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 obligate intracellular eukaryotic parasites that infect nearly all animal groups, including humans. 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%). 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.

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 and certain protist species. 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, Microsporidia can infect any organ system; the most reported 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, since Microsporidia are studied mostly as zoonotic and/or waterborne agents of human disease or veterinary parasites. 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 of both morphological and genomic characteristics. The classical microsporidian cell is characterized by mitochondria 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 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 identification (Xu & Weiss, 2005). Increasingly, the description of new species is being supplemented by molecular data (Nishikori et al., 2018; Sokolova & Overstreet, 2018; Vávra, Fiala, Krylová, Petrusek, & Hyliš, 2019).

The standard Microsporidia detection methods 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). On the one hand, the staining for light microscopy rarely enables species identification. 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 nested- or quantitive-PCR techniques 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). Microsporidia detection in natural populations, on the other hand, base on end-point PCR amplification of 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 Microsporidia-specific (Weiss & Vossbrinck, 1998) or semi-specific primer sets (e.g. Emsen et al., 2016; Grabner, 2017; Grabner et al., 2015; Quiles et al., 2019). Presently, the primer pair V1F (Zhu et al., 1993) and 530R (Baker, Vossbrinck, Didier, Maddox, & Shadduck, 1995) seems 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 other species of insects, crustaceans, and vertebrates (Andreadis, 2007; Becnel, White, & Shapiro, 2005; Vossbrinck, Andreadis, Vávra, & Becnel, 2004).

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 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.

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*.

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 collected at night using human-landing catches. 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 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

We compared the performance of our new metabarcoding approach with the standard PCR-based detection method. 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) 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.) 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.

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.

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, where the amplicons were invisible on the gel, to 1 µl in case of brighter bands. The rare samples which had very high intensity were diluted 100-fold 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), 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 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

295 published in GenBank under the accession numbers MT001301-MT001427, MT015707-
296 MT015901 and MT075548-MT075550 (Table S1).

297

298 ***Phylogenetic analyses***

299 To confirm the taxonomic position of the Microsporidia detected in field-collected
300 mosquitoes we used 122 ssu rDNA sequences representing all known clades of the classical
301 Microsporidia (74), Metchnikovellida (4) and Chytridopsida (1). As close outgroups, we used
302 Rozellomycota (6) and Fungi (28). For details concerning all ingroup and outgroup taxa see
303 Table S2. Sequences were aligned using the L–INS–I algorithm in MAFFT v7.388 (Katoh,
304 Misawa, Kuma, & Miyata, 2002; Katoh & Standley, 2013) as implemented in Geneious
305 R11.1.5. The final alignment consisted of 2801 nucleotide positions (nps). The best fit model
306 of DNA evolution (GTR+I+G) was chosen by PartitionFinder2 (Lanfear, Calcott, Ho, &
307 Guindon, 2012). Phylogenetic trees were reconstructed using Maximum Likelihood (ML) in
308 Garli v.2.0 (Zwickl, 2006) and Bayesian inference (BI) in MrBayes 3.2.6 (Ronquist et al.,
309 2012). Each BI run of four independent chains was performed in 2 × 10,000,000 generations,
310 and the trees were sampled every 1000 generations. The final consensus tree was
311 generated after discarding the burn-in fraction of 0.25% of initial trees; the average standard
312 deviation of split frequencies dropped below 0.003. Bootstrap support for the ML tree was
313 calculated by using 1000 data replicates as implemented in Garli. The trees were edited in
314 FigTree 1.4.4 (Rambaut, 2018) and further in Corel Draw X4.

315

316 ***Statistical analyses***

317 Sequence reads from microsporidia were normalized by OTUTAB_RARE algorithm
318 (Edgar & Flyvbjerg, 2018) to compare sample diversities. The diversity of OTUs in individual
319 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 (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.

345 None of the negative control samples passed our sequencing quality threshold of having at
346 least 10 reads per OTU. Sequences generated from the DNAs extracted from cultured
347 microsporidians (ca. 18,500) showed three OTUs that corresponded to the microsporidian
348 species from which DNA samples were extracted: *A*. *algerae*, *V*. *culicis*, and *E. bieneusi*.

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

355 Analysis of the relative sequence read abundance in sequencing results from DNA
356 samples being a mixture of microsporidian species showed that DNAs from species present
357 in smaller quantities in the sample (*E. cuniculi* and *E. hellem* vs *E*. *intestinalis*) were
358 successfully detected in each mixture of spores in all technical replicates (Figure 2). DNAs
359 from *E. cuniculi* and *E. hellem* spores were detected in the sample even when they were
360 mixed in a ratio of 1 to 1000 with *E. intestinalis*. In this sample, they were represented by
361 about 0.1% of all reads which corresponded to the assumed share of the spores
362 representing each species. The repeatability of the experiment was supported by Kruskal–
363 Wallis test ($H < 0.008$; $p > 0.05$).

364 To test the new method on colony mosquitoes, 213 samples consisting of the V5
365 region amplicons from *A. aegypti*, *A. quadrimaculatus*, *C. quinquefasciatus*, *U. lowii* and
366 negative controls (13) were sequenced in one experiment. Negative controls included PCRs
367 performed on the DNA extracted from preservation medium for mosquitoes, blank DNA
368 extractions, and blank PCRs. After quality filtering, 480,307 reads were used in the OTU
369 analysis. None of the negative control samples passed our sequencing quality threshold to
370 generate OTUs. Less than 9% of the whole sequence data were of non-microsporidian

origin. From these non-microsporidian sequences, 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 for only 12% of all quality filtered reads; most of them were of host origin (11.9%) and coded ssu rRNA genes (9.8%) or a collagen alpha-1 chain gene (2.1%; 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 which we named Microsporidium sp. 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, S1). Rarefaction curve analysis showed that the read depth was sufficient to recover all microsporidian species in the tested individuals (Figure S2). The analysis revealed that 10,000 reads per sample 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 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. 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. PL01 (69.3% 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 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 (4.7%) was *Nosema* sp. CHW-2007a, which was recorded in five of seven examined host species (Figure 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. 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 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%); this relationship concerned all microsporidian and host species (Figure 5, Table 2). There were no statistically significant relationships between co-occurring microsporidians and mosquito species ($\chi^2 = 1.2$; $p > 0.05$). Microsporidia considered as incidental – *E. hellem*, *E. artemiae*, and *N. ceranae* – were found with the most abundant Microsporidium sp. PL01 in four mosquito individuals; additionally, in a single case, *N. ceranae* was noticed with *N. chrysorrhoeae* and/or *N. portugal*. The co-occurrence rate remained high in all host species, even omitting the incidental microsporidians. 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. PL01 (76.2%), the highest convergence rate of co-infections concerned microsporidians of the genus *Nosema*. Among 39 cases of the presence of *Nosema* DNAs in

447 mosquitoes, 23 of them (59%) were observed in co-occurrence with DNA representing the
448 other microsporidian species. The chi-squared statistic showed that *Nosema* spp. occurred
449 more frequently in mosquitoes with another microsporidian species than individually (χ^2 =
450 25.15; $p < 0.05$).

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

456

457 ***Comparison of metabarcoding and standard molecular approach***

458 The Kolmogorov-Smirnov plots for the microsporidian V1-V3 and V5 sequence
459 alignments showed that the proposed V5 marker is only 4% less variable than the standard
460 sequence covering V1-V3 regions (about 40% and 36% of different nps, respectively) (Figure
461 7). The extraction of duplicate reads from the alignment comprising the V5 region
462 representing 607 microsporidian species belonging to 120 genera indicated that the marker
463 enables the proper identification of almost all species in the dataset (596/607, >98%).
464 Several sequences that were found in duplicates represented species of mainly of the genus
465 *Nosema*, e.g. *N. chrysorrhoeae* and *N. portugal* or a group consisting of *N. antheraeae, N.*
466 *trichoplusiae, N. spodopterae*, and *N. philosamiae* showed no variation in the V5 region.
467 However, in these sample groups extremely low or no variation can also be observed in
468 other regions of the ssu rDNA, including V1-V3 marker (data not shown). In three cases, the
469 same V5 region sequences were found in species representing different genera
470 (*Conglomerata obtusa* and *Berwaldia schaefernai*; *Ameson portunus*, *A. pulvis* and
471 *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 S3, Table 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 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. PL01. The Microsporidium sp. PL01 populations 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. 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. 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 all types 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 V5 region 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, Terresporidia, and Marinosporidia (Vossbrinck & Debrunner-Vossbrinck, 2005) (Figure 4). However, recently published first sequence data for the two remaining microsporidian lineages – metchnikovellid *Amphiamblys* sp. (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 Metchnikovellida and the Chytridiopsida 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, clinical samples 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 overcome these difficulties saving the analysis time and costs. In addition, the metabarcoding of concentrated water samples could be standardized which can help to develop good laboratory methods for more accurate 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

549 and ranged from 1% to 9.6% (Andreadis, 1999). On the other hand, in the study concerning
550 a life cycle and ecology of *Amblyospora khaliulini*, prevalence rates of *A*. *khaliulini* infections
551 in adult mosquito females ranged from 16.4% to 50% (Andreadis, Thomas, & Shepard,
552 2018). In our study, all mosquito species showed a much higher level of microsporidian
553 positive individuals, ranging from 54.0% to 92.3% (Table 1). However, in the studies
554 conducted by Andreadis et al. (1999, 2018), Microsporidia were identified by microscopic
555 examination of infected tissues; mosquitoes were considered infected if either the vegetative
556 or spore developmental stage of the parasite was observed.

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

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

575 knowledge, the remaining microsporidian species, found by us in field-collected mosquitoes,
576 have been recorded in Culicidae for the first time.

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

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

596 The new microsporidian ssu rDNA sequence found in this study is the most similar to
597 ssu rDNA of Microsporidium sp. 1199 which was recorded in freshwater populations of
598 *Gammarus duebeni* (Crustacea, Amphipoda) in a rivulet from Wales, Holyhead Island, during
599 research on molecular characterization of the microsporidians of this host across its natural
600 range (Krebes, Blank, Frankowski, & Bastrop, 2010). Our phylogenetic analysis results

strongly support that Microsporidium sp. 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 this clade 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%) 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. PL01 are evidence of mixed infection by these species.

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 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.

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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-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.

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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 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 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. 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 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 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 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 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. 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. 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.