Natural distribution and co-infection patterns of microsporidia parasites in the *Daphnia longispina* complex

S. WEIGL[1], H. KÖRNER[2], A. PETRUSEK[3], J. SEDA[4] *and* J. WOLINSKA[1]*

[1] *Ludwig-Maximilians-Universität München, Department Biologie II, Großhaderner Strasse 2, 82152 Planegg-Martinsried, Germany*
[2] *Department of Neuroimmunology, Institute for Multiple Sclerosis Research, University Medical Center Göttingen and The Hertie Foundation, Göttingen, Germany*
[3] *Charles University in Prague, Faculty of Science, Department of Ecology, Viničná 7, 12844 Prague, Czech Republic*
[4] *Biological Centre AS CR, Institute of Hydrobiology, Na Sádkách 7, 37005 České Budějovice, Czech Republic*

(*Received 27 September 2011; revised 10 November, 13 December 2011 and 20 January 2012; accepted 30 January 2012; first published online 12 March 2012*)

SUMMARY

Microsporidia are intracellular parasites, frequently infecting the planktonic crustacean *Daphnia*. Questioning the ability to detect and identify microsporidia with conventional microscopic techniques, we applied molecular methods in order to investigate the distribution and co-infection patterns of this parasite among 8 communities of the *Daphnia longispina* hybrid complex. Eight microsporidian taxa were detected, including 3 that previously had not been characterized genetically. Microsporidian communities from nearby lakes were found to be more similar to each other, apparently due to short distance dispersal via secondary hosts. Moreover, we detected seasonal (but not interannual) changes in microsporidian community structure. With some microsporidia being host-specific, these changes might have resulted from seasonal changes in host taxon and clonal composition. The 2 dominant and closely related parasite species were found mainly in single infections, whereas another pair of related microsporidians was found predominantly in co-infections; suggesting species-level differences in the ability to colonize infected hosts. By applying molecular methods, we were not only able to unambiguously identify parasite taxa but also to reveal multiple infections that otherwise would have remained undetected. Given the increased level of accuracy and sensitivity, we highly recommend molecular approaches in future parasite surveys of *Daphnia* infections.

Key words: co-infection, *Daphnia*, microsporidia, multiple infections, parasite distribution.

INTRODUCTION

Microsporidia are a unique and diverse group of obligatory intracellular parasites (Keeling, 2009), now regarded as derived from fungi or fungi-like ancestors (Hirt *et al*. 1999; Keeling and Fast, 2002). Microsporidia have been reported to infect almost all invertebrate phyla as well as most major vertebrate groups. Their ubiquitous presence and significant adaptations to a parasitic life, such as the reduction of mitochondria to vestigial organelles called mitosomes (Williams *et al*. 2002), support the argument that the origin of parasitism in this group is ancient (Vossbrinck *et al*. 1987). Amongst *Daphnia*, an ecologically important genus of planktonic crustaceans inhabiting lakes and ponds, they constitute one of the most common groups of parasites (Green, 1974; Stirnadel and Ebert, 1997; Ebert, 2005). Field studies on the occurrence of microsporidia in natural *Daphnia* communities have found that they usually first appear in late spring when host density is high and persist throughout summer and autumn, with prevalences in some cases rising up to 100% (Brambilla, 1983; Bengtsson and Ebert, 1998; Ebert *et al*. 2001; Wolinska *et al*. 2011*a*). Microsporidia infect a variety of *Daphnia* species across different habitats, ranging from tiny rock pools (Ebert *et al*. 2001) and temporary ponds (Decaestecker *et al*. 2005), to permanent lakes (Wolinska *et al*. 2011*a*; Yan and Larsson, 1988).

Until now only microscopic techniques have been used to assess microsporidian infection patterns in natural *Daphnia* communities. Thus, the prevalence of microsporidia may have been largely underestimated, due to their minute size and intracellular lifestyle (Ebert, 2005). In particular, the early stages of infection are easily missed, as during this phase there are no visible signs of infection. Moreover, some microsporidian species are similar in appearance within the *Daphnia* host, making it difficult to discriminate species based on light microscopy (Stirnadel and Ebert, 1997). Lastly, co-infections by different parasite species are common in *Daphnia* (Decaestecker *et al*. 2005; Stirnadel and Ebert, 1997; Wolinska *et al*. 2007). As a result of the underestimation of prevalences and/or misclassifications it is difficult to compare epidemic patterns across

* Corresponding author: Ludwig-Maximilians-Universität München, Department Biologie II, Großhaderner Strasse 2, 82152 Planegg-Martinsried, Germany. Tel: +49 (0) 89 2180 74 201. Fax: +49 (0) 89 2180 204. E-mail: wolinska@bio.lmu.de

doi:10.1017/S0031182012000303

different habitats or between studies. For further detailed studies of this host-parasite system, it is thus crucial to obtain more powerful tools to detect and properly identify different microsporidian species. These methods should be applicable for screening a large sample of host individuals from a given population. Although the above-mentioned limitations would make microsporidia prime candidates for using a molecular approach for their identification, molecular techniques have scarcely been used to assess infection patterns in natural host populations (e.g. Hogg *et al*. 2002; McClymont *et al*. 2005), and never in *Daphnia*.

The purpose of the present study was to describe microsporidian infection patterns across *Daphnia* communities inhabiting 8 different permanent water bodies, focusing on spatial and temporal variation. In addition, we wanted to determine the frequency of microsporidian co-infections within single hosts. Thus, we designed general and species-specific primers, amplifying a fragment of the microsporidian small subunit ribosomal DNA (hereafter referred to as SSU rDNA) and applied them to apparently infected *Daphnia*. In addition, some PCR products were sequenced to verify the specificity of the newly designed primers, as well as to confirm detected co-infections. As we detected some lineages that had never been sequenced before, we performed phylogenetic analyses to reveal their relationship to other microsporidian parasites of *Daphnia*.

MATERIALS AND METHODS

Origin of specimens and symptoms of infection

The infected *Daphnia* were selected from ethanol-preserved zooplankton samples originating from 8 man-made reservoirs in the Czech Republic. All sampled reservoirs have a similar canyon-shaped morphology, and were created between years 1934 and 1982. Stanovice and Žlutice (21 km apart) are located in the west of the country, Seč and Trnávka (47 km apart) in the centre, Brno and Vír (40 km apart) in the east, and Římov and Vranov (97 km apart) in the south (see Fig. 1). The *Daphnia* populations of these reservoirs consist mainly of common members from the European *D. longispina* complex (Petrusek *et al*. 2008*a*): *D. cucullata*, *D. galeata* and *D. longispina*, as well as their interspecific hybrids (Seda *et al*. 2007*b*; Petrusek *et al*. 2008*b*). The samples were collected in summer (July) and autumn (late September to October) of the years 2004 and 2005 (Table 1). Additionally, we analysed 2 more samples: from autumn 2009 (Vranov) and summer 2010 (Seč). The samples were collected from the entire water column, by hauling a plankton net (mesh size 170 μm) at 3 sampling stations along the reservoir's horizontal axis: near the river inflow, in the middle of the reservoir, and near the dam (see Table 1).

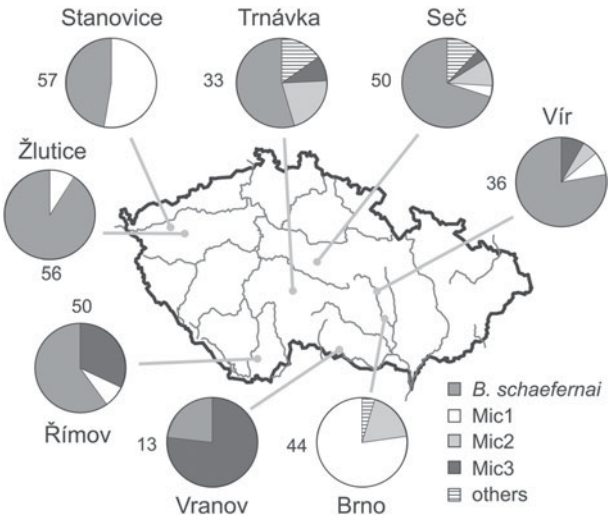

Fig. 1. Location of reservoirs in the Czech Republic and taxon composition of microsporidian communities infecting the *Daphnia longispina* complex (samples from different dates are pooled). The 'other' group consists of 4 rare taxa (*Gurleya vavrai*, Mic4, Mic5 and Mic6). Total number of screened microsporidia is given next to the respective pie chart.

The ethanol-preserved samples (96% ethanol) were screened for *Daphnia* with visible signs of microsporidian infections, using a stereomicroscope at X 50 magnification. Under the microscope, microsporidian infections appear as a fuzzy and opaque mass within the body cavity of their host (Fig. 2; see also Vossbrinck *et al*. 2004; Refardt *et al*. 2008). In an attempt to link the visible signs of infection to a particular parasite species or clade, infected *Daphnia* were photographed and assigned into categories based on the shape of the parasite mass. After molecular identification of parasites (as described below), their appearance was evaluated against parasite identity. To allow for the specificity of the assignment, only *Daphnia* infected with a single parasite species were taken into consideration for this comparison.

DNA extraction and primer test

Genomic DNA was isolated by overnight incubation of entire, single infected *Daphnia* with 100 μg/ml proteinase K (Merck KgaA, Darmstadt, Germany) in proteinase K buffer (10 mM Tris (pH 8·0), 100 mM NaCl, 25 mM EDTA (pH 8·0) and 0·1% SDS) at 55 °C. After heat inactivation of proteinase K for 12 min at 95 °C, chitinous debris was removed and the DNA was precipitated from the supernatant using isopropanol. The DNA was then dissolved in 20–50 μl of sterile water (see Wolinska *et al*. 2009). One general and four species-specific primer pairs (see Table 2) were designed, all amplifying a segment from the SSU rDNA of 4 microsporidia taxa

Table 1. Number of analysed hosts infected with microsporidia, per reservoir and sampling season

(Unless stated otherwise the samples were taken from the dam stations.)

	Sampling season						
	Summer 2004	Autumn 2004	Summer 2005	Autumn 2005	Autumn 2009	Summer 2010	Total
Brno	na	36	na	na	na	na	36
Římov	14	18[a]	na	na	na	na	32
Seč	13	na	18	7	na	9	47
Stanovice	19[b]	21[c]	15	na	na	na	55
Trnávka	na	na	8[d]	18[e]	na	na	26
Vír	na	na	14	17[f]	na	na	31
Vranov	na	na	na	na	11[g]	na	11
Žlutice	23	na	16	13	na	na	52

[a–g] Some or all analysed *Daphnia* individuals originated from inflow stations of the respective reservoirs (a: 18/18 individuals, b: 12/19, c: 21/21, d: 3/8, e: 15/18, f: 15/17), or from a middle station (g: 11/11).
na, Not analysed (due to low prevalence in 2004–2005, and due to unavailability of samples in 2009–2010).

Fig. 2. Picture of *Daphnia* infected with *Berwaldia schaefernai*, with (left; arrow) and without (right) spore mass proliferating into the host's head.

identified in a previous study (Wolinska *et al*. 2009), namely *Berwaldia schaefernai*, and unidentified lineages provisionally labelled as Mic1, Mic2 and Mic3. Primer specificity was evaluated using DNA isolated from several hosts infected by any of the 4 microsporidian taxa, obtained by Wolinska *et al*. (2009), see Supplementary Table 1, online version only. In addition, negative controls were included: DNA extracted from laboratory cultivated (i) uninfected *Daphnia*; (ii) *Daphnia* infected with the protozoan *Caullerya mesnili*, and (iii) *Daphnia* infected with the parasitic yeast *Metschnikowia* sp. These 2 microparasites frequently infect natural communities of the *D. longispina* complex (Wolinska *et al*. 2007, 2011*a*). Detection sensitivity of the primers in the case of co-infections was tested on artificially mixed parasite DNA, in various ratios (as obtained from Wolinska *et al*. 2009) as well as by using parasite DNA isolated from naturally co-infected *Daphnia* (only available for *B. schaefernai* and Mic3 infections, from Wolinska *et al*. 2009).

Application of primers on field samples

First, the general primers were used to verify microsporidian infections in *Daphnia* previously selected by microscopic examination (Table 1). Amplicon size in PCR varied between 438 and 494 bp, depending on the parasite taxon (Table 2). Then, species-specific primers were applied to those samples for which PCR products of the aforementioned size range were detected. PCRs were performed in a 20 μl reaction volume, including 2 μl of genomic DNA, Dream*Taq* buffer (resulting in a 2 mM concentration of $MgCl_2$), 0·25 mM deoxynucleoside triphosphates (both from Fermentas, St. Leon-Rot, Germany) and 0·5 μM of each primer pair (Metabion, Martinsried, Germany). Different amounts of Dream*Taq* polymerase were used: 0·1 U/μl for the general primer, 0·0625 U/μl for *Berwaldia* and Mic1 primers and 0·125 U/μl for Mic2 and Mic3 primers. Furthermore, for Mic2 and Mic3 primers a final $MgCl_2$ concentration of 4 mM was used and 10% DMSO were added to the PCR reactions to improve PCR efficiency. Primer sequences, annealing temperatures, and PCR cycle settings are given in Table 2.

Sequencing

There were 2 main objectives for sequencing: (i) to verify whether the appropriately-sized bands obtained with the general primers, but not with any of the specific primers, represent microsporidian infections and, if so, to identify the parasite (attempted for all 59 such cases), (ii) to check the reliability of identification by species-specific primers (Table 2; in total 44 PCR products were sequenced, comprising representatives of all 4 focal microsporidian species and 27·7% of all detected co-infections). Before sequencing, PCR amplicons obtained with the general primers were cut from the gel and eluted using the QIAquick gel extraction kit (Qiagen,

Table 2. Description of primers used to amplify partial SSU rDNA regions from microsporidian parasite taxa

Primer name[a]	Microsporidian species	Primer sequence (5′–3′)	Product length	Tm (°C)
16SMicGen_361 For	Microsporidia spp.	CTGGTGCCAGCAGCCGCGGTAAT	438–494 bp[b]	65
16SMicGen_869 Rev		TTKTCCCGCGTTGAGTCAAATTAA		
16SBERW_655 For	*B. schaefernai*	GGATTGCAAGCTATGTGATTCA	204 bp	58
16SBERW_867 Rev		AGCATCATCAGAACATAGAACTA		
16SMIC1_655 For	Mic1	GGAATGCAAGGAATGTGTTCCG	204 bp	59
16SMIC1_867 Rev		AACGCTATCAGAATACAGATCAC		
16SMIC2_ 543 For	Mic2	TTCTTAAATAAAGGACGATAGTT	339 bp	50
16SMIC2_944 Rev		AAATGATCTCACTGGTTTCAAC		
16SMIC3_527 For	Mic3	TGTGTGCCACGACGAGTGTGAT	350 bp	62
16SMIC3_843 Rev		CCTTCTCAGTTCTCCACACATC		

[a] The PCR cycle was identical for all primers: initial denaturation of 3 min at 95 °C, followed by 35 cycles of 95 °C (30 sec), primer specific annealing temperature Tm (30 sec) and 72 °C (30 sec) with a final extension of 7 min at 72 °C.
[b] Product length of the general primers varied depending on parasite taxa; 455 bp for *B. schaefernai* and Mic1, 451 bp for Mic2a and b variant likewise, 461 bp for Mic3a and 494 bp for Mic3b (variants of Mic2 and Mic3 were not considered separately, for further information on a and b variants, see Wolinska *et al*. 2009). *Gurleya vavrai* had a product length of 456 bp. The bands of the newly sequenced taxa, Mic4, Mic5 and Mic6, were of the following length: 461 bp, 438 bp and 466 bp.

Hilden, Germany). The amplicons obtained with the species-specific primers were purified (using exonuclease I and shrimp alkaline phosphatase, both from Fermentas, St. Leon-Rot, Germany) and directly sequenced. The sequencing reactions were carried out using BigDye v1.1 sequencing mix and an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA). The resulting chromatograms were carefully inspected for occasional polymorphic sites (i.e. those with overlapping or secondary peaks) indicating potential intragenomic (Wolinska *et al*. 2009, 2011*b*) or intraspecific (in case of co-infection by different parasite strains) variation in the respective microsporidian taxon. Nucleotide composition of such polymorphic sites was expressed by the respective IUPAC codes. Representative sequences of newly characterized lineages (Mic4 to Mic6) have been deposited in GenBank (Accession numbers: JQ012755, JQ012756, JQ012757).

Data analysis

Spatial and temporal patterns in microsporidian communities. Differences in microsporidian taxon composition across and within the lakes (i.e. between different years or seasons) were determined with a *G* test of independence (Sokal and Rohlf, 1995). A sequential Bonferroni correction (Rice, 1989) was used to assess significance in case of multiple tests performed within the same lake. In addition, to evaluate similarities in parasite community composition among different host populations, the Euclidean distance between each pair of lakes was computed from the presence/absence data as well as from the frequency data on microsporidian taxa composition (taking into consideration only the microsporidian taxa *B. schaefernai*, Mic1, Mic2 and Mic3, as the additionally recorded taxa occurred at low frequencies only). The correlation between pairwise geographical distances and Euclidean distances of community composition was evaluated using a Mantel test (5000 permutations) as incorporated in Past v2.07 (Hammer *et al*. 2001).

Molecular identification and phylogeny reconstruction. Obtained sequences (one representing each lineage) were compared with those deposited in the NCBI nucleotide database. First, we used similarity searches (BLAST) to check for the presence of corresponding sequences, and calculated the sequence divergence (using Kimura 2-parameter distance) of lineages that did not have any close match (<1% divergence) in GenBank from their most similar counterparts. Then we constructed a tree in order to reveal the relationships of these lineages. We included in the analysis sequences of related microsporidians known to infect *Daphnia*, as well as closely related microsporidian lineages infecting other hosts for each clade (Table 3). The selection of taxa was based on similarity searches and previously published microsporidia phylogenies (Refardt *et al*. 2002, 2008; Slothouber Galbreath *et al*. 2003; Franzen *et al*. 2005; Vossbrinck and Debrunner-Vossbrinck 2005; Wolinska *et al*. 2009). A sequence of the basal fungus *Basidiobolus ranarum* was used as an outgroup. Sequences were aligned by MUSCLE (Edgar, 2004) incorporated in MEGA5 (Tamura *et al*. 2011). The poorly aligned, non-conserved and highly divergent regions were eliminated by Gblocks 0.91b (Castresana, 2000) using less-stringent settings, resulting in 402 bp long alignment. The relationship among taxa was inferred by the maximum likelihood method

Table 3. List of microsporidia and outgroup sequences used for the SSU rDNA phylogenetic analysis

(GenBank Accession numbers and additional information on host family and order, site of infection and transmission mode (if known or assumed) are given for each microsporidian taxon. Unless indicated otherwise the respective parasites are found in aquatic habitats.)

Organism	Host order/and family	*Daphnia* species	Most heavily infected tissue	Transmission	GenBank Acc. No.	References
Binucleata daphniae	Daphniidae/Cladocera	*D. magna*	epidermis	direct, horizontal	EU075347	Refardt *et al*. (2008)
Berwaldia schaefernai	Daphniidae/Cladocera	*D. galeata*	ovaries, fat body	presumably indirect	AY090042	Vávra and Larsson (1994)
Encephalitozoon cuniculi[a]	diverse vertebrate families	–	white blood cells, brain, inner organs	horizontal and vertical	DQ453123	Murphy *et al*. (2007); Vivarès and Méténier (2001)
Fibrillanosema crangonycis	Crangonyctidae/ Amphipoda	–	gonads of adult hosts	direct, vertical (horizontal probable)	AY364089	Slothouber *et al*. (2003)
Glugoides intestinalis	Daphniidae/Cladocera	*D. magna, D. pulex*	gut epithelium	direct, horizontal	AF394525	Larsson *et al*. (1996)
Gurleya daphniae	Daphniidae/Cladocera	*D. pulex*	epidermis	presumably indirect	AF439320	Friedrich *et al*. (1996)
Gurleya vavrai	Daphniidae/Cladocera	*D. pulex*, *D. longispina*	epidermis	presumably indirect	AF394526	Green (1974)
Larssonia obtusa	Daphniidae/Cladocera	*D. pulex*, *D. longispina*	ovaries, fat body, muscle	presumably indirect	AF394527	Vidtman and Sokolova (1994); Ebert *et al*. (2001)
Marssoniella elegans	Cyclopidae/Cyclopoida	–	ovaries	presumably indirect	AY090041	Vávra *et al*. (2005)
Microsporidium sp. "Ängskärs-klubben 126"	Daphniidae/Cladocera	*D. longispina*	unknown	–	EU075352	Refardt *et al*. (2008)
Microsporidium sp. BVER2	Eulimnogammaridae/ Amphipoda	–	–	–	FJ756170	Qiu *et al*. (*unpublished*)
Microsporidium sp. CRANFA	Crangonyctidae/ Amphipoda	–	multiple tissues	direct, vertical and horizontal suggested	AJ966723	Slothouber Glabreath *et al*. (2010)
Microsporidium sp. DP-1-19	Daphniidae/Cladocera	*D. pulex*	gut epithelium	direct, horizontal	AF394528	Refardt *et al*. (2002)
Microsporidium sp."Ripley Pond I"	Daphniidae/Cladocera	*D. pulicaria*	epidermis	–	EU075355	Refardt *et al*. (2008)
Microsporidium sp. Mic1	Daphniidae/Cladocera	*D. longispina* complex	–	–	FJ794863	Wolinska *et al*. (2009)
Microsporidium sp. Mic2a	Daphniidae/Cladocera	*D. longispina* complex	–	–	FJ794870	Wolinska *et al*. (2009)
Microsporidium sp. Mic3a	Daphniidae/Cladocera	*D. longispina* complex	–	–	FJ794876	Wolinska *et al*. (2009)
Ordospora Colligate	Daphniidae/Cladocera	*D. magna*	gut epithelium	direct, horizontal	AF394529	Larsson *et al*. (1997)
Senoma globulifera[a]	Culicidae/Diptera	–	midgut epithelium	horizontal suggested	DQ641245	Franzen *et al*. (*unpublished*)

[a] Microsporidia parasites found in terrestrial habitats or terrestrial life phase of the respective hosts.

computed in PAUP 4.10b (Swofford, 2000) under the TIM3 + I + G model selected by Akaike information criterion in jModeltest 0.1.1 (Posada, 2008). Branch support in the resulting tree was estimated by nonparametric bootstrapping with 200 pseudoreplicates. Furthermore, we performed a Bayesian inference of phylogeny in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with equivalent settings (6 substitution types, gamma-distributed rate variation with invariant sites) run for 100 000 generations, with 2 parallel runs of 4 chains, tree samples every 100 generations, and first 250 trees eliminated as the burn-in.

RESULTS

Visible symptoms of microsporidia infections

In an attempt to link external signs of infection to a particular parasite species or clade, 238 microphotographs were analysed to classify visible symptoms of infections, but only 2 categories could be identified independently by 2 researchers (J.W. and S.W.): infections (i) with or (ii) without a spore mass proliferating into the host's head (Fig. 2). In 96·2% of cases, the assignment of symptoms into 2 categories matched between the investigators. All *Daphnia* that were assigned to the category (i) by either researcher (28 and 36 individuals, respectively) were infected with a single microsporidian taxon, *Berwaldia schaefernai*. However, *Daphnia* infected with *B. schaefernai* were also frequently classified into the category (ii), i.e. with no spore mass proliferating within the host's head (108 and 100 individuals, respectively; together with the 7 other microsporidian species uncovered by the genetic analysis).

Primer specificity

The general primers designed to amplify different microsporidian species (amplicon size of 438–494 bp; Table 2) also amplified *Daphnia* DNA with a PCR product of approximately 800 bp, and occasionally DNA of other microorganisms, some of which resulted in bands similar in size to those of microsporidia. We attempted to sequence all PCR products of the expected size, whose identity could not be revealed by species-specific primers (see Materials and Methods).The sequencing was successful for 41 of 59 such cases. In 34 cases, the sequenced bands represented microsporidia different from *B. schaefernai*, Mic1, Mic2 and Mic3, while in 7 cases they originated from other organisms (e.g. another *Daphnia* parasite, a bacterium *Spirobacillus cienkowskii*, as revealed by 100% match to sequences available in GenBank). In contrast, all species-specific primers were shown to amplify exclusively the respective microsporidian taxon and to perform reliably for co-infections as well.

Identification of microsporidia taxa

Across 290 *Daphnia* hosts that were identified as carriers of microsporidian parasites (based on the results obtained with species-specific primers and/or sequencing), 339 microsporidian infections were detected comprising 8 different taxa (see Supplementary Table 2; online version only). Apart from *B. schaefernai* previously studied in detail (Wolinska *et al*. 2011*b*), we could identify 1 additional lineage, *Gurleya vavrai*, to the species level. Although the analysed SSU rDNA fragment matched sequences assigned to 2 species: *G. daphniae* (with a complete match) and *G. vavrai* (differing by a single point mutation i.e. 99·8% match), we identified the microsporidium detected by us as the latter species because of the host taxon (*Daphnia longispina* complex); *G. daphniae* was described from *D. pulex* (Friedrich *et al*. 1996). Furthermore, the results of Refardt *et al*. (2002) suggest that the two names might actually be synonymous (i.e., referring to the same species), in which case *G. vavrai* would take precedence.

Then, we detected taxa provisionally labelled Mic1, Mic2 and Mic3 in a previous study on *Daphnia* microparasites (Wolinska *et al*. 2009). Three remaining lineages, labelled Mic4, Mic5 and Mic6, did not have an exact match in the NCBI sequence databases. The sequence of rarely found Mic5 (only 2 detections in Seč reservoir), however, was closely related to *Glugoides intestinalis*, from which it diverged by 1·3% (Kimura 2-parameter distance) and 2 single-nucleotide indels (not considered in divergence calculations).

Mic4 was related to 2 *Daphnia* parasites, *Ordospora colligata* and previously recorded Mic3, diverging from both to a similar extent (8·6 and 9·2%, respectively). Despite originating from 2 different reservoirs (Brno and Trnávka), the 5 available sequences of Mic4 were almost uniform (a single nucleotide position was polymorphic in 1 isolate but fixed in all 4 others).

Similarly, 5 sequences of Mic6 from 2 reservoirs (Seč and Trnávka) showed little intraspecific variation (1 fixed point mutation in a single isolate, and 3 other positions with occasional polymorphisms). The sequence best-matching to Mic6 was relatively divergent (on average 12·9%) and belonged to *Fibrillanosema crangonycis*, a parasite of amphipods (Slothouber Galbreath *et al*. 2003).

The relatedness of these taxa to other selected microsporidians infecting *Daphnia* with available sequence data was assessed using maximum likelihood and Bayesian inference methods; both resulting in trees with identical topology (Fig. 3). Three major clades were identified. Clade I contained a number of relatively closely related lineages infecting *Daphnia* (including *Berwaldia schaefernai, Gurleya vavrai*, and Mic1) among which also the mosquito parasite

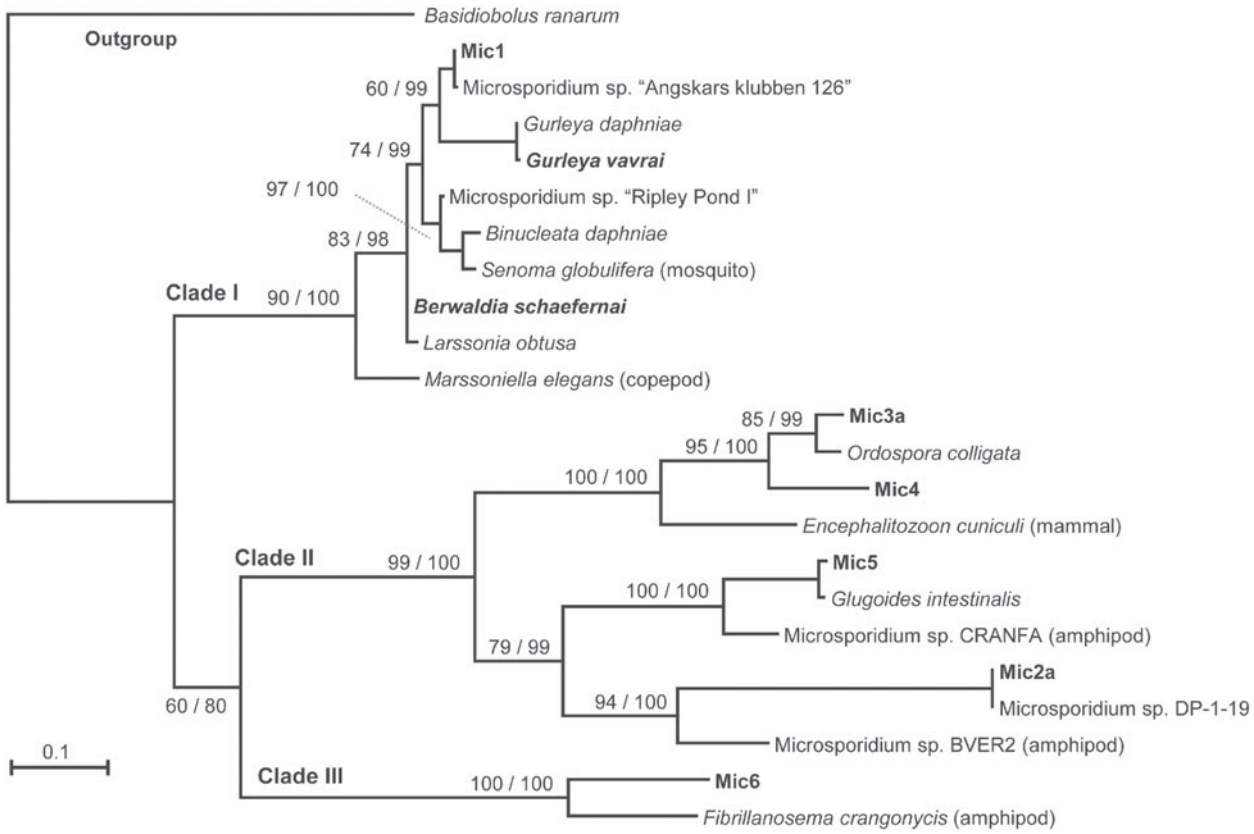

Fig. 3. Relationship of the selected microsporidian taxa (estimated by the maximum likelihood method based on partial SSU rDNA sequences), including 8 microsporidians detected in the present study (highlighted in bold). For species infecting hosts other than *Daphnia*, the host taxa are given in parentheses. The numbers at selected branches indicate bootstrap values for the maximum likelihood, and posterior probability of the Bayesian inference. Branch lengths are based on the expected numbers of nucleotide substitutions per site.

Senoma globulifera was clustered. The 2 other clades consisted of more divergent lineages. Clade II was further divided into 3 well-supported subclades, including species known to infect *Daphnia* gut epithelium (*Ordospora colligata, Glugoides intestinalis*) and 4 microsporidian lineages detected in the reservoirs (Mic2 to Mic5). Finally, a divergent Clade III included the newly sequenced microsporidian Mic6 together with *Fibrillanosema crangonycis*.

Spatial and temporal patterns in microsporidian communities

Microsporidian taxon composition differed significantly across *Daphnia* communities originating from the 8 reservoirs ($G = 306$; D.F. $= 28$; $P < 0{\cdot}001$), with either *B. schaefernai* (5 reservoirs), Mic1 (2 reservoirs) or Mic3 (1 reservoir) being the dominant taxon (Fig. 1). The total number of microsporidian taxa detected in the different reservoirs ranged from 2 to 7 (from 1 to 5 in samples collected on the same date; Supplementary Table 2, online version only). In general, *B. schaefernai* was the most abundant taxon (56·6% of all detected infections) with Brno being the only reservoir in which this species was not detected. In the Seč and Vír reservoirs, all 4 common taxa (i.e. *B. schaefernai*, Mic1, Mic2 and Mic3) coexisted. Among the rare taxa, Mic4 was detected 5 times (in Brno and Trnávka), as was Mic6 (in Seč and Trnávka), whereas Mic5 was detected only twice (in Seč), and *G. vavrai* was detected only once (in Seč).

There were seasonal but not interannual changes in taxon composition (none of the 3 summer-to-summer comparisons, whereas 3 of 6 summer-to-autumn comparisons resulted in significant differences: Fig. 4). Specifically, we observed either changes in parasite taxon proportions (in Stanovice) or a complete replacement of particular taxa (in Římov and Seč). A Mantel test revealed a significant relationship between geographical distance of the reservoirs and microsporidian taxon composition, when calculated for presence/absence ($R^2 = 0{\cdot}13$, $P = 0{\cdot}027$) but not for frequency data ($R^2 < 0{\cdot}01$, $P = 0{\cdot}414$). This contrasting result was due to differences in relative proportions of the same taxa in different reservoirs. For example, Stanovice and Žlutice, being only 21 km apart, were inhabited exclusively by the same 2 microsporidian taxa (*B. schaefernai* and Mic1); however, these occurred at different frequencies (Fig. 1).

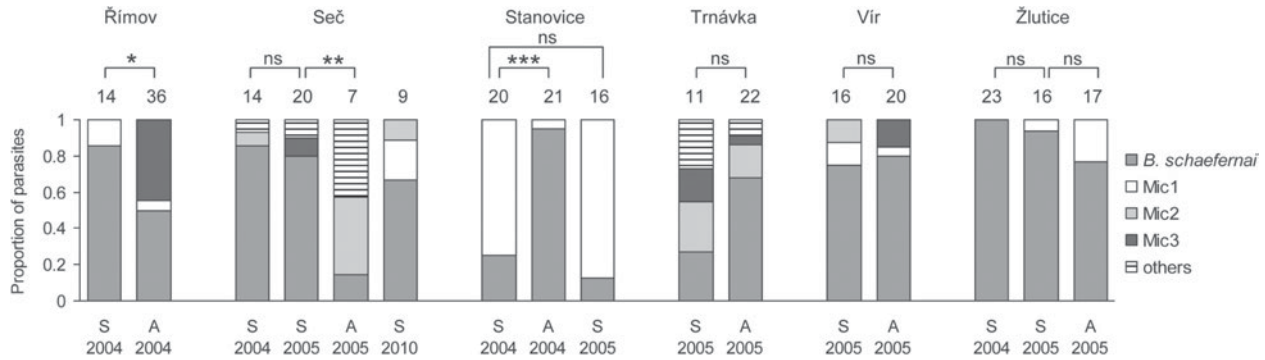

Fig. 4. Taxon composition of microsporidian communities infecting the *Daphnia longispina* complex across 8 reservoirs in the Czech Republic and across sampling seasons. Other sampling seasons were not taken into consideration due to low prevalence values. Brno and Vranov reservoirs are not shown as the samples were collected in a single season. The 'other' group consists of 4 rare taxa (Mic4, Mic5, Mic6 and *Gurleya vavrai*). Sample size is given at the top of each bar. A, Autumn; S, Summer (*$P<0{\cdot}05$; **$P<0{\cdot}01$; ***$P<0{\cdot}001$; ns, not significant).

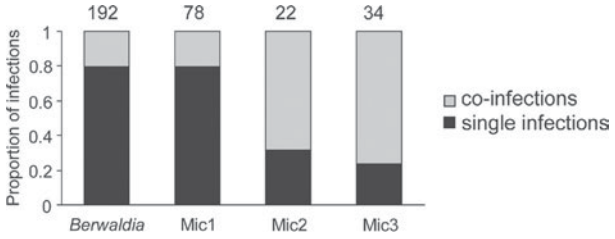

Fig. 5. Proportions of each parasite occurring in single or co-infections. Sample size is given at the top of each bar.

Co-infection pattern within host individuals

In total, 47 out of 290 analysed *Daphnia* (i.e. 16·2%) were infected with more than 1 microsporidian taxon; 45 double-infections and 2 triple-infections were detected. *Berwaldia schaefernai* and Mic1 mostly occurred in single infections, whereas Mic2 and Mic3 mostly occurred in co-infections (Fig. 5). Co-infections rarely happened between closely related taxa; we detected only 8 cases of *B. schaefernai*–Mic1 co-infections (despite these being the 2 most commonly detected taxa), and a single case of Mic2–Mic3 co-infection.

DISCUSSION

Identification of microsporidia taxa

In the present study, using sequences of the SSU rDNA gene, we detected 8 taxa of microsporidian parasites across 8 investigated communities of the *Daphnia longispina* hybrid complex. The patterns of sequence similarity summarized in Fig. 3 were consistent with previously published phylogenies for microsporidia (Refardt *et al*. 2002; Slothouber Galbreath *et al*. 2003; Franzen *et al*. 2005;Vossbrinck and Debrunner-Vossbrinck, 2005; Wolinska *et al*. 2009). In addition, our study discovered at least 2 microsporidian taxa, which had not been genetically analysed before (Mic4, Mic6, and possibly Mic5; see below).

The most common parasites were *Berwaldia schaefernai* and the unidentified microsporidium Mic1. The Mic1 lineage is closely related to *B. schaefernai*, *Larssonia obtusa*, and *Gurleya vavrai* (Wolinska *et al*. 2009), all shown to negatively impact host fecundity (Vávra and Larsson, 1994; Bengtsson and Ebert, 1998). These species probably have indirect life cycles, as their maintenance in the laboratory has failed repeatedly (J.W., *personal observation* and Vávra, 1964), and because they are closely related to the family Amblyosporidae (Refardt *et al*. 2002), a group of microsporidia which infect mosquitoes and use copepods as intermediate hosts (Sweeney *et al*. 1985; Vossbrinck *et al*. 2004). The previously observed low genetic differentiation between *B. schaefernai* infecting the different *Daphnia* populations (Wolinska *et al*. 2011*b*) also supports the hypothesis that this parasite is transmitted through a mobile vector, probably insects. However, another well-studied species belonging to this clade, *Binucleata daphniae*, does have a direct life cycle (Refardt *et al*. 2008).

The lineages Mic2 to Mic5 detected in the present study belong to a relatively diverse group (clade II in Fig. 3) containing taxa infecting *Daphnia* gut epithelium (*Ordospora colligata*, *Glugoides intestinalis*, and Microsporidium sp. DP-1-19 possibly conspecific with Mic2). However, as all investigated taxa appeared in our study as clear fuzzy and opaque mass within the body cavity of *Daphnia*, it seems that multiple host tissues are infected within the *Daphnia* by these parasites (Mic2 to Mic5). The identity of Mic5 requires further analyses – although the sequence data clearly indicate that it is closely related to *Glugoides intestinalis*, the latter has been reported from other host species (*D. magna* and *D. pulex*) and with different infection symptoms (unlike Mic5, *G. intestinalis* infections seem to be limited to *Daphnia* gut epithelium; Larsson *et al*. 1996). Mic5 might thus be either a sister species to *G. intestinalis* or it is conspecific but its infection progresses differently in an alternative host (*D. longispina* complex).

Spatial and temporal patterns in microsporidian communities

The taxon composition of microsporidian communities differed among the 8 studied reservoirs, with the lakes in closer geographical proximity hosting more similar assemblages of microsporidian taxa. Aggregated distributions are likely to be caused by dispersal between neighbouring habitats (e.g. Bengtsson and Ebert, 1998). We suppose that at least some of the observed parasites have good dispersal capabilities via airborne vectors, as discussed above for *B. schaefernai* and its relatives (see also Wolinska *et al*. 2011*b*). Brno is the only reservoir in which we did not detect *B. schaefernai* infections; however, comparisons of limnological and hydrological parameters, as well as *Daphnia* host community structure, did not reveal any major differences between Brno and the other reservoirs (see Seda *et al*. 2007*b*). Thus, the reasons for the variation in parasite community structure remain uncertain.

Within reservoirs, the parasite community composition was similar between consecutive summers. Thus, microsporidians were able to overcome periods of low host density (i.e. in winter; Wolinska *et al*. 2004). This could be facilitated by forming resistant spores deposited in lake sediments (Ebert, 1995), by surviving unfavourable periods in alternate hosts (Becnel and Andreadis, 1999) or within the host's diapausing stages themselves (Canning *et al*. 1985). In contrast, parasite composition changed within years, from summer to autumn. Seasonal changes in microsporidia abundance is a commonly observed phenomenon in *Daphnia* (Green, 1974; Brambilla, 1983; Lass and Ebert, 2006), and density-dependent transmission (associated with host life cycle), environmental factors (especially temperature) and the ability to survive outside the host have been argued to shape the observed patterns (Ebert, 1995; Lass and Ebert, 2006). However, with some microsporidian parasites being host species-specific (Solter and Maddox, 1998; Stirnadel and Ebert, 1997) or even genotype-specific (Little and Ebert, 1999), seasonal changes in parasite community structure may also be related to changes in taxon and/or clonal composition of the host population. Such seasonal changes in *D. longispina* communities have been frequently observed (Keller *et al*. 2007; Seda *et al*. 2007*a*; Spaak, 1996), including in the reservoirs studied here (A.P. and J.S., unpublished data). Conversely, no major interannual changes in host community structure were detected between the consecutive summers of 2004 and 2005; host distribution patterns were relatively stable in the 8 studied Czech reservoirs (Petrusek *et al*. 2008*b*). These findings support the idea of an interdependency of host and parasite occurrence. Nonetheless, further studies are required to test this hypothesis. Specifically, by simultaneously following the changes in host community structure (e.g. by using microsatellite markers, Yin *et al*. 2010) and parasite community structure (using the species-specific primers developed here), one would be able to test for the dependence of specific parasite taxa on certain hosts.

Co-infection patterns within host individuals

Co-infection of a host with multiple parasite species is not the exception but rather a rule in nature (Lipsitch and Moxon, 1997; Petney and Andrews, 1998). The 2 pairs of related microsporidian species (i.e. *B. schaefernai* with Mic1, and Mic2 with Mic3) showed similar proportions of single and co-infections, respectively. Here, relatedness may result in similar abilities to invade and seize host resources. Moreover, closely related parasites were rarely found in co-infections with one another. With the immune response being pathogen-specific (Kurtz and Armitage, 2006), relatedness could prove a disadvantage to a parasite invading an already infected host; such a parasite may encounter an already activated immune system. This mechanism may result in strong priority effects (e.g. de Roode *et al*. 2005) and apparent competition between related species. However, experimental infection studies (similar to those of Ben-Ami *et al*. 2011 and Lohr *et al*. 2010) are required to reveal the true levels of competition between parasites.

Conclusions

Despite the existence of some visible morphological characteristics, mainly *B. schaefernai* showing spore mass proliferation within the host's head, discrimination between the 8 microsporidian taxa is not possible using the traditional screening method of light microscopy. By applying a molecular approach, we were able to overcome not only the problem of unambiguous identification but also to detect otherwise unseen multiple infections. What still remains to be tested is the sensitivity of the applied methods and hence their applicability to detect infections at early stages. Nevertheless, when seeking to investigate large host samples for microsporidian infection patterns, to gain more detailed insights into the diversity of parasites or to compare findings among studies and investigators, molecular methods should be considered in future parasite surveys of *Daphnia* infections.

ACKNOWLEDGEMENT

We thank Rita Jaenichen and Sabine Radetzki for assistance with the molecular work, and Jennifer Lohr, Mingbo Yin, Volker Witte and anonymous referees who provided valuable comments that helped to improve the manuscript.

FINANCIAL SUPPORT

This work was supported in part by the German Research Foundation (WO 1587 / 2–1, SPP 1399), the Grant Agency of the Academy of Sciences of the Czech Republic (IAA600960901), the STRESSFLEA project of the European Science Foundation EUROCORES Programme EuroEEFG (funded through the Czech Science Foundation project EEF/10/E022), and by departmental research money provided by Wilfried Gabriel.

REFERENCES

Becnel, J. J. and Andreadis, T. G. (1999). Microsporidia in insects. In *The Microsporidia and Microsporidiosis* (ed. Wittner, M. and Weiss, L. M.), pp. 172–195. ASM Press, Washington, USA.

Ben-Ami, F., Rigaud, T. and Ebert, D. (2011). The expression of virulence during double infections by different parasites with conflicting host exploitation and transmission strategies. *Journal of Evolutionary Biology* **24**, 1307–1316. doi: 10.1111/j.1420-9101.2011.02264.x.

Bengtsson, J. and Ebert, D. (1998). Distributions and impacts of microparasites on *Daphnia* in a rockpool metapopulation. *Oecologia* **115**, 213–221. doi: 10.1007/s004420050510.

Brambilla, D. J. (1983). Microsporidiosis in a *Daphnia pulex* population. *Hydrobiologia* **99**, 175–188. doi: 10.1007/BF00008769.

Canning, E. U., Barker, R. J., Page, A. M. and Nicholas, J. P. (1985). Transmission of microsporidia, especially *Orthosoma operophterae* (Canning, 1960) between generations of winter moth *Operophtera brumata* (Lepidoptera: Geometridae). *Parasitology* **90**, 11–19. doi: 10.1017/S0031182000048976.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**, 540–552.

de Roode, J. C., Helinski, M. E. H., Anwar, M. A. and Read, A. F. (2005). Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *American Naturalist* **166**, 531–542.

Decaestecker, E., Declerck, S., De Meester, L. and Ebert, D. (2005). Ecological implications of parasites in natural *Daphnia* populations. *Oecologia* **144**, 382–390. doi: 10.1007/s00442-005-0083-7.

Ebert, D. (1995). The ecological interactions between a microsporidian parasite and its host *Daphnia magna*. *Journal of Animal Ecology* **64**, 361–369.

Ebert, D. (2005). *Ecology, Epidemiology, and Evolution of Parasitism in Daphnia*. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information. Available from: http://www.ncbi.nlm.nih.gov/books/NBK2036/.

Ebert, D., Hottinger, J. W. and Pajunen, V. I. (2001). Temporal and spatial dynamics of parasite richness in a *Daphnia* metapopulation. *Ecology* **82**, 3417–3434.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797. doi: 10.1093/nar/gkh340.

Franzen, C., Fischer, S., Schroeder, J., Schölmerich, J. and Schneuwly, S. (2005). Morphological and molecular investigations of *Tubulinosema ratisbonensis* gen. nov., sp. nov. (Microsporidia: Tubulinosematidae fam. nov.), a parasite infecting a laboratory colony of *Drosophila melanogaster* (Diptera: Drosophilidae). *Journal of Eukaryotic Microbiology* **52**, 141–152. doi: 10.1111/j.1550-7408.2005.04-3324.x.

Friedrich, C., Winder, O., Schaffler, K. and Reinthaler, F. F. (1996). Light and electron microscope study on *Gurleya daphniae* sp. nov. (Microspora, Gurleyidae), a parasite of *Daphnia pulex* (Crustacea, Phyllopoda). *European Journal of Protistology* **32**, 116–122.

Green, J. (1974). Parasites and epibionts of Cladocera. *Transactions of the Zoological Society of London* **32**, 417–515.

Hammer, Ø., Harper, D. A. T. and Ryan, P. D. (2001). PAST paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**, 1–9.

Hirt, R. P., Logsdon, J. M., Healy, B., Dorey, M. W., Doolittle, W. F. and Embley, T. M. (1999). Microsporidia are related to fungi: evidence from the largest subunit of RNA polymerase II and other proteins. *Proceedings of the National Academy of Sciences, USA* **96**, 580–585. doi: 10.1073/pnas.96.2.580

Hogg, J. C., Ironside, J. E., Sharpe, R. G., Hatcher, M. J., Smith, J. and Dunn, A. M. (2002). Infection of *Gammarus duebeni* populations by two vertically transmitted microsporidia; parasite detection and discrimination by PCR. *Parasitology* **125**, 59–63. doi:10.1017/S0031182002001774.

Keeling, P. (2009). Five questions about microsporidia. *PLoS Pathology* **5**, e1000489. doi: 10.1371/journal.ppat.1000489.

Keeling, P. J. and Fast, N. M. (2002). Microsporidia: Biology and evolution of highly reduced intracellular parasites. *Annual Review of Microbiology* **56**, 93–116. doi:10.1146/annurev.micro.56.012302.160854.

Keller, B., Wolinska, J., Tellenbach, C. and Spaak, P. (2007). Reproductive isolation keeps hybridizing *Daphnia* species distinct. *Limnology and Oceanography* **52**, 984–991.

Kurtz, J. and Armitage, S. A. O. (2006). Alternative adaptive immunity in invertebrates. *Trends in Immunology* **27**, 493–496. doi: 10.1016/j.it.2006.09.001.

Larsson, J. I. R., Ebert, D. and Vávra, J. (1997). Ultrastructural study and description of *Ordospora colligata* gen. et sp. nov. (Microspora, Ordosporidae fam. nov.), a new microsporidian parasite of *Daphnia magna* (Crustacea, Cladocera). *European Journal of Protistology* **33**, 432–443.

Larsson, J. I. R., Ebert, D., Vávra, J. and Voronin, V. N. (1996). Redescription of *Pleistophora intestinalis* Chatton, 1907, a microsporidian parasite of *Daphnia magna* and *Daphnia pulex*, with establishment of the new genus *Glugoides* (Microspora, Glugeidae). *European Journal of Protistology* **32**, 251–261.

Lass, S. and Ebert, D. (2006). Apparent seasonality of parasite dynamics: analysis of cyclic prevalence patterns. *Proceedings of the Royal Society of London, B* **273**, 199–206. doi: 10.1098/rspb.2005.3310.

Lipsitch, M. and Moxon, E. R. (1997). Virulence and transmissibility of pathogens: what is the relationship? *Trends in Microbiology* **5**, 31–37. doi: 10.1016/s0966-842x(97)81772-6.

Little, T. J. and Ebert, D. (1999). Associations between parasitism and host genotype in natural populations of *Daphnia* (Crustacea: Cladocera). *Journal of Animal Ecology* **68**, 134–149. doi: 10.1046/j.1365-2656.1999.00271.x.

Lohr, J., Yin, M. and Wolinska, J. (2010). Prior residency does not always pay off – coinfections in *Daphnia*. *Parasitology* **137**, 1493–1500.

McClymont, E. H., Dunn, A. M., Terry, R. S., Rollinson, D., Littlewood, D. T. J. and Smith, J. E. (2005). Molecular data suggest that microsporidian parasites in freshwater snails are diverse. *International Journal for Parasitology* **35**, 1071–1078. doi: 10.1016/j.ijpara.2005.05.008.

Murphy, T. M., Walochnik, J., Hassl, A., Moriarty, J., Mooney, J., Toolan, D., Sanchez-Miguel, C., O'Loughlin, A. and McAuliffe, A. (2007). Study on the prevalence of *Toxoplasma gondii* and *Neospora caninum* and molecular evidence of *Encephalitozoon cuniculi* and *Encephalitozoon* (*Septata*) *intestinalis* infections in red foxes (*Vulpes vulpes*) in rural Ireland. *Veterinary Parasitology* **146**, 227–234. doi: 10.1016/j.vetpar.2007.02.017.

Petney, T. N. and Andrews, R. H. (1998). Multiparasite communities in animals and humans: frequency, structure and pathogenic significance. *International Journal for Parasitology* **28**, 377–393. doi: 10.1016/s0020-7519(97)00189-6.

Petrusek, A., Hobaek, A., Nilssen, J. P., Skage, M., Černý, M., Brede, N. and Schwenk, K. (2008*a*). A taxonomic reappraisal of the European *Daphnia longispina* complex (Crustacea, Cladocera, Anomopoda). *Zoologica Scripta* **37**, 507–519. doi: 10.1111/j.1463-6409.2008.00336.x.

Petrusek, A., Seda, J., Macháček, J., Ruthová, Š. and Šmilauer, P. (2008*b*). *Daphnia* hybridization along ecological gradients in pelagic environments: the potential for the presence of hybrid zones in plankton. *Philosophical Transactions of the Royal Society, B: Biological Sciences* **363**, 2931–2941. doi: 10.1098/rstb.2008.0026.

Posada, D. (2008). jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253–1256. doi: 10.1093/molbev/msn083.

Refardt, D., Canning, E., Mathis, A., Cheney, S. A., Lafranchi-Tristem, N. J. and Ebert, D. (2002). Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera). *Parasitology* **124**, 381–389. doi:10.1017/S0031182001001305.

Refardt, D., Decaestecker, E., Johnson, P. T. J. and Vávra, J. (2008). Morphology, molecular phylogeny, and ecology of *Binucleata daphniae* n. g., n. sp. (Fungi: Microsporidia), a parasite of *Daphnia magna* Straus, 1820 (Crustacea: Branchiopoda). *Journal of Eukaryotic Microbiology* **55**, 393–408. doi: 10.1111/j.1550-7408.2008.00341.x.

Rice, W. R. (1989). Analyzing tables of statistical tests. *Evolution* **43**, 223–225.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574. doi: 10.1093/bioinformatics/btg180.

Seda, J., Kolarova, K., Petrusek, A. and Machacek, J. (2007*a*). *Daphnia galeata* in the deep hypolimnion: spatial differentiation of a "typical epilimnetic" species. *Hydrobiologia* **594**, 47–57. doi: 10.1007/s10750-007-9075-4.

Seda, J., Petrusek, A., Machacek, J. and Smilauer, P. (2007*b*). Spatial distribution of the *Daphnia longispina* species complex and other planktonic crustaceans in the heterogeneous environment of canyon-shaped reservoirs. *Journal of Plankton Research* **29**, 619–628. doi: 10.1093/plankt/fbm044.

Slothouber Galbreath, J., Smith, J. E., Becnel, J. J., Butlin, R. and Dunn, A. M. (2010). Reduction in post-invasion genetic diversity in *Crangonyx pseudogracilis* (Amphipoda: Crustacea): a genetic bottleneck or the work of hitchhiking vertically transmitted microparasites? *Biological Invasions* **12**, 191–209. doi: 10.1007/s10530-009-9442-3.

Slothouber Galbreath, J., Smith, J. E., Terry, R. S., Becnel, J. J. and Dunn, A. M. (2003). Invasion success of *Fibrillanosema crangonycis*, n.sp., n.g.: a novel vertically transmitted microsporidian parasite from the invasive amphipod host *Crangonyx pseudogracilis*. *International Journal for Parasitology* **34**, 235–244. doi: 10.1016/j.ijpara.2003.10.009.

Sokal, R. R. and Rohlf, F. J. (1995). *Biometry: the Principles and Practice of Statistics in Biological Research*, 3rd Edn. W.H. Freeman and Co, San Francisco, CA, USA.

Solter, L. F. and Maddox, J. V. (1998). Physiological host specificity of microsporidia as an indicator of ecological host specificity. *Journal of Invertebrate Pathology* **71**, 207–216. doi: 10.1006/jipa.1997.4740.

Spaak, P. (1996). Temporal changes in the genetic structure of the *Daphnia* species complex in Tjeukemeer, with evidence for backcrossing. *Heredity* **76**, 539–548. doi: 10.1038/hdy.1996.77.

Stirnadel, H. A. and Ebert, D. (1997). Prevalence, host specificity and impact on host fecundity of microparasites and epibionts in three sympatric *Daphnia* species. *Journal of Animal Ecology* **66**, 212–222.

Sweeney, A. W., Hazard, E. I. and Graham, M. F. (1985). Intermediate host for an *Amblyospora* sp. (Microspora) infecting the mosquito, *Culex annulirostris*. *Journal of Invertebrate Pathology* **46**, 98–102. doi: 10.1016/0022-2011(85)90133-8.

Swofford, D. L. (2000). *PAUP*: Phylogenetic Analysis using Parsimony (and Other Methods). Version 4*. Sinauer Associates, Sunderland, MA, USA.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731–2739. doi: 10.1093/molbev/msr121.

Vávra, J. (1964). A failure to produce artificial infection in cladoceran microsporidia. *Journal of Protozoology* **11**, 35–36.

Vávra, J., Hyliš, M., Oborník, M. and Vossbrinck, C. R. (2005). Microsporidia in aquatic microcrustacea: the copepod microsporidium *Marssoniella elegans* Lemmermann, 1900 revisited. *Folia Parasitologica* **52**, 163–172.

Vávra, J. and Larsson, J. I. R. (1994). *Berwaldia schaefernai* (Jírovec, 1937) comb. n. (Protozoa, Microsporida), fine-structure, life-cycle, and relationship to *Berwaldia singularis* Larsson, 1981. *European Journal of Protistology* 30, 45–54.

Vidtman, S. S. and Sokolova, Y. Y. (1994). The description of the new genus *Larssonia* gen. n. based on the ultrastructural analysis of *Microsporidium (Pleistophora) obtusa* from *Daphnia pulex*. *Parazitologyia* **28**, 202–213.

Vivarès, C. P. and Méténier, G. (2001). The microsporidian *Encephalitozoon*. *BioEssays* **23**, 194–202. doi: 10.1002/1521-1878(200102)23:2<194::aid-bies1027>3·0.co;2-d.

Vossbrinck, C. R., Andreadis, T. G., Vávra, J. and Becnel, J. J. (2004). Molecular phylogeny and evolution of mosquito parasitic microsporidia (Microsporidia: Amblyosporidae). *Journal of Eukaryotic Microbiology* **51**, 259–261. doi: 10.1111/j.1550-7408.2004.tb00167.x.

Vossbrinck, C. R. and Debrunner-Vossbrinck, B. A. (2005). Molecular phylogeny of the microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitologica* **52**, 131–142.

Vossbrinck, C. R., Maddox, J. V., Friedman, S., Debrunner-Vossbrinck, B. A. and Woese, C. R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature, London* **326**, 411–414. doi: 10.1038/326411a0.

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

Wolinska, J., Giessler, S. and Koerner, H. (2009). Molecular identification and hidden diversity of novel *Daphnia* parasites from European lakes. *Applied and Environmental Microbiology* **75**, 7051–7059. doi: 10.1128/AEM.01306-09.

Wolinska, J., Keller, B., Bittner, K., Lass, S. and Spaak, P. (2004). Do parasites lower *Daphnia* hybrid fitness? *Limnology and Oceanography* **49**, 1401–1407.

Wolinska, J., Keller, B., Manca, M. and Spaak, P. (2007). Parasite survey of a *Daphnia* hybrid complex: host-specificity and environment determine infection. *Journal of Animal Ecology* **76**, 191–200. doi: 10.1111/j.1365-2656.2006.01177.x.

Wolinska, J., Seda, J., Koerner, H., Smilauer, P. and Petrusek, A. (2011a). Spatial variation of *Daphnia* parasite load within individual waterbodies. *Journal of Plankton Research* **33**, 1284–1294. doi: 10.1093/plankt/fbr016.

Wolinska, J., Spaak, P., Koerner, H., Petrusek, A., Seda, J. and Giessler, S. (2011b). Transmission mode affects the population genetic structure of *Daphnia* parasites. *Journal of Evolutionary Biology* **24**, 265–273. doi: 10.1111/j.1420-9101.2010.02163.x.

Yan, N. D. and Larsson, J. I. R. (1988). Prevalence and inferred effects of microsporidia of *Holopedium gibberum* (Crustacea:Cladocera) in a Canadian Shield lake. *Journal of Plankton Research* **10**, 875–886. doi: 10.1093/plankt/10.5.875.

Yin, M., Wolinska, J. and Gießler, S. (2010). Clonal diversity, clonal persistence and rapid taxon replacement in natural populations of species and hybrids of the *Daphnia longispina* complex. *Molecular Ecology* **19**, 4168–4178. doi: 10.1111/j.1365-294X.2010.04807.x.