoh & Standley, 2013) as implemented in Geneious R11.1.5. Primers were manually designed to cover about 200-bp fragment coding for the helices 27 to 34 in *Heterosporis anguillarum* ssu rDNA secondary structure (Tsai, Kou, Lo, & Wang, 2002); according to Neefs, Van de Peer, De Rijk, Chapelle & de Wachter (1993) this region covers the V5 hypervariable region of ssu rRNA (V5 region). The primer sequences were analyzed in Oligo Analyzer version 3.1 (Integrated DNA Technologies Inc.) ~~at https://eu.idtdna.com/calc/analyzer~~ to check the difference of melting temperatures and to search for possible primer secondary structures.

The percentage identities of the aligned V1-V3 and V5 regions were estimated using Kolmogorov-Smirnov statistical test in GeneDoc version 2.7 sequence editing tool (Nicholas Jr. & McClain, 1995). The final dataset for V1-V3 and V5 alignments used in the analysis consisted of 649 and 607 available microsporidian sequences, respectively. Duplicate reads were extracted from the dataset using the default settings in Dedupe Duplicate Read Remover version 37.64 implemented in Geneious R11.1.5.

~~Amplicon production~~Amplification of V5 region and mini-COI for NGS sequencing

Microsporidian V5 region was amplified using CM-V5F (GATTAGANACCNNNGTAGTTC) and CM-V5R (TAANCAGCACAMTCCACTC) primers developed in this study. Mosquito species were determined by DNA-barcoding using the shortened (373 bp) fragment of the mitochondrial COI gene (henceforth: mini-COI) covering 5' fragment of the standard DNA-barcode. The mini-COI was amplified using a primer pair: bcdF01 (CATTTTCHACTAAYCATAARGATATTGG) (Dabert, Witalinski, Kazmierski, Olszanowski, & Dabert, 2010) and bcdR06 (GGDGGRTAHACAGTYCAHCCNGT) developed in this study. All PCR primers for NGS sequencing used in this study were tailed at 5' ends with dual-indexed Ion Torrent adapters (forward tail 5'-

CCATCTCATCCCTGCGTGTCTCCGACTCAG-index-GAT, reverse tail 5'-CCTCTCTATGGGCAGTCGGTGAT-index) for sequencing using Ion Torrent system (Life Technologies, USA).

The V5 region was amplified in two technical replications, each in a total volume of 10 µl containing Hot FIREPol DNA Polymerase, 0.25 µM of each tailed primer and 1 µl of template DNA. PCR program was as follows: 12 min at 95˚C, followed by 40 cycles of 15 s at 95˚C, 30 s at 50˚C and 30 s at 72˚C, with a final extension step at 72˚C for 5 min.

PCR amplification of mini-COI was performed in a volume of 5 µl containing Hot FIREPol DNA Polymerase, 0.25 µM of each tailed primer and 1 µl of template DNA. PCR program was as follows: 12 min at 95˚C, followed by 35 cycles of 15 s at 95˚C, 30 s at 50˚C and 45 s at 72˚C, with a final extension step at 72˚C for 5 min.

~~*NGS libraries and amplicon*~~*Library construction and NGS* *sequencing*

The V5 region and mini-COI libraries were prepared separately (Figure 1). For each PCR reaction, 3 µl were electrophoresed on a 2% agarose gel to check amplification efficiency. The V5 region amplicons were pooled based on DNA band intensities. ~~:~~The sample volumes ranged from 5 µl, ~~while~~ where the amplicons were ~~not~~ invisible on the gel, to 1 µl in case of brighter bands. The ~~:~~ rare samples which had very high intensity were diluted 100-fold ~~diluted~~ with sterile water before pooling. For the mini-COI library, all samples were pooled using 1 µl of each PCR reaction. The V5 region and mini-COI libraries were purified separately using 2% E-Gel SizeSelect II Agarose Gels system (Invitrogen, USA), ~~with regard~~according to the manufacturer's instructions. DNA concentration and fragment length distribution of the libraries were established with the use of High Sensitivity D1000 Screen Tape assay on 2200 Tape Station system (Life Technologies, USA).

Sequence data used in this study were generated in several independent sequencing experiments. Clonal template amplifications were performed using the Ion Torrent One Touch System II and the Ion Torrent OT2 Kit ~~with regard~~according to manufacturer's instructions. For the emulsion PCR, the V5 region and mini-COI libraries were pooled in a 10:1 ratio. Sequencing was carried out using Hi-Q View Sequencing Kit and Ion PGM system on Ion 314 and Ion 318 chips or Ion 520 & Ion 530 Kit-OT2 and S5 system on Ion 530 chip (Life Technologies) according to the manufacturer's instructions. Samples were pooled for each sequencing to get at least 100,000 reads per sample. Negative controls from blank DNA extractions and PCR reagents were included in each PCR and sequencing experiment.

Read processing and data analysis

Raw sequence data were pre-filtered by Ion Torrent Suite software version 5.10.1 (Life Technologies, USA) to remove polyclonal and low quality sequences. Further bioinformatic analysis was conducted using fastq data and custom workflow. Sequence reads shorter than180-bp were removed from the dataset. Leading and trailing low-quality bases or Ns were removed using trimmomatic version 0.39 (Bolger, Lohse, & Usadel, 2014). Fastx toolkit (Hannon, 2010) was used to extract sequences with the minimum of 50% of bases with a quality score ≥25. Quality filtered sequences were separated into individual combinations of indexes in Geneious R11.1.5. Chimeras were removed using the default settings in UCHIME2 version 4.2.40 (Edgar, 2016) and SILVA database for ARB for small subunit ribosomal RNAs version 132 (Glöckner et al., 2017; Quast et al. 2013; Yilmaz et al., 2014) as implemented in Geneious R11.1.5. Next, the sequences were trimmed at 5' and 3' ends to exclude PCR primers.

Operational taxonomic unit (OTU) clustering was done in USEARCH version 11.0.667 (Edgar, 2010). Singletons (<10 reads) were removed, then OTUs were clustered from the

sequences whose abundance exceeded a threshold of 10 counts using the CLUSTER_OTUS algorithm (Edgar, 2013). The OTU consensus sequences were compared to GenBank using BLASTN (Zhang, Schwartz, Wagner, & Miller, 2000) optimized for highly similar sequences (megablast algorithm) (Morgulis et al., 2008).

We used 97% identity threshold to determine mosquito species, and 100% identities for the identification of microsporidian species. Sequences generated in this study were published in GenBank under the accession numbers MT001301-MT001427, MT015707-MT015901 and MT075548-MT075550 (Table S1).

Phylogenetic analyses

To confirm the taxonomic position of the Microsporidia detected in field-collected mosquitoes we used 122 ssu rDNA sequences representing all known clades of the classical Microsporidia (74), Metchnikovellida (4) and Chytridopsida (1). As close outgroups, we used Rozellomycota (6) and Fungi (28). For details concerning all ingroup and outgroup taxa see Table S2. Sequences were aligned using the L–INS–I algorithm in MAFFT v7.388 (Katoh, Misawa, Kuma, & Miyata, 2002; Katoh & Standley, 2013) as implemented in Geneious R11.1.5. The final alignment consisted of 2801 nucleotide positions (nps). The best fit model of DNA evolution (GTR+I+G) was chosen by PartitionFinder2 (Lanfear, Calcott, Ho, & Guindon, 2012). Phylogenetic trees were reconstructed using Maximum Likelihood (ML) in Garli v.2.0 (Zwickl, 2006) and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al., 2012). Each BI run of four independent chains was performed in 2 × 10,000,000 generations, and the trees were sampled every 1000 generations. The final consensus tree was generated after discarding the burn-in fraction of 0.25% of initial trees; the average standard deviation of split frequencies dropped below 0.003. Bootstrap support for the ML tree was

calculated by using 1000 data replicates as implemented in Garli. The trees were edited in FigTree 1.4.4 (Rambaut, 2018) and further in Corel Draw X4.

Statistical analyses

Sequence reads from microsporidia were normalized by OTUTAB_RARE algorithm (Edgar & Flyvbjerg, 2018) to compare sample diversities. The diversity of OTUs in individual samples were calculated using ALPHA_DIV algorithm (Edgar & Flyvbjerg, 2018). Due to the lack of near-normal distribution in any sample ($p < 0.05$) in the initial Shapiro-Wilk test (Shapiro & Wilk, 1965), the nonparametric Kruskal–Wallis test (Kruskal & Wallis, 1952) was used to compare technical repetitions series of spore dilutions and control co-infections, as well as to compare microsporidian species detected in mosquitoes. Rarefaction curves were generated using Past software version 3.23 (Hammer, Harper, & Ryan, 2001). The level of significance of the microsporidian infection frequencies was tested with the Dunn test (Dunn, 1964) constituting the post-hoc test for Q-Cochran analysis (Cochran, 1950). Heatmap was prepared using Heatmapper tools (Babicki et al., 2016). The chi-squared test statistic (Pearson, 1900) were used to evaluate whether there is an association between the detected microsporidia and their occurrence in different host species or in mixed-infections, and the relationship between numbers of sequence reads per OTU in the metabarcoding approach and a successful amplification of the V1-V3 region. Pearson's correlation coefficient was used to determine the correlation between numbers of reads of individual microsporidians~~um~~ (Pearson, 1895). The results of Pearson's correlation were visualized in Gephi software version 0.9.2 (Bastian, Heymann, & Jacomy, 2009).

Microsporidian DNA was considered as incidental in field-collected mosquitoes when noticed in less than 1% of all analyzed individuals, its OTU was coveraged by less than 50 sequences and the species has not been previously reported from mosquitoes.

Results

Proof-of-concept experiment: positive and negative samples and mini-COI barcoding of the hosts

Microsporidia-positive samples including DNA extracts from spores (36), mixture of spores (36) and cultured microsporidia (3), as well as negative controls including blank DNA extractions (3) and blank PCRs (6) yielded 1,413,000 sequence reads after quality filtering. None of the negative control samples passed our sequencing quality threshold of having at least 10 reads per OTU. Sequences generated from the DNAs extracted from cultured microsporidians (ca. 18,500) showed three OTUs that corresponded to the microsporidian species from which DNA samples were extracted: *A*. *algerae*, *V*. *culicis*, and *E. bieneusi*.

Almost 106,000 sequence reads were obtained from the series of ten-fold dilution of *E. cuniculi*, *E. hellem*, and *E. intestinalis* spores. Samples containing 100, 1000 and 10,000 spores per mL were represented by, respectively, 673 (SD = 94), 1499 (SD = 407) and 9524 (SD = 862) sequence reads. The method was reproducible for all tested species to the level of 100 spores per 1 mL ($R^2 > 0.99$) (Figure ~~2~~S1). The repeatability of the method was supported by Kruskal–Wallis test ($H < 0.006$; $p > 0.05$).

Analysis of the relative sequence read abundance in sequencing results from DNA samples being a mixture of microsporidian species showed that DNAs from species present in smaller quantities in the sample (*E. cuniculi* and *E. hellem* vs *E*. *intestinalis*) were successfully detected in each mixture of spores in all technical replicates (Figure ~~3~~2). DNAs from *E. cuniculi* and *E. hellem* spores were detected ~~even~~ in the sample even when~~where~~ they were mixed in a ratio of 1 to 1000 with *E. intestinalis*. In this sample, they were represented by about 0.1% of all reads which corresponded to the assumed share of the

spores representing each species. The repeatability of the experiment was supported by Kruskal–Wallis test ($H < 0.008$; $p > 0.05$).

To test the new method on colony mosquitoes, 213 samples consisting of the V5 region amplicons from *A. aegypti*, *A. quadrimaculatus*, *C. quinquefasciatus*, *U. lowii* and negative controls (13) were sequenced in one experiment. Negative controls included PCRs performed on the DNA extracted from preservation medium for mosquitoes, blank DNA extractions, and blank PCRs. After quality filtering, 480,307 reads were used in the OTU analysis. None of the negative control samples passed our sequencing quality threshold to generate OTUs. Less than 9% of the whole sequence data were of non-microsporidian origin. From these non-microsporidian sequences~~these data~~, we reconstructed one OTU showing 96% of identities to acetylcholinesterase (Ace1) gene from *A. aegypti* (GenBank acc. no. BK006052). This OTU was found in almost all mosquito individuals (181/200). However, the number of reads for this OTU never exceeded 785 per individual (median = 312). Among colony mosquitoes, we found microsporidian DNA in two individuals belonging to *A. quadrimaculatus*. The OTU we found in this species matched Microsporidium sp. OB1 (GenBank acc. no. MG456597) originally reported from geometer moth *Operophtera bruceata* (Lepidoptera).

Based on mini-COI data, all mosquitoes were unambiguously assigned to the proper species. The COI sequence reads in a mosquito individual positive for microsporidia amounted to 7% (SD = 4) while the remaining reads represented the V5 region library. This result corresponded to the molar ratio of 1:10 used for pooling the libraries for sequencing.

Applying the metabarcoding method for field-collected mosquitoes

Using mini-COI data, all field-collected mosquitoes were unambiguously assigned to seven species: *Aedes cinereus* (13), *A. vexans* (19), *Coquillettidia richiardii* (16),

Ochlerotatus annulipes (63), *O. cantans* (77), *O. punctor* (11) and *O. sticticus* (13) (Table 1, Table S1).

In total, the V5 region library of the field-collected mosquitos (212) and negative controls (5) generated about 1,330,000 reads after quality filtering. Negative controls yielded no sequence data using our default threshold. In microsporidia-free mosquitoes the number of reads never exceeded 626 per sample (median = 101). Together, non-microsporidian sequences accounted ~~only~~ for only 12% of all quality filtered reads; most of them were of host origin (11.9~~4~~%) and coded ssu rRNA genes (9.8~~76~~%) or a collagen alpha-1 chain gene (2.1~~8~~%; 100% identities with GenBank acc. no. XM_021840458). The less abundant OTUs represented ssu rDNA fragments from fungi (in three mosquito individuals; <0.03% of sequence reads), gregarine (in one individual; <0.002%) and human (in one individual; <0.001%) (Table S1).

In field-collected mosquitoes, microsporidian DNA was found in almost 60% (127/212) of individuals representing all analyzed species (Table 1). The OTU clustering across microsporidia-positive samples produced 11 unique OTUs that represented: a new microsporidian ssu rDNA gene sequence~~species~~ which we named Microsporidium sp. ~~nov.~~ PL01, *Amblyospora salinaria, Amblyospora* sp. (identical to AY090055), *Encephalitozoon hellem*, *Enterocytospora artemiae*, *Nosema adaliae*, *N. ceranae*, *N. pieriae*, *N. thomsoni*, *Nosema* sp. CHW-2007a, and indistinguishable in this sequence fragment *N. chrysorrhoeae* and/or *N. portugal* (Tables 1, ~~S3~~S1). Rarefaction curve analysis showed that the read depth was sufficient to recover all microsporidian species in the tested individuals (Figure ~~S1~~S2). The analysis revealed that 10,000 reads per sample ~~are~~ is required to identify all microsporidian diversity in the tested host.

Except for *A. cinereus* and *O. punctor* that hosted two microsporidian species, all the remaining mosquito species were positive for at least four different microsporidians that could potentially ~~could~~ infect them (Table 1). The highest richness of different Microsporidia

was observed in *O. annulipes*; mosquitoes belonging to this species potentially hosted six different microsporidians: Microsporidium sp. ~~nov.~~ PL01, *Amblyospora salinaria* and at least four species from the genus *Nosema*. The presence of *E. hellem*, *E. artemiae* and *N. ceranae* was recognized as incidental because each of OTUs was noticed only in one host species and was found in <1% of all mosquito individuals.

The predominant microsporidium that was noticed in all mosquito species was Microsporidium sp. ~~nov.~~ PL01 (69.3~~3~~% of all infected individuals) (Table 1). This species occurred significantly more often than any of the other microsporidians ($p < 0.01$) in each of the analyzed mosquito species (Figure ~~4~~3); Berger-Parker (0.69) and Simpson (0.51) dominance indexes supported this observation. In addition, high frequency of *N. chrysorrhoeae* and/or *N*. *portugal* was observed (15.3%, $p < 0.05$), especially in *O. cantans*; this microsporidian DNA was also detected in mosquitoes belonging to the *A. vexans*, *C. richiardii* and *O. annulipes* species. The third most common detected microsporidian~~um~~ (4.7%) was *Nosema* sp. CHW-2007a, which was recorded in five of seven examined host species (Figure ~~4~~3). In the remaining possible microsporidian pairs, there were no significant differences in their frequency ($p > 0.05$).

Results of the phylogenetic analysis showed that the Microsporidium sp. ~~nov.~~ PL01 forms clade with Microsporidium sp. 1199 (GenBank acc. no. FN610845.1) and represents another species in the same genus nested in the microsporidian Clade IV (Figure ~~5~~4).

Co-occurrence of different Microsporidia in single mosquito individuals

The co-occurrence of DNAs representing different microsporidian species in one host individual was recorded in 20 field-collected mosquitoes (9.4~~3~~%); this relationship concerned all microsporidian and host species (Figure ~~6~~5, Table 2). There were no statistically significant relationships between co-occurring microsporidians and mosquito species (χ^2 =

1.2; p > 0.05). Microsporidia considered as incidental – *E. hellem*, *E. artemiae*, and *N. ceranae* – were found with the most abundant Microsporidium sp. ~~nov.~~ PL01 in four mosquito individuals; additionally, in a single case, *N. ceranae* was noticed with *N. chrysorrhoeae* and/or *N. portugal*. ~~Even if the incidental Microsporidia were omitted, t~~The co-occurrence rate remained high in all host species, even omitting the incidental microsporidians. ~~and m~~More than two microsporidian species were found in 12.6% of all microsporidia-positive mosquitoes.

Although most microsporidian co-infections occurred with the most abundant Microsporidium sp. ~~nov.~~ PL01 (76.~~19~~2%), the highest convergence rate of co-infections concerned microsporidians of the genus *Nosema*. Among 39 cases of the presence of *Nosema* DNAs in mosquitoes, 23 of them (~~58~~59~~.97~~%) were observed in co-occurrence with DNA representing the other microsporidian species. The chi-squared statistic showed that *Nosema* spp. occurred more frequently ~~occurred~~ in mosquitoes with another microsporidian species~~um~~ than individually (χ^2 = 25.15; p < 0.05).

A statistically significant relationship (p < 0.05) was found between the numbers of reads representing a given co-infecting microsporidian species. An almost complete correlation was observed in increasing numbers of sequence reads between *N. chrysorrhoeae*/*portugal* and *Nosema* sp. CHW-2007a (r = 0.9); also, similarly high correlation was observed between *Amblyospora* sp. and *A. salinaria* (r = 0.51) (Figure ~~7~~6, Table S3).

Comparison of metabarcoding and standard molecular approach

The Kolmogorov-Smirnov plots for the microsporidian V1-V3 and V5 sequence alignments showed that the proposed V5 marker is only 4% less variable than the standard sequence covering V1-V3 regions (about 40% and 36% of different nps, respectively) (Figure ~~8~~7). The extraction of duplicate reads from the alignment comprising the V5 region

representing 607 microsporidian species belonging to 120 genera indicated that the marker enables the proper identification of almost all species in the dataset (596/607, >98%). Several sequences that were found in duplicates represented species of ~~The results of the extraction of duplicate reads clearly indicated that the nucleotide sequence of the V5 region enabled species identification in almost all Microsporidia known from the ssu rDNA. Several sequences that were found in duplicated sequences concerned mainly~~ mainly of the genus *Nosema*, e.g.~~, e.g. the ssu rDNA sequences published for~~ *N. chrysorrhoeae* and *N. portugal* or a group consisting of *N. antheraeae, N. trichoplusiae, N. spodopterae*, and *N. philosamiae* showed no variation in the V5 region. However, in these sample groups extremely low or no variation can also be observed in other regions of the ssu rDNA, including V1-V3 marker (data not shown). In three cases, the same V5 region sequences were found in species representing different genera (*Conglomerata obtusa* and *Berwaldia schaefernai*; *Ameson portunus*, *A. pulvis* and *Nadelospora canceri*; *Tetramicra brevifilum* and *Microgemma caulleryi*).

In total, 42 microsporidia-positive samples were obtained using the standard V1F/530R primer set and DNA extracts from field-collected mosquitoes previously used to test the metabarcoding method (Figure ~~S2~~S3, Table ~~S2~~S1). Using the same DNA templates we successfully amplified full-length COI-barcode (about 670 bp), which means that all DNA isolates were suitable for PCR amplification (Figure ~~S3~~S4). As PCR products were found among 127 microsporidia-positive mosquitoes identified previously by the metabarcoding method, the standard PCR approach gave almost 67% false-negative samples (Table S4). We found a statistically significant correlation ($\chi 2 = 68.59$, $p < 0.05$) between the detection of microsporidia using the standard molecular approach and the obtaining at least 300 reads per OTU found by the metabarcoding method. Direct Sanger sequencing of the V1-V3 amplicons revealed two samples with unreadable chromatograms, one sample positive for *Amblyospora* sp. which was in agreement with the metabarcoding result, and 39 samples

positive for the Microsporidium sp. ~~nov.~~ PL01. All populations of ~~Microsporidium sp. nov. PL01~~this microsporidians infecting different mosquito host species shared the same V1-V3 sequence. Two unreadable chromatograms were found in mosquitoes that were highly co-infected with Microsporidium sp. ~~nov.~~ PL01 and two *Nosema* spp. (samples AT.p02.E06 and AT.p03.D07, bolded in Table 2). On the other hand, direct V1-V3 amplicon sequencing failed to show four mixed samples where the Microsporidium sp. ~~nov.~~ PL01 significantly dominated over other co-infecting species (highlighted in grey in Table 2).

Discussion

Effectiveness of metabarcoding microsporidia and their hosts

The metabarcoding approach proposed in this study can be used to fast, accurate and sensitive detection of microsporidia in any kind of DNA samples. Our data show that the new marker covering the hypervariable V5 region of ssu rDNA allows proper identification of almost all classical microsporidian species known from rDNA sequence data.

~~The comparison of our new method with a standard PCR-based approach clearly shows that the metabarcoding is more sensitive and accurate in detecting microsporidian infections. Our results show that direct amplicon sequencing would be impractical in co-infected samples where one microsporidium was present at a much higher level of infection than the other co-infecting species. Although amplicon cloning and Sanger sequencing of several clones can detect mixed samples in cases of relatively balanced co-infections, the dominance of one microsporidian species would require sequencing a large number of clones, which is usually not assumed a priori.~~

The ~~hypervariable~~ V5 region ~~of ssu rDNA~~ is flanked by group-conserved sequences that allowed us to design the CM-V5F/CM-V5R primer set with great specificity towards microsporidian ssu rDNA. Indeed, the specificity of our primers seems to be higher than the

commonly used V1F/530R pair applied by Williams et al. (2018), as in our experiments the non-target sequences never exceeded 12% of quality-filtered sequence data. Results of our phylogenetic analysis, which included the V5 region sequences found in this study, support that the new primer set enables the amplification of ssu rDNA in different microsporidian evolutionary lineages, including Aquasporidia, Terresporisdia, and Marinosporidia (Vossbrinck & Debrunner-Vossbrinck, 2005) (Figure 54). However, recently published first sequence data for the two remaining microsporidian lineages – for metchnikovellid *Amphiamblys* sp. *Metchnikovella* (Galindo et al., 2018; Mikhailov, Simdyanov, & Aleoshin, 2017) and chytridiopsid *Chytridiopsis typographi* (Corsaro et al., 2019) – suggest that the CM-V5F/CM-V5R primer set is rather group-specific for the classical Microsporidia. This observation indicates that there may be a need to develop additional primers that will be group-specific for both the mMetchnikovellidas and the Cchytridiopsidsa to study species diversity in the whole phylum.

Our metabarcoding approach allows detecting of 100 spores per 1 mL which is comparable with other PCR-based methods (Menotti et al., 2003; Rinder et al., 1998; Wolk, Schneider, Wengenack, Sloan, & Rosenblatt, 2002) or microarray techniques developed for *Encephalitozoon* spp. in clinical samples (Wang, Orlandi, & Stenger, 2005). Usually, cClinical samples usually are hardly accessible; therefore, the screening of different microsporidian species in the same sample at the same time may be of clinical benefit. The advantage of the metabarcoding is that it allows simultaneous screening of all species, without the need to detect particular species in separate PCR reactions. For example, methods commonly used for microsporidia detection in water are based on spore staining in smears from concentrated water samples, followed by the PCR amplification of marker sequences using species-specific primers (e.g. Ben Ayed et al., 2012; Izquierdo et al., 2011; Li et al., 2012). Metabarcoding approach could help in overcomeing these difficulties saving saving the analysis time and costs of the analysis. In addition, the metabarcoding of concentrated water

samples could be standardized which can help to develop good laboratory methods for more accurate ~~monitoring of~~ waterborne disease risk assessment.

The comparison of our new method with a standard PCR-based approach clearly shows that the metabarcoding is more sensitive and accurate in detecting microsporidian infections. Our results show that direct amplicon sequencing would be impractical in co-infected samples where one microsporidian species was present at a much higher level of infection than the other co-infecting species. Although amplicon cloning in a plasmid vector and subsequent Sanger sequencing of several clones can detect mixed samples in cases of relatively balanced co-infections, the dominance of one microsporidian species would require sequencing a large number of clones, which is usually not assumed a priori.

Basing on the same mosquito DNA samples we successfully amplified both the full-length COI-barcode (about 670 bp) and the mini-COI fragment (373 bp) for the metabarcoding analysis. COI sequence analysis showed that both fragments allowed to unambiguously assign all mosquitoes to the proper species. Therefore, the mini-COI fragment can be successfully used in NGS approaches to determine microsporidian host species.

Microsporidia in field-collected mosquitoes

More than half of field-collected mosquitoes analyzed in this study were positive for microsporidian DNA. Reports about the prevalence of microsporidia in adult mosquito populations in nature are sparse and focused on particular microsporidians and their primary mosquito hosts. In one experiment conducted for almost 20 years, the prevalence of *Amblyospora stimuli* infections in *A. stimulans* adult female populations was relatively low and ranged from 1% to 9.6% (Andreadis, 1999). On the other hand, in the study concerning a life cycle and ecology of *Amblyospora khaliulini*, prevalence rates of *A*. *khaliulini* infections

in adult mosquito females ranged from 16.4% to 50% (Andreadis, Thomas, & Shepard, 2018). In our study, all mosquito species showed a much higher level of microsporidian positive individuals, ranging from 54.0% to 92.3% (Table 1). However, in the studies conducted by Andreadis ~~Andrealis~~ et al. (1999, 2018), Microsporidia were identified by microscopic examination of infected tissues; ~~and~~ mosquitoes were ~~scored~~ considered ~~as~~ infected if ~~any~~ either the ~~developmental stage (~~vegetative or spore developmental stage of the parasite~~)~~ was observed.

~~In addition, b~~Both Andreadis' studies (1999, 2018) ~~were~~ based on a single collection of mosquitoes during the end of April or May, which are not the warmest months of the year in the studied areas (public data of the National Weather Service, National Oceanic and Atmospheric Administration; www.weather.gov). ~~We~~ For the present study, ~~collected~~ mosquitoes were collected in July and August, which are the warmest months in Poland (public data of the National Research Institute, Institute of Meteorology and Water Management; www.imgw.pl). Our preliminary results of a phenological survey of microsporidian infections in mosquito natural populations suggest that during the warmest months of the year the prevalence of microsporidians is higher and can reach about 60% of microsporidia-positive individuals, than in other seasons (about 30%) (Trzebny et al. in prep.). Thus, the differences between prevalence noticed in our and the previous studies could result from both the season of mosquito capturing and the use of a much more sensitive method for microsporidia detection.

The presence of microsporidian DNA does not necessarily result from infection. ~~– –~~ ~~this~~ This assumption led us to exclude *E. hellem*, *E. artemiae* and *N. ceranae* as infecting factors because they had been noticed in less than 1% of all analyzed individuals and their OTUs were coveraged by low numbers of reads. However, there is no empirical basis to exclude *Nosema* spp. or *Amblyospora* spp. even though in the present study ~~they were~~we recorded them ~~recorded~~ in single host individuals and/or their OTUs were represented by low

numbers of reads. To our knowledge, the remaining microsporidian species, found by us in field-collected mosquitoes, have been~~, even excluding incidental ones, are~~ recorded in Culicidae for the first time.

~~However, our~~Our observations basing on numbers of reads representing the newly recorded microsporidian species ~~their~~ OTUs or their occurrence ~~of the newly recorded microsporidians~~ in multiple mosquito individuals support the hypothesis that these species indeed can infect mosquitoes. A wide range of hosts for members of the *Nosema* genus also supports this hypothesis. For example, *N. thomsoni* has been noticed in *Andrena vaga* (Hymenoptera) (Ravoet et al., 2014), *Harmonia axyridis* (Coleoptera) (Vilcinskas, Stoecker, Schmidtberg, Röhrich, & Vogel, 2013) and *Choristoneura conflictana* (Lepidoptera) (Kyei-Poku, Gauthier, & Van Frankenhuyzen, 2008).

Among the other microsporidians detected in ~~this~~ our study, *N. adaliae* was found in *Adalia bipunctata* (Coleoptera) (Steele & Bjørnson, 2014), while *N. pieriae*, *N. chrysorrhoeae*, *N. portugal* and *Nosema* sp. CHW-2007a were noticed in different lepidopteran hosts (Huang et al., 2008; Hyliš et al., 2006; Maddox et al., 1999; Yaman, Bekircan, Radek, & Linde, 2014). *Amblyospora* sp. ~~found in field-collected mosquitoes~~recorded in our study ~~has~~ had been ~~known~~ previously ~~only from rDNA sequences~~ found in *Cyclops strenuous* (Copepoda) from the Czech Republic (Vossbrinck, Andreadis, Vávra, & Becnel, 2004). The dominant presence of one microsporidian species - Microsporidium sp. ~~nov.~~ PL01 - in all mosquito species analyzed in this study suggests that there may be no "primary host" in microsporidia-mosquito relationships and the observed prevalence reflects only the abundance of a generalist microsporidian species infecting mosquito larvae in the water environment. Nevertheless, ultrastructural assessment, based on histology and transmission electron microscopy should be carried on to confirm the actual infection.

The new microsporidian ~~species~~ ssu rDNA sequence found in this study~~, Microsporidium sp. nov. PL01,~~ is the most similar to ssu rDNA of Microsporidium sp. 1199

which was recorded in freshwater populations of *Gammarus duebeni* (Crustacea, Amphipoda) in a rivulet from Wales, Holyhead Island, during research on molecular characterization of the microsporidians of this host across its natural range (Krebes, Blank, Frankowski, & Bastrop, 2010). Our ~~results of~~ phylogenetic analysis results strongly support that Microsporidium sp. ~~nov.~~ PL01 and Microsporidium sp. 1199 represent different species in the same genus, which belongs to one of the two main clades in the Terresporidia. The members of this clade mostly parasitize on terrestrial arthropods, but it groups also microsporidians that can infect vertebrates, including humans (e.g. *Enterocytozoon bieneusi*).

Microsporidia in co-infections

Our metabarcoding method allows us to detect a relatively high level (9.4~~3~~%) of co-occurrence of DNAs representing different microsporidian species in the same host individual. We cannot exclude accidental intake of spores in food; however, high numbers of sequence reads representing *N. thomsoni* or *N. chrysorrhoeae*/*portugal* in samples where they co-occurred with Microsporidium sp. ~~nov.~~ PL01 are evidence of mixed infection by these species.

~~There are many reports about mixed-infections by different pathogens in mosquitoes (Bian, Xu, Lu, Xie, & Xi, 2010; Blanford et al., 2005; Moreira et al., 2009; Scholte et al., 2005) and that the co-infecting organisms interact with each other (Bano, 1958; Bargielowski & Koella, 2009; Turell, Rossignol, Spielman, Rossi, & Bailey, 1984; Vaughan & Turell, 1996). For example, an infection of the microsporidium *Vavraia culicis* elicits an immune response from *A. aegypti* larvae (Biron et al., 2005) or suppresses *Plasmodium* spp. (Apicomplexa) infections in mosquitoes (Bano, 1958; Bargielowski & Koella, 2009). In addition, the analysis of the interaction of multi capsid nuclear polyhedrosis virus (LdNPV) and *N. portugal* in gypsy~~

~~moth larvae has shown that these pathogens can influence each other in a synergistic or antagonistic way. If LdNPV and *N. portugal* were orally inoculated at the same time or the LdNPV infection preceded the inoculation of *N. portugal* by several days, median infectious dose for the virus was lower in microsporidia-infected larvae. If the *N. portugal* inoculation preceded the LdNPV inoculation, the larvae also died earlier, but virus and spore production was also lower in co-infected insects (Bauer, Miller, Maddox, & McManus, 1998).~~

It is noteworthy that we found *Nosema* spp. in field-collected mosquitoes mostly in co-infections. Furthermore, we noticed that three out of five *Nosema* species – *N. adaliae*, *N. pieriae*, and *N. thomsoni* – in our study were found only in co-infections. Additionally, we found a positive correlation between numbers of reads for OTUs representing *N. chrysorrhoeae* and/or *N. portugal* and *Nosema* sp. CHW-2007a which suggests that these species support each other. A similar relationship was found for the *Amblyospora* spp. where the occurrence of *A. salinaria* was associated with the occurrence of *Amblyospora* sp.; although, in this case the numbers of reads suggest a lower level of infection. However, quantitative analyses should be carried out to confirm these relationships. To conclude, we ~~assume~~ believe that our observations based on the microsporidian DNA occurrences reflect the actual microsporidian diversity in a tested sample; however, the hypotheses concerning infections and the mutual relationships of co-infecting species should be tested using more intensive host sampling with the use of ultrastructure data.

Conclusions

In this paper, we have proposed a new molecular approach for the detection of microsporidian DNA in samples extracted from their hosts. The method uses NGS sequence data from the hypervariable V5 region of ssu rDNA and the shortened fragment of the

standard metazoan DNA-barcode, i.e. the mitochondrial COI gene, for microsporidian and host species identification, respectively.

We have tested this metabarcoding approach on microsporidians infecting mosquitoes; however, the method can be applied to all types of DNA extracts, including medical and environmental samples. We compared our new method with the standard molecular approach for microsporidian DNA detection, which uses end-point PCR and Sanger sequencing. This comparison showed that the metabarcoding is more sensitive and accurate than the traditional method.

An additional advantage of the new method is its effectiveness in identifying microsporidian species that co-infect the same host individual. Moreover, our data concerning numbers of sequence reads for the microsporidian OTUs found in co-infected hosts suggest that the occurrence of some microsporidian species in mixed-infections could benefit them; however, this observation should be re-tested using more intensive host sampling.

~~We propose a new, fast and sensitive method for the detection of microsporidian DNA in any kind of DNA isolates. Our method bases on PCR amplification of the hypervariable V5 region of ssu rDNA using the new microsporidia-specific primer set CM-V5F/CM-V5R fused with double indexed adapters and further NGS sequencing of the amplicons. In addition, we propose to use a shorter COI sequence fragment to determine microsporidian host species. The new metabarcoding approach allows detecting mixed-infections with two or more different microsporidian species in the same host individual. A comparison of the new method and a standard molecular approach for the detection of microsporidian DNA showed the metabarcoding as more sensitive. A standard PCR-based approach confirmed only 37% of the microsporidia-positive samples found using the NGS-based method. Moreover, direct Sanger sequencing failed to show mixed samples where one microsporidium significantly dominated over the other co-infecting species. Applying the metabarcoding to study~~

~~microsporidian diversity in mosquitoes from natural populations, we found a new species, Microsporidium sp. nov. PL01 that dominated in all mosquito species. This result suggests that the observed prevalence of the dominant species infecting mosquitoes reflects the abundance of a generalist microsporidium infecting mosquito larvae in the water environment. Our data concerning numbers of sequence reads for the microsporidian OTUs found in co-infected hosts suggest that the occurrence of some microsporidian species in mixed-infections could benefit them; however, all that observations should be tested using more intensive host sampling.~~

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Data accessibility

Sequences generated in this study were published in GenBank under the accession numbers MT001301-MT001427, MT015707-MT015901 and MT075548-MT075550. Other details concerning our data are available in supplementary Figures S1-~~S3~~ S4 and Tables S1-S4. All data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

AT, ASK, MD designed the research; ASK, JB, NS provided the material; AT performed experiments and analyzed data; AT, MD co-wrote the first draft of the manuscript. All authors interpreted results and contributed to the final manuscript.

References

Andreadis, T. G. (1999). Epizootiology of Amblyospora stimuli (Microsporidiida: Amblyosporidae) Infections in Field Populations of a Univoltine Mosquito, Aedes stimulans (Diptera: Culicidae), Inhabiting a Temporary Vernal Pool. Journal of Invertebrate Pathology, 74(2), 198–205. doi:10.1006/jipa.1999.4875

Andreadis, T. G. (2007). Microsporidian Parasites of Mosquitoes. Journal of the American Mosquito Control Association, 23(2), 3–29. doi:10.2987/8756-971X(2007)23[3:MPOM]2.0.CO;2

Andreadis, T. G., Thomas, M. C., & Shepard, J. J. (2018). Amblyospora khaliulini (Microsporidia: Amblyosporidae): Investigations on its life cycle and ecology in Aedes communis (Diptera: Culicidae) and Acanthocyclops vernalis (Copepoda: Cyclopidae) with redescription of the species. Journal of Invertebrate Pathology, 151, 113–125. doi:10.1016/j.jip.2017.11.007

Babicki, S., Arndt, D., Marcu, A., Liang, Y., Grant, J. R., Maciejewski, A., & Wishart, D. S. (2016). Heatmapper: web-enabled heat mapping for all. Nucleic Acids Research, 44(1), 147–153. doi:10.1093/nar/gkw419

Baker, M. D., Vossbrinck, C. R., Didier, E. S., Maddox, J. V., & Shadduck, J. A. (1995). Small Subunit Ribosomal DNA Phylogeny of Various Microsporidia with Emphasis on AIDS Related Forms. Journal of Eukaryotic Microbiology, 42(5), 564–570. doi:10.1111/j.1550-7408.1995.tb05906.x

~~Bano, L. (1958). Partial inhibitory effect of Plistophora culicis on the sporogonic cycle of plasmodium cynomolgi in anopheles stephensi. Nature. doi:10.1038/181430a0~~

~~Bargielowski, I., & Koella, J. C. (2009). A possible mechanism for the suppression of Plasmodium berghei development in the mosquito Anopheles gambiae by the Microsporidian Vavraia culicis. PLoS ONE, 4(3), e4676. doi:10.1371/journal.pone.0004676~~

Bass, D., Czech, L., Williams, B. A. P., Berney, C., Dunthorn, M., Mahé, F., … Williams, T. A. (2018). Clarifying the Relationships between Microsporidia and Cryptomycota. Journal of Eukaryotic Microbiology, 65(6), 773–782. doi:10.1111/jeu.12519

Bastian, M., Heymann, S., & Jacomy, M. (2009). Gephi: an open source software for exploring and manipulating networks. Proceedings of International AAAI Conference on Weblogs and Social Media, 361–362.

~~Bauer, L. S., Miller, D. L., Maddox, J. V, & McManus, M. L. (1998). Interactions between a Nosema sp. (Microspora: Nosematidae) and Nuclear Polyhedrosis Virus Infecting the Gypsy Moth, Lymantria dispar (Lepidoptera: Lymantriidae). Journal of Invertebrate Pathology, 72(2), 147–153. doi:10.1006/jipa.1998.4773~~

Becnel, J. J., Takvorian, P. M., & Cali, A. (2014). Checklist of Available Generic Names for Microsporidia with Type Species and Type Hosts. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity. Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi:10.1002/9781118395264.app1

Becnel, J. J., White, S. E., & Shapiro, A. M. (2005). Review of microsporidia-mosquito relationships: From the simple to the complex. Folia Parasitologica, 52(1–2), 41–50. doi:10.14411/fp.2005.006

Ben Ayed, L., Yang, W., Widmer, G., Cama, V., Ortega, Y., & Xiao, L. (2012). Survey and genetic characterization of wastewater in Tunisia for Cryptosporidium spp., Giardia duodenalis, Enterocytozoon bieneusi, Cyclospora cayetanensis and Eimeria spp. Journal of Water and Health, 10(3), 431–444. doi:10.2166/wh.2012.204

Beznoussenko, G. V., Dolgikh, V. V., Seliverstova, E. V., Semenov, P. B., Tokarev, Y. S., Trucco, A., … Mironov, A. A. (2007). Analogs of the Golgi complex in microsporidia: structure and avesicular mechanisms of function. Journal of Cell Science, 120(7), 1288–1298. doi:10.1242/jcs.03402

~~Bian, G., Xu, Y., Lu, P., Xie, Y., & Xi, Z. (2010). The Endosymbiotic Bacterium Wolbachia Induces Resistance to Dengue Virus in Aedes aegypti. PLoS Pathogens, 6(4), e1000833. doi:10.1371/journal.ppat.1000833~~

~~Biron, D. G., Agnew, P., Marché, L., Renault, L., Sidobre, C., & Michalakis, Y. (2005). Proteome of Aedes aegypti larvae in response to infection by the intracellular parasite Vavraia culicis. International Journal for Parasitology, 35(13), 1385–1397. doi:10.1016/j.ijpara.2005.05.015~~

~~Blanford, S., Chan, B. H. K., Jenkins, N., Sim, D., Turner, R. J., Read, A. F., & Thomas, M. B. (2005). Fungal pathogen reduces potential for malaria transmission. Science, 308(5728), 1638–1641. doi:10.1126/science.1108423~~

Bojko, J., Dunn, A. M., Stebbing, P. D., Ross, S. H., Kerr, R. C., & Stentiford, G. D. (2015). Cucumispora ornata n. sp. (Fungi: Microsporidia) infecting invasive “demon shrimp” (Dikerogammarus haemobaphes) in the United Kingdom. Journal of Invertebrate Pathology, 128, 22–30. doi: 10.1016/j.jip.2015.04.005

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics, 30(15), 2114–2120. doi:10.1093/bioinformatics/btu170

Cochran, W. G. (1950). The Comparison of Percentages in Matched Samples. Biometrika, 37(3/4), 256–266. doi:10.2307/2332378

Corsaro, D., Wylezich, C., Venditti, D., Michel, R., Walochnik, J., & Wegensteiner, R. (2019). Filling gaps in the microsporidian tree: rDNA phylogeny of Chytridiopsis typographi (Microsporidia: Chytridiopsida). Parasitology Research, 118(1), 169–180. doi:10.1007/s00436-018-6130-1

Dabert, M., Witalinski, W., Kazmierski, A., Olszanowski, Z., & Dabert, J. (2010). Molecular phylogeny of acariform mites (Acari, Arachnida): Strong conflict between phylogenetic signal and long-branch attraction artifacts. Molecular Phylogenetics and Evolution, 56(1), 222–241. doi:10.1016/J.YMPEV.2009.12.020

del Aguila, C., Croppo, G. P., Moura, H., Da Silva, A. J., Leitch, G. J., Moss, D. M., … Visvesvara, G. S. (1998). Ultrastructure, immunofluorescence, western blot, and PCR analysis of eight isolates of Encephalitozoon (Septata) intestinalis established in culture

from sputum and urine samples and duodenal aspirates of five patients with AIDS. Journal of clinical microbiology, 36(5), 1201-1208

~~Duncan, A. B., Agnew, P., Noel, V., Demettre, E., Seveno, M., brizard, J. P., & Michalakis, Y. (2012). Proteome of Aedes aegypti in response to infection and coinfection with microsporidian parasites. Ecology and Evolution, 2(4), 681–694. doi:10.1002/ece3.199~~

Dunn, O. J. (1964). Multiple Comparisons Using Rank Sums. Technometrics, 6(3), 241–252. doi:10.1007/978-1-349-95810-8_109

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics, 26(19), 2460–2461. doi:10.1093/bioinformatics/btq461

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature Methods, 10(10), 996–998. doi:10.1038/nmeth.2604

Edgar, R. C. (2016). UCHIME2 : improved chimera prediction for amplicon sequencing. doi:10.1101/074252

Edgar, R. C., & Flyvbjerg, H. (2018). Octave plots for visualizing diversity of microbial OTUs. doi:10.1101/389833

Emsen, B., Guzman-Novoa, E., Hamiduzzaman, M. M., Eccles, L., Lacey, B., Ruiz-Pérez, R. A., & Nasr, M. (2016). Higher prevalence and levels of Nosema ceranae than Nosema apis infections in Canadian honey bee colonies. Parasitology Research, 115(1), 175–181. doi:10.1007/s00436-015-4733-3

Evans, A. N., Llanos, J. E. M., Kunin, W. E., & Evison, S. E. F. (2018). Indirect effects of agricultural pesticide use on parasite prevalence in wild pollinators. Agriculture, Ecosystems and Environment, 258, 40–48. doi: 10.1016/j.agee.2018.02.002

Fast, N. M., Law, J. S., Williams, B. A. P., & Keeling, P. J. (2003). Bacterial Catalase in the Microsporidian Nosema locustae : Implications for Microsporidian Metabolism and Genome Evolution. Eukaryotic Cell, 2(5), 1069–1075. doi:10.1128/ec.2.5.1069-1075.2003

Fayer, R., & Santin-Duran, M. (2014). Epidemiology of Microsporidia in Human Infections. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity: First Edition (pp. 135–164). Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi:10.1002/9781118395264.ch3

Field, A. S., Hing, M. C., Milliken, S. T., & Marriott, D. J. (1993). Microsporidia in the small intestine of HIV-infected patients. A new diagnostic technique and a new species. Medical Journal of Australia, 158(6), 390–394. doi:10.5694/J.1326-5377.1993.TB121832.X

Franzen, C. (2004). Microsporidia: How can they invade other cells? Trends in Parasitology, 20(6), 275–279. doi:10.1016/j.pt.2004.04.009

Franzen, C., & Müller, A. (2001). Microsporidiosis: Human diseases and diagnosis. Microbes and Infection, 3(5), 389–400. doi:10.1016/S1286-4579(01)01395-8

Furuya, K., Miwa, S., Omura, M., Asakura, T., Yamano, K., Takatori, K., & Kudo, S. (2008). Mouse Monoclonal Immunoglobulin E Antibodies Specific for the Microsporidian Encephalitozoon cuniculi Polar Tube Protein 1. Hybridoma, 27(3), 153–157. doi:10.1089/hyb.2007.0564

Galindo, L. J., Torruella, G., Moreira, D., Timpano, H., Paskerova, G., Smirnov, A., … López-García, P. (2018). Evolutionary genomics of metchnikovella incurvata (metchnikovellidae): An early branching microsporidium. Genome Biology and Evolution, 10(10), 2736–2748. doi:10.1093/gbe/evy205

Ghosh K., Schwartz D., & Weiss L. M. (2014). Laboratory Diagnosis of Microsporidia. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity: First Edition (pp. 135–164). Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi:10.1002/9781118395264.ch17

Ghosh, K., & Weiss, L. M. (2009). Molecular Diagnostic Tests for Microsporidia. Interdisciplinary Perspectives on Infectious Diseases. doi: 10.1155/2009/926521

Ghoyounchi, R., Mahami-Oskouei, M., Rezamand, A., Spotin, A., Aminisani, N., Nami, S., … Madadi, S. (2019). Molecular Phylodiagnosis of Enterocytozoon bieneusi and Encephalitozoon intestinalis in Children with Cancer: Microsporidia in Malignancies as an Emerging Opportunistic Infection. Acta Parasitologica, 64(1), 103–111. doi:10.2478/s11686-018-00012-w

Glöckner, F. O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., … Ludwig, W. (2017). 25 years of serving the community with ribosomal RNA gene reference databases and tools. Journal of Biotechnology, 261, 169–176. doi: 10.1016/j.jbiotec.2017.06.1198

Grabner, D. S. (2017). Hidden diversity: parasites of stream arthropods. Freshwater Biology, 62(1), 52–64. doi: 10.1111/fwb.12848

Grabner, D. S., Weigand, A. M., Leese, F., Winking, C., Hering, D., Tollrian, R., & Sures, B. (2015). Invaders, natives and their enemies: distribution patterns of amphipods and their microsporidian parasites in the Ruhr Metropolis, Germany. Parasites and Vectors, 8, 419. doi: 10.1186/s13071-015-1036-6

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica, 4(1), 1-9. doi:10.1016/j.bcp.2008.05.025

Hannon, G.J. (2010) FASTX-Toolkit. http://hannonlab.cshl.edu

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences, 270, 313–321. doi: 10.1098/rspb.2002.2218

Hollister, W. S., Canning, E. U., & Willcox, A. (1991). Evidence for widespread occurrence of antibodies to Encephalitozoon cuniculi (Microspora) in man provided by ELISA and other serological tests. Parasitology, 102, 33–43. doi:10.1017/S0031182000060315

Huang, W. F., Bocquet, M., Lee, K. C., Sung, I. H., Jiang, J. H., Chen, Y. W., & Wang, C. H. (2008). The comparison of rDNA spacer regions of Nosema ceranae isolates from different hosts and locations. Journal of Invertebrate Pathology, 97(1), 9–13. doi: 10.1016/j.jip.2007.07.001

Huibers, M. H. W., Moons, P., Maseko, N., Gushu, M. B., Iwajomo, O. H., Heyderman, R. S., … Calis, J. C. J. (2018). Multiplex Real-Time PCR Detection of Intestinal Protozoa in HIV-Infected Children in Malawi, Enterocytozoon Bieneusi is Common and Associated with Gastrointestinal Complaints and May Delay BMI (Nutritional Status) Recovery. The Pediatric Infectious Disease Journal, 37(9), 910–915. doi:10.1097/inf.0000000000001924

Hyliš, M., Pilarska, D. K., Oborník, M., Vávra, J., Solter, L. F., Weiser, J., … McManus, M. L. (2006). Nosema chrysorrhoeae n. sp. (Microsporidia), isolated from browntail moth (Euproctis chrysorrhoea L.) (Lepidoptera, Lymantriidae) in Bulgaria: Characterization and phylogenetic relationships. Journal of Invertebrate Pathology, 91(2), 105–114. doi: 10.1016/j.jip.2005.11.006

Ignatius, R., Henschel, S., Liesenfeld, O., Mansmann, U., Schmidt, W., Köppe, S., … Ullrich, R. (1997). Comparative evaluation of modified trichrome and Uvitex 2B stains for detection of low numbers of microsporidial spores in stool specimens. Journal of Clinical Microbiology, 35(9), 2266–2269.

Ishihara, R., & Hayashi, Y. (1968). Some properties of ribosomes from the sporoplasm of Nosema bombycis. Journal of Invertebrate Pathology, 11(3), 377–385. doi:10.1016/0022-2011(68)90186-9

Izquierdo, F., Castro-Hermida, J. A., Fenoy, S., Mezo, M., González-Warleta, M., & del Aguila, C. (2011). Detection of microsporidia in drinking water, wastewater and recreational rivers. Water Research, 45(16), 4837–4843. doi:10.1016/J.WATRES.2011.06.033

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., … Vilgalys, R. (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature, 443, 818–822. doi: 10.1038/nature05110

James, T. Y., Pelin, A., Bonen, L., Ahrendt, S., Sain, D., Corradi, N., & Stajich, J. E. (2013). Shared signatures of parasitism and phylogenomics unite cryptomycota and microsporidia. Current Biology, 23(16), 1548–1553. doi: 10.1016/j.cub.2013.06.057

Javanmard, E., Mirjalali, H., Niyyati, M., Jalilzadeh, E., Seyed Tabaei, S. J., Asadzadeh Aghdaei, H., … Zali, M. R. (2018). Molecular and phylogenetic evidences of dispersion of human-infecting microsporidia to vegetable farms via irrigation with treated wastewater: One-year follow up. International Journal of Hygiene and Environmental Health, 221(4), 642–651. doi:10.1016/j.ijheh.2018.03.007

Karpov, S. A., Mamkaeva, M. A., Aleoshin, V. V., Nassonova, E., Lilje, O., & Gleason, F. H. (2014). Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. Frontiers in Microbiology 5, 1–11. doi: 10.3389/fmicb.2014.00112

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution, 30(4), 772–780. doi:10.1093/molbev/mst010

Katoh, K., Misawa, K., Kuma, K., & Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research, 30(14), 3059–3066. doi: 10.1093/nar/gkf436

Krebes, L., Blank, M., Frankowski, J., & Bastrop, R. (2010). Molecular characterisation of the Microsporidia of the amphipod Gammarus duebeni across its natural range revealed hidden diversity, wide-ranging prevalence and potential for co-evolution. Infection, Genetics and Evolution, 10(7), 1027–1038. doi:10.1016/J.MEEGID.2010.06.011

Kruskal, W. H., & Wallis, W. A. (1952). Use of Ranks in One-Criterion Variance Analysis. Journal of the American Statistical Association 47(260), 583-621. doi:10.1080/01621459.1952.10483441

966 Kučerová-Pospišilová, Z., & Ditrich, O. (1998). The serological surveillance of several groups
967 of patients using antigens of Encephalitozoon hellem and E. cuniculi antibodies to
968 microsporidia in patients. Folia Parasitologica, 45(2), 108–112.

969 Kyei-Poku, G., Gauthier, D., & Van Frankenhuyzen, K. (2008). Molecular data and phylogeny
970 of Nosema infecting lepidopteran forest defoliators in the genera Choristoneura and
971 Malacosoma. Journal of Eukaryotic Microbiology, 55(1), 51–58. doi:10.1111/j.1550-
972 7408.2007.00302.x

973 Lanfear, R., Calcott, B., Ho, S. Y. W., & Guindon, S. (2012). PartitionFinder: Combined
974 selection of partitioning schemes and substitution models for phylogenetic analyses.
975 Molecular Biology and Evolution, 29(6), 1695–1701. doi: 10.1093/molbev/mss020

976 Li, N., Xiao, L., Wang, L., Zhao, S., Zhao, X., Duan, L., … Feng, Y. (2012). Molecular
977 Surveillance of Cryptosporidium spp., Giardia duodenalis, and Enterocytozoon bieneusi
978 by Genotyping and Subtyping Parasites in Wastewater. PLoS Neglected Tropical
979 Diseases, 6(9), e1809. doi:10.1371/journal.pntd.0001809

980 Luján, H. D., Conrad, J. T., Clark, C. G., Touz, M. C., Delbac, F., Vivares, C. P., & Nash, T.
981 E. (1998). Detection of Microsporidia Spore-Specific Antigens by Monoclonal Antibodies.
982 Hybridoma, 17(3), 237–243. doi:10.1089/hyb.1998.17.237

983 Maddox, J. V., Baker, M. D., Jeffords, M. R., Kuras, M., Linde, A., Solter, L. F., …
984 Vossbrinck, C. R. (1999). Nosema portugal, N. SP., Isolated from Gypsy Moths
985 (Lymantria dispar L.) Collected in Portugal. Journal of Invertebrate Pathology, 73(1), 1–
986 14. doi:10.1006/jipa.1998.4817

987 Menotti, J., Cassinat, B., Porcher, R., Sarfati, C., Derouin, F., & Molina, J-M. (2003).
988 Development of a Real-Time Polymerase-Chain-Reaction Assay for Quantitative
989 Detection of Enterocytozoon bieneusi DNA in Stool Specimens from
990 Immunocompromised Patients with Intestinal Microsporidiosis. The Journal of Infectious
991 Diseases, 187(9), 1469–1474. doi: 10.1086/374620

992 Mikhailov, K. V., Simdyanov, T. G., & Aleoshin, V. V. (2017). Genomic survey of a
993 hyperparasitic microsporidian Amphiamblys sp. (Metchnikovellidae). Genome Biology
994 and Evolution, 9(3), 454–467. doi: 10.1093/gbe/evw235

995 ~~Moreira, L. A., Iturbe-Ormaetxe, I., Jeffery, J. A., Lu, G., Pyke, A. T., Hedges, L. M., …~~
996 ~~O'Neill, S. L. (2009). A Wolbachia Symbiont in Aedes aegypti Limits Infection with~~
997 ~~Dengue, Chikungunya, and Plasmodium. Cell, 139(7), 1268–1278.~~
998 ~~doi:10.1016/j.cell.2009.11.042~~

999 Morgulis, A., Coulouris, G., Raytselis, Y., Madden, T. L., Agarwala, R., & Schäffer, A. A.
1000 (2008). Database indexing for production MegaBLAST searches. Bioinformatics, 24(16),
1001 1757–1764. doi:10.1093/bioinformatics/btn322

1002 Moura, H., Schwartz, D. A., Bornay-Llinares, F. J., Sodré, F. C., Wallace, S., & Visvesvara,
1003 G. S. (1997). A new and improved "quick-hot Gram-chromotrope" technique that
1004 differentially stains microsporidian spores in clinical samples, including paraffin-
1005 embedded tissue sections. Archives of Pathology and Laboratory Medicine, 121(8),
1006 888–893.

1007 Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S., & De Wachter, R. (1993).
1008 Compilation of small ribosomal subunit RNA structures. Nucleic Acids Research, 21(13),
1009 3025–3049. doi: 10.1093/nar/21.13.3025

1010 Nicholas Jr., H. B., & McClain, W. H. (1995). Searching tRNA sequences for relatedness to
1011 aminoacyl-tRNA synthetase families. Journal of Molecular Evolution, 40, 482–486. doi:
1012 10.1007/BF00166616

1013 Nishikori, K., Setiamarga, D. H. E., Tanji, T., Kuroda, E., Shiraishi, H., & Ohashi-Kobayashi,
1014 A. (2018). A new microsporidium Percutemincola moriokae gen. nov., sp. nov. from
1015 Oscheius tipulae: A novel model of microsporidia–nematode associations. Parasitology,
1016 145(14), 1853–1864. doi:10.1017/s0031182018000628

1017 Pearson, K. (1895). Note on Regression and Inheritance in the Case of Two Parents.
1018 Proceedings of the Royal Society of London, 58(347-352), 240–242.
1019 doi:10.1098/rspl.1895.0041

1020 Pearson, K. (1900). On the criterion that a given system of deviations from the probable in
1021 the case of a correlated system of variables is such that it can be reasonably supposed
1022 to have arisen from random sampling. The London, Edinburgh, and Dublin Philosophical
1023 Magazine and Journal of Science 5, 50, 157–175. doi:10.1080/14786440009463897

1024 Peterson, T. S., Spitsbergen, J. M., Feist, S. W., & Kent, M. L. (2011). Luna stain, an
1025 improved selective stain for detection of microsporidian spores in histologic sections.
1026 Diseases of Aquatic Organisms, 95(2), 175–180. doi:10.3354/dao02346

1027 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O.
1028 (2013). The SILVA ribosomal RNA gene database project: Improved data processing
1029 and web-based tools. Nucleic Acids Research, 41(D1), 590–596.
1030 doi:10.1093/nar/gks1219

1031 Quiles, A., Bacela-Spychalska, K., Teixeira, M., Lambin, N., Grabowski, M., Rigaud, T., &
1032 Wattier, R. A. (2019). Microsporidian infections in the species complex Gammarus
1033 roeselii (Amphipoda) over its geographical range: Evidence for both host-parasite co-
1034 diversification and recent host shifts. Parasites and Vectors, 12, 327. doi:
1035 10.1186/s13071-019-3571-z

1036 Radek, R., Kariton, M., Dabert, J., & Alberti, G. (2015). Ultrastructural characterization of
1037 Acarispora falculifera n.gen., n.sp., a new microsporidium (Opisthokonta: Chytridiopsida)
1038 from the feather mite Falculifer rostratus (Astigmata: Pterolichoidea). Acta
1039 Parasitologica, 60(2), 200–210. doi:10.1515/ap-2015-0029

1040 Rambaut, A. (2018). FigTree v. 1.4.4. https://github.com/rambaut/figtree

1041 Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T., & de Graaf, D. C. (2014).
1042 Widespread occurrence of honey bee pathogens in solitary bees. Journal of Invertebrate
1043 Pathology, 122, 55–58. doi:10.1016/j.jip.2014.08.007

1044 Ribeiro, M. F. B., & Passos, L. M. F. (2006). Natural co-infection of Babesia caballi and
1045 Encephalitozoon -like microsporidia in the tick Anocentor nitens (Acari : Ixodidae). 93(3),
1046 183–185. doi: 10.1016/j.jip.2006.07.003

1047 Rijpkema, S., & Bruinink, H. (1996). Detection of Borrelia burgdorferi sensu lato by PCR in
1048 questing Ixodes ricinus larvae from the Dutch North Sea island of Ameland.
1049 Experimental and Applied Acarology, 20, 381–385. doi:10.1007/BF00130550

1050 Rinder, H., Janitschke, K., Aspöck, H., Da Silva, A. J., Deplazes, P., Fedorko, D. P., … the
1051 Diagnostic Multicenter Study Group on Microsporidia (1998). Blinded, externally
1052 controlled multicenter evaluation of light microscopy and PCR for detection of
1053 microsporidia in stool specimens. Journal of Clinical Microbiology, 36(6), 1814–1818.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., … Huelsenbeck, J. P. (2012). Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Systematic Biology, 61(3), 539–542. doi: 10.1093/sysbio/sys029

~~Scholte, E. J., Ng'Habi, K., Kihonda, J., Takken, W., Paaijmans, K., Abdulla, S., … Knols, B. G. J. (2005). An entomopathogenic fungus for control of adult African malaria mosquitoes. Science, 308(5728), 1641–1642. doi:10.1126/science.1108639~~

Shapiro, S. S., & Wilk, M. B. (1965). An Analysis of Variance Test for Normality. Biometrika, 52(3-4), 591–611. doi:10.1093/biomet/52.3-4.591

Sharma, S., Balne, P. K., & Das, S. (2014). Ocular Microsporidiosis. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity: First Edition (pp. 403-419). Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi: 10.1002/9781118395264.ch16

Simakova, A. V., Tokarev, Y. S., & Issi, I. V. (2018). A new microsporidium Fibrillaspora daphniae g. n. sp. n. infecting Daphnia magna (Crustacea: Cladocera) in Siberia and its taxonomic placing within a new family Fibrillasporidae and new superfamily Tubulinosematoidea (Opisthosporidia: Microsporidia). Parasitology Research, 117(3), 759-766. doi: 10.1007/s00436-018-5749-2

Singh, I., Sheoran, A. S., Zhang, Q., Carville, A., & Tzipori, S. (2005). Sensitivity and Specificity of a Monoclonal Antibody-Based Fluorescence Assay for Detecting Enterocytozoon bieneusi Spores in Feces of Simian Immunodeficiency Virus-Infected Macaques. Clinical and Vaccine Immunology, 12(10), 1141–1144. doi:10.1128/cdli.12.10.1141-1144.2005

Sokolova, Y. Y., & Overstreet, R. M. (2018). A new microsporidium, Apotaspora heleios n. g., n. sp., from the Riverine grass shrimp Palaemonetes paludosus (Decapoda: Caridea: Palaemonidae). Journal of Invertebrate Pathology, 157, 125–135. doi:10.1016/j.jip.2018.05.007

Steele, T., & Bjørnson, S. (2014). Nosema adaliae sp. nov., a new microsporidian pathogen from the two-spotted lady beetle, Adalia bipunctata L. (Coleoptera: Coccinellidae) and its relationship to microsporidia that infect other coccinellids. Journal of Invertebrate Pathology, 115, 108–115. doi:10.1016/j.jip.2013.09.008

Stentiford, G. D., Becnel, J. J., Weiss, L. M., Keeling, P. J., Didier, E. S., Williams, B. A. P., … Solter, L. (2016). Microsporidia – Emergent Pathogens in the Global Food Chain. Trends in Parasitology, 32(4), 336–348. doi:10.1016/j.pt.2015.12.004

Tsai, S. J., Kou, G. H., Lo, C. F., & Wang, C. H. (2002). Complete sequence and structure of ribosomal RNA gene of Heterosporis anguillarum. Diseases of Aquatic Organisms, 49(3), 199–206. doi:10.3354/dao049199

~~Turell, M. J., Rossignol, P. A., Spielman, A., Rossi, C. A., & Bailey, C. L. (1984). Enhanced arboviral transmission by mosquitoes that concurrently ingested microfilariae. Science, 225(4666), 1039–1041. doi:10.1126/science.6474165~~

van Gool, T., Snijders, F., Reiss, P., Eeftinck Schattenkerk, J. K., van den Bergh Weerman, M. A., Bartelsman, J. F., … Dankert, J. (1993). Diagnosis of intestinal and disseminated microsporidial infections in patients with HIV by a new rapid fluorescence technique. Journal of Clinical Pathology, 46(8), 694–699. doi:10.1136/jcp.46.8.694

van Gool, T., Vetter, J. C. M., Weinmayr, B., Van Dam, A., Derouin, F., & Dankert, J. (1997). High Seroprevalence of Encephalitozoon Species in Immunocompetent Subjects. The Journal of Infectious Diseases, 175(4), 1020–1024. doi:10.1086/513963

~~Vaughan, J. A., & Turell, M. J. (1996). Facilitation of Rift Valley fever virus transmission by Plasmodium berghei sporozoites in Anopheles stephensi mosquitoes. Am J Trop Med Hyg, 55(4), 407–409. doi:10.4269/ajtmh.1996.55.407~~

Vávra, J. (2005). “Polar vesicles” of microsporidia are mitochondrial remnants (“mitosomes”)? Folia Parasitologica, 52(1–2), 193–195. doi:10.14411/fp.2005.024

Vávra, J., & Lukeš, J. (2013). Microsporidia and ‘The Art of Living Together.’ In D. Rollinson (Ed.), Advances in Parasitology (Vol. 82, pp. 253–319). Cambridge, Massachusetts, United States: Academic Press. doi:10.1016/B978-0-12-407706-5.00004-6

Vávra, J., Fiala, I., Krylová, P., Petrusek, A., & Hyliš, M. (2019). Establishment of a new microsporidian genus and species, Pseudoberwaldia daphnia (Microsporidia, Opisthosporidia), a common parasites of the Daphnia longispina complex in Europe. Journal of Invertebrate Pathology, 162, 43–54. doi:10.1016/j.jip.2019.02.004

Verweij, J. J., Ten Hove, R., Brienen, E. A., & van Lieshout, L. (2007). Multiplex detection of Enterocytozoon bieneusi and Encephalitozoon spp. in fecal samples using real-time PCR. Diagnostic Microbiology and Infectious Disease, 57(2), 163–167. doi:10.1016/j.diagmicrobio.2006.08.009

Vilcinskas, A., Stoecker, K., Schmidtberg, H., Röhrich, C. R., & Vogel, H. (2013). Invasive harlequin ladybird carries biological weapons against native competitors. Science, 340(6134), 862–863. doi: 10.1126/science.1234032

Vossbrinck, C. R., & Debrunner-Vossbrinck, B. A. (2005). Molecular phylogeny of the Microsporidia: Ecological, ultrastructural and taxonomic considerations. Folia Parasitologica, 52(1–2), 131–142. doi:10.14411/fp.2005.017

Vossbrinck, C. R., Andreadis, T. G., Vávra, J., & Becnel, J. J. (2004). Molecular Phylogeny and Evolution of Mosquito Parasitic Microsporidia (Microsporidia: Amblyosporidae). Journal of Eukaryotic Microbiology, 51(1), 88–95. doi:10.1111/j.1550-7408.2004.tb00167.x

Vossbrinck, C. R., Debrunner-Vossbrinck, B. A., & Weiss, L. M. (2014). Phylogeny of the Microsporidia. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity: First Edition (pp. 203–220). Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi:10.1002/9781118395264.ch6

Wang, Z. D., Liu, Q., Liu, H. H., Li, S., Zhang, L., Zhao, Y. K., & Zhu, X. Q. (2018). Prevalence of Cryptosporidium, microsporidia and Isospora infection in HIV-infected people: A global systematic review and meta-analysis. Parasites and Vectors, 11, 28. doi:10.1186/s13071-017-2558-x

Wang, Z., Orlandi, P. A., & Stenger, D. A. (2005). Simultaneous detection of four human pathogenic microsporidian species from clinical samples by oligonucleotide microarray. Journal of Clinical Microbiology, 43(8), 4121–4128. doi:10.1128/JCM.43.8.4121-4128.2005

Wattier, R. A., Haine, E. R., Beguet, J., Martin, G., Bollache, L., Muskó, I. B., … Rigaud, T. (2007). No genetic bottleneck or associated microparasite loss in invasive populations of a freshwater amphipod. Oikos, 116(11), 1941–1953. doi: 10.1111/j.2007.0030-1299.15921.x

Weber, R., Bryan, R. T., Schwartz, D. A., & Owen, R. L. (1994). Human microsporidial infections. Clinical Microbiology Reviews, 7(4), 426–461. doi:10.1128/CMR.7.4.426

Weiss, L. M. (2014). Clinical Syndromes Associated with Microsporidiosis. In L. M. Weiss & J. J. Becnel (Eds.), Microsporidia: Pathogens of Opportunity: First Edition (pp. 371–401). Hoboken, New Jersey, United States: John Wiley & Sons, Inc. doi:10.1002/9781118395264.ch15

Weiss, L. M., & Schwartz, D. A. (2015). Microsporidiosis. In J. E. Bennett, R. Dolin, & M. J. Blaser (Eds.), Principles and Practice of Infectious Diseases (8th ed., Vol. 2, pp. 3031–3044). Amsterdam, Netherlands: Elsevier Inc. doi:10.1016/C2012-1-00075-6

Weiss, L. M., & Vossbrinck, C. R. (1998). Microsporidiosis: molecular and diagnostic aspects. Advances in Parasitology, 40, 351–395. doi: 10.1016/S0065-308X(08)60127-X

Williams, B. A. P., Hamilton, K. M., Jones, M. D., & Bass, D. (2018). Group-specific environmental sequencing reveals high levels of ecological heterogeneity across the microsporidian radiation. Environmental Microbiology Reports, 10(3), 328–336. doi:10.1111/1758-2229.12642

Williams, B. A. P., Hirt, R. P., Lucocq, J. M., & Embley, T. M. (2002). A mitochondrial remnant in the microsporidian Trachipleistophora hominis. Nature, 418, 865–869. doi:10.1038/nature00949

Wolk, D. M., Schneider, S. K., Wengenack, N. L., Sloan, L. M., & Rosenblatt, J. E. (2002). Real-time PCR method for detection of Encephalitozoon intestinalis from stool specimens. Journal of Clinical Microbiology, 40(11), 3922–3928. doi:10.1128/JCM.40.11.3922-3928.2002

Xu, Y., & Weiss, L. M. (2005). The microsporidian polar tube: A highly specialised invasion organelle. International Journal for Parasitology, 35(9), 941–953. doi:10.1016/j.ijpara.2005.04.003

Yaman, M., Bekircan, Ç., Radek, R., & Linde, A. (2014). Nosema pieriae sp. n. (Microsporida, Nosematidae): A new microsporidian pathogen of the cabbage butterfly Pieris brassicae L. (Lepidoptera: Pieridae). Acta Protozoologica, 53(2), 223–232. doi:10.4467/16890027AP.14.019.1600

Yilmaz, P., Wegener Parfrey, L., Yarza, P., Gerken, J., Pruesse, E., Quast, C., … Glöckner, F. O. (2014). The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. Nucleic Acids Research, 42(D1), 643–648. doi: 10.1093/nar/gkt1209

Zhang, Q., Singh, I., Sheoran, A., Feng, X., Nunnari, J., Carville, A., & Tzipori, S. (2005). Production and characterization of monoclonal antibodies against Enterocytozoon bieneusi purified from rhesus macaques. Infection and Immunity, 73(8), 5166–5172. doi:10.1128/IAI.73.8.5166-5172.2005

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A Greedy Algorithm for Aligning DNA Sequences. Journal of Computational Biology, 7(1–2), 203–214. doi:10.1089/10665270050081478

Zhu, X., Wittner, M., Tanowitz, H. B., Kotler, D., Cali, A., Weiss, L. M., … Weiss, L. M. (1993). Small Subunit rRNA Sequence of Enterocytozoon bieneusi and Its Potential Diagnostic Role with Use of the Polymerase Chain Reaction. Journal of Infectious Diseases, 168(6), 1570–1575. doi:10.1093/infdis/168.6.1570

1186 Zwickl D. M. (2006). Genetic algorithm approaches for the phylogenetic analysis of large
1187 biological sequence datasets under the maximum likelihood criterion. Dissertation, The
1188 University of Texas at Austin, 1–125.

Figure captions

Figure 1.

Protocol for metabarcoding of microsporidia and their hosts. Total genomic DNA is extracted from each previously-washed host specimen and from the medium used for host preservation and washing. Mini-COI barcode and V5 region of ssu rDNA are amplified using PCR primers fused with double-indexed NGS adaptors. The mini-COI and V5 libraries are prepared separately, and after quality control, pooled in a ratio 1:10, respectively. The libraries are NGS sequenced using a threshold of at least 10,000 reads per sample. After data quality filtering, the OTUs are clustered, and then compared to databases with reference sequences.

~~**Figure 2.**~~

~~Relationship between the numbers of microsporidian spores used for DNA extraction and the numbers of reads in quality-filtered sequence data. The method was reproducible for all tested species to the level of 100 spores per 1 mL.~~

Figure ~~3~~2.

Relationship between the numbers of spores of different species in mixed samples and their relative sequence read abundance in quality-filtered sequence data. DNAs from *E. cuniculi* and *E. hellem* spores were detected even when they were mixed in a ratio of 1 to 1000 (10:10:10,000) with *E. intestinalis*.

Figure ~~4~~3.

Heatmap showing microsporidian species detected in field-collected mosquitoes. Blue colour darkens as the number of infected individuals in each mosquito species increases. Abbreviations of microsporidian species: *A*. sp. — *Amblyospora* sp., *A*. *s*. — *A. salinaria*, *E*. *h*. — *Encephalitozoon hellem*, *E*. *a*. — *Enterocytospora artemiae*, M. sp. PL01 — Microsporidium sp. ~~nov.~~ PL01, *N*. *a*. — *Nosema adaliae*, *N*. *c*. — *N. ceranae*, *N*. *p*. — *N. pieriae*, *N*. *t*. — *N. thomsoni*, *N*. *ch./p*. — *N. chrysorrhoeae* and/or *N*. *portugal*, *N*. sp. — *Nosema* sp. CHW-2007a.

Figure ~~5~~4.

Phylogenetic tree of Microsporidia inferred from BI and ML analyses of concatenated ssu rDNA sequence data. Values near branches show Bayesian posterior probabilities (PP) and bootstrap supports (BS) (PP/BS). Black circles: maximally supported; empty circles: supported >0.95 PP and >75% BS. Sequences found in this study are in bold; the new species found in this study is in red.

Figure ~~6~~5.

Co-infection network of microsporidian species detected in field-collected mosquitoes. Thick lines — species previously noted in mosquitoes; thin lines — species that potentially can infect mosquitoes; dashed lines — species recognized as incidental because each of OTUs was noticed only in one host species and was found in <1% of all mosquito individuals; shaded in colour — co-infections with three different species. Abbreviations of mosquito species: Ac — *Aedes cinereus*, Av — *A. vexans*, Cr — *Coquillettidia richiardii*, Oa — *Ochlerotatus annulipes*, Oc — *O. cantans*, Op — *O. punctor*, Os — *O. sticticus*.

Figure ~~7~~6.

Correlation network between the numbers of reads representing each microsporidian species found in co-infected mosquitoes. The blue lines indicate statistically significant correlations. Thicker line indicates higher correlation (for correlation values see Table S3).

Figure ~~8~~7.

Percentage of identities of the aligned V1-V3 and V5 regions of microsporidian ssu rDNA calculated using Kolmogorov-Smirnov statistical test. The plots show that about 0.75 fraction of the V1-V3 and V5 aligned sequences has less than 60% and 64% of identical nucleotide positions, respectively.

Table 1.

Microsporidia found in field-collected mosquitoes using the metabarcoding. The total number of infected mosquito specimens is higher due to the co-infections with two or more microsporidian species. M-P – microsporidia-positive mosquitoes.

Table 2.

Numbers of sequence reads representing microsporidian OTUs in co-infected mosquitoes. Samples that were unreadable in direct Sanger sequencing of V1-V3 amplicons are in bold; samples where direct Sanger sequencing of V1-V3 amplicons detected only Microsporidium sp. ~~nov.~~ PL01 are marked with a grey background. Abbreviations of microsporidian species: *A. s.* — *Amblyospora salinaria*, *A.* sp. — *Amblyospora* sp., *E. h.* — *Encephalitozoon hellem*, *E. a.* — *Enterocytospora artemiae*, M. sp. PL01 — Microsporidium sp. ~~nov.~~ PL01, *N. a.* —

Nosema adaliae, *N. c*. — *N. ceranae*, *N. ch./p*. — *N. chrysorrhoeae* and/or *N. portugal*, *N. p*. — *N. pieriae*, *N. t*. — *N. thomsoni*, *N*. sp. — *Nosema* sp. CHW-2007a.