Subterranean Biology 33: 71–85 (2020)
doi: 10.3897/subtbiol.33.48633
http://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Updates to the sporadic knowledge on microsporidian infections in groundwater amphipods (Crustacea, Amphipoda, Niphargidae)

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Academic editor: *O. T. Moldovan* | Received 20 November 2019 | Accepted 20 January 2020 | Published 13 February 2020

http://zoobank.org/78CFBE17-0918-455A-8813-C92324DBFCFE

Citation: Grabner D, Weber D, Weigand AM (2020) Updates to the sporadic knowledge on microsporidian infections in groundwater amphipods (Crustacea, Amphipoda, Niphargidae). Subterranean Biology 33: 71–85. https://doi.org/10.3897/subtbiol.33.48633

Abstract

A set of 69 specimens from 19 groundwater species of the genera *Niphargus*, *Niphargellus*, *Microniphargus* and *Crangonyx* was genetically screened for microsporidian infections. Samples mostly originated from groundwater-dependent spring environments (71%), natural caves (9%) and artificial caverns/tunnels (13%). Amphipod hosts were identified by morphology and/or molecular data, whereas microsporidian parasites were characterised by a genetic screening assay targeting a section of the small subunit rRNA gene.

Five microsporidian species (*Dictyocoela duebenum*; *Nosema* sp.; *Hyperspora aquatica* and two undescribed *Microsporidium* spp.) were revealed from 13 host specimens (*Niphargus schellenbergi*; *N. aquilex* lineages B, F and G; *Niphargellus arndti*). In particular *N. schellenbergi* was frequently infected with *D. duebenum* as well as a new and potentially niphargid-specific *Nosema* sp. identified in *Niphargellus arndti*.

Our results shed further light on the still largely unknown diversity and specificity of microsporidian parasites in groundwater amphipods and subterranean animals in general.

Keywords

parasites, stygobionts, ecological network, transmission pathways, SSU rDNA, COI, 28S

Introduction

Microsporidians are microparasites that belong to the taxon Opisthosporidia, a sister group of the Fungi (Karpov et al. 2014). Depending on the microsporidian species, they can develop in various host tissues where they form spores that are infective for the next host (horizontal transmission). Some microsporidians are transmitted vertically from the mother to the offspring (Dunn and Smith 2001, Smith 2009). Microsporidians can influence the host population by causing mortality of infected individuals, or by modulating the sex ratio towards a female-biased population in the case of vertical transmission (Dunn and Smith 2001).

Studies on microsporidian diversity in freshwater amphipods have a long history and are steadily increasing (see Bulnheim 1975 for review, Ironside et al. 2003, Haine et al. 2004, Terry et al. 2004, Krebes et al. 2010, Wilkinson et al. 2011, Bacela-Spychalska et al. 2012, 2018, Stentiford et al. 2013, Stentiford and Dunn 2014, Grabner et al. 2015, Madyarova et al. 2015, Weigand et al. 2016, Dimova et al. 2018, Quiles et al. 2019), but knowledge on microsporidians in groundwater amphipods is very scarce. Early last century, Poisson (1924) was the first reporting *Niphargus stygius* (today regarded as a species-group) to be infected with *Microsporidium vandeli* (originally referred to as *Mrazekia niphargi*, later *Bacillidium niphargi*) and *Microsporidium niphargi* (former *Thelohania vandeli*). Almost fifty years later, Bulnheim (1971) stated that *Pleistophora mülleri* (described as *Stempellia mülleri*) was detected in *Niphargus ilidzensis*. Since then, it has become more and more clear that the identification and delineation of microsporidian species as well as of groundwater amphipod hosts had been far from consistent. Again, almost 50 years after Bulnheim's publication, Weigand et al. (2016) were the first addressing microsporidian diversity in a *Niphargus* population by genetically analysing the parasites as well as the host species. The authors revealed *Nosema granulosis*, *Orthosomella* sp., *Microsporidium* sp. I and *Microsporidium* sp. BPAR3 as well as some unclassified infections for the target species *Niphargus schellenbergi*. Notably, all microsporidian infections were shared by a sympatrically occurring population of *Gammarus fossarum* lineage 13. This lead to the assumption that groundwater amphipods could enable transmission of microsporidians between surface habitats that are only connected by groundwater (Weigand et al. 2016).

In the present study, we intended to take another step in improving our sporadic knowledge on microsporidian diversity in a variety of groundwater-dependent environments in Central Europe using different niphargids (genera *Niphargus*, *Niphargellus*, *Microniphargus*) as target hosts.

Material and methods

Sample material

In total, 58 *Niphargus* specimens, 9 *Niphargellus*, 1 *Microniphargus leruthi* and 1 *Crangonyx* sp. have been analysed for microsporidian infections (Table 1; for further infor-

mation see Suppl. material 1). Specimens have been collected in the period between 2015–2018, representing the morphospecies *Niphargus aquilex*, *Niphargus glenniei*, *Niphargus irlandicus*, *Niphargus kochianus*, *Niphargus puteanus*, *Niphargus schellenbergi*, *Niphargellus nolli* and *Niphargellus arndti*, as well as some undetermined *Niphargus* sp. Most of the samples originate from Central Europe (here, Germany, Belgium, Luxembourg and the East of France), fewer from surrounding areas (Poland, Great Britain, Ireland, The Netherlands, Czech Republic and the rest of France). The most frequently sampled aquatic habitats are springs, followed by subterranean water bodies in natural caves and artificial caverns (Table 1).

Host barcoding and parasite detection

One to two molecular markers were investigated for molecular species identification of amphipods, thus to a) allow a genetic cross-validation of the often morphologically hard to identify niphargid specimens, b) identify also juvenile specimens and c) enable a more precise taxonomic identification in case of cryptic species complexes (e.g. for *Niphargus aquilex*) (Fišer et al. 2009). The mitochondrial cytochrome *c* oxidase subunit I (COI) marker and the nuclear 28S rDNA marker (28S) were targeted. DNA was extracted from whole specimens according to the DNeasy Blood & Tissue Kit (Qiagen) and the NucleoSpin Tissue Kit (Macherey-Nagel) manufacturers' protocols. The COI marker was amplified using the primer pair LCO1490-JJ (5'-CHA CWA AYC ATA AAG ATA TYG G-3') and HCO2198-JJ (5'-AWA CTT CVG GRT GVC CAA ARA ATC A-3') of Astin and Stüben (2008). The PCR mix contained 1 µL DNA extract of variable concentration, 0.8 µL of each primer (10 pmol/µL), 5 µL of DreamTaq DNA Polymerase Master Mix (Thermo Scientific) and 2.4 µL of ultrapure water. PCR cycling conditions were 3 min denaturation at 94°C, 36 cycles of 20 s denaturation at 94°C, 45 s annealing at 50°C, and 60 s extension at 65°C; final elongation of 2 min at 65°C. Bi-directional Sanger-sequencing was performed at Genoscreen (Lille, France) using the PCR primer pair. The 28S nuclear fragment was amplified using the primer pair Niph15 (5'-CAA GTA CCG TGA GGG AAA GTT-3') and Niph16 (5'-AGG GAA ACT TCG GAG GGA ACC-3') of Verovnik et al. (2005). The PCR mix contained 2 µL of DNA extract of variable concentration, 1 µL of each primer (10 pmol/µL), 0.2 µL of REDTaq Polymerase (Sigma-Aldrich), 5 µL REDTaq reaction buffer and 15.8 µL ultrapure water. PCR cycling conditions for 28S were an initial 3 min denaturation at 95°C, 56 cycles of 30 s denaturation at 94°C, 60 s annealing at 45°C, and 90 s extension at 72°C. Bi-directional Sanger-sequencing was performed using three primers: Niph 15, Niph 20 (5'-AAA CAC GGG CCA AGG AGT AT-3') and Niph 21 (5'-TAT ACT CCT TGG CCC GTG TT-3') (Flot et al. 2010). All PCR results were visualised on a 1.2% agarose gel prior to sequencing. COI-based molecular species identification was performed against the Barcode of Life Data System (BOLD, Ratnasingham and Hebert 2007) and the 28S marker compared to sequences stored in NCBI GenBank. Additional but so far unpublished COI and 28S sequences as part of an ongoing doctoral thesis were integrated for molecular species identification.

Table 1. Overview of host amphipod species and microsporidian infections. Further information can be retrieved from Supplementary material 1.

Taxonomy	Country	Locality	date	habitat	microsporidium infection
Crangonyx sp.	Germany	Bavaria, Mömlingen, Interstitial Mümling	4/28/2017	interstitial	
Microniphargus leruthi	Ireland	County Clare, Ballyvaghan, Polldubh Cave	10/21/2017	cave	
Niphargellus arndti	Czech Republic	Málkov, spring near Málkov	4/13/2018	spring	
	Germany	Bavaria, Münchberg, Förmitzquelle	3/14/2018	spring	
	Germany	Bavaria, Hainstetten, Rotbühlquelle	4/15/2018	spring	*Hyperspora aquatica* (99.8% to KX364284)
	Germany	Bavaria, Hainstetten, Fensterbachquelle	4/15/2018	spring	
	Germany	Bavaria, Hainstetten, Boiwiequelle 1	4/15/2018	spring	
	Poland	Szczawno-Zdrój, Jaskinia Daisy (former Liebichauer Höhle)	7/7/2018	cave	*Nosema* sp. (97.2% to KM977840)
	Poland	Szczawno-Zdrój, Jaskinia Daisy (former Liebichauer Höhle)	7/7/2018	cave	*Microsporidium* sp. (97.5% similar to KX137915)
	Germany	Saxony, Wüstenbrand, Obere Jungfernquelle	5/28/2017	spring	
Niphargellus nolli	Germany	Bavaria, Mömlingen, Interstitial Mümling	4/28/2017	interstitial	
Niphargus aquilex A	Germany	Rhineland-Palatinate, Grünstadt, Queckbrunnen	1/10/2017	spring	
	Germany	Rhineland-Palatinate, Waldleiningen, Felsenbrunnen	12/14/2016	spring	
	Germany	Rhineland-Palatinate, Lambrecht, Bürgermeister-Hermann-Schneider-Brunnen	4/17/2016	spring	
Niphargus aquilex B	Germany	Hesse, Quelle Heidtränktal bei Mündung Schellbach	5/5/2018	spring	
	Germany	Hesse, Hanswagnersborn	5/6/2018	spring	
	Germany	Hesse, Quelle 12 im Krofdorfer Forst	5/7/2018	spring	*Dictyocoela duebenum* (99.45% similar to MH753359)
	Germany	Rhineland-Palatinate, Höhn, Trinkwasserquelle Hilpischmühle	8/14/2018	spring	
	Germany	Saarland, Saarhölzbach, Schankbur	12/1/2016	spring	
Niphargus aquilex F	Belgium	Wallonia, Stablo, Interstitial l'Eau Rouge	10/20/2018	interstitial	*Dictyocoela duebenum* (99.45% similar to MH753359)
	Germany	Saxony, Geising, Barbara-Stollen Geising	3/18/2018	artificial cavern	
	Germany	Saxony, Geising, Barbara-Stollen Geising	3/18/2018	artificial cavern	
	Germany	Bavaria, Kasendorf, Friesenquelle	3/14/2018	spring	
	Germany	Thuringia, Sankt Ganglof, Tesse	3/15/2018	spring	

Taxonomy	Country	Locality	date	habitat	microsporidium infection
Niphargus aquilex-complex lineage H	France	Meurthe-et-Moselle, Haroué, Drainage Haroué	4/5/2018	spring	
Niphargus aquilex-complex lineage I	France	Haut Rhin, Source Mitteleck	1/14/2018	spring	
Niphargus aquilex-complex lineage M	France	Calvados, Saint-Vaast-en-Auge, Carrière souterrain de Saint-Vaast-en-Auge	5/26/2018	artificial cavern	
Niphargus aquilex-complex, lineage G	Germany	Saarland, Nunkirchen, Zillas Keller	1/10/2015	artificial cavern	
	Germany	Saarland, Steinkopfstollen	3/12/2018	artificial cavern	
	Germany	Saarland, Nunkirchen, Zillas Keller	12/30/2017	artificial cavern	*Microsporidium* sp. (93.1% similarity to FJ755996)
	Germany	Baden-Wurttemberg, Blaubeuren, Interstitial Blau	8/3/2018	spring	
	Luxembourg	Minette, Esch sur Alzette, Minière Langegronn	1/1/2016	artificial cavern	
Niphargus aquilex-complex, lineage J	France	Loir et Cher, 35 Pleine-Fougères	n.a.	n.a.	
Niphargus aquilex-complex, lineage K	Belgium	Wallonia, Péruwelz, Source Edouard Simon	9/14/2017	spring	
Niphargus aquilex-complex, lineage L	Belgium	Flandres, Kleine Spouwen, Bron in Kleine Spouwen	10/20/2018	spring	
Niphargus cf. *aquilex*	Germany	Hesse, Wettenberg, Quelle 37 im Krofdorfer Forst	22.07.2016	spring	
Niphargus glenniei	United Kingdom	Devon, Ashburton, Pridhamsleigh Caverh	9/7/2016	cave	
Niphargus irlandicus	Ireland	County Clare, Ballyvaghan, Aillwee Cave	10/22/2017	cave	
Niphargus kochianus-complex (not A-D)	the Netherlands	Stokhem, Dorpstratwell	5/20/2017	well	
Niphargus puteanus	Germany	Baden-Wurttemberg, Schlattstall, Wasserhäuschen Schwarze Lauter	6/13/2017	spring	
Niphargus schellenbergi	Belgium	Wallonia, Felenne, Source abbrevoir Felenne	6/10/2017	spring	
	Belgium	Wallonia, Rotheux-Rimière, Source des Amoureux	2/25/2017	spring	
	Belgium	Wallonia, Baionville, Source sous arbre	3/8/2018	spring	
	Belgium	Wallonia, Lomprez, Source près des Dames	3/10/2018	spring	*Dictyocoela duebenum* (99.45% similar to MH753359)

Taxonomy	Country	Locality	date	habitat	microsporidium infection
Niphargus schellenbergi	Belgium	Wallonia, Clermont, Fontaine de Saint-Jean	5/19/2017	spring	
	France	Vosges, Valfroicourt, Lavoir de Valfroicourt	4/7/2018	spring	
	Germany	North Rhine-Westphalia, Behlingen, spring near Behlingen	8/10/2017	spring	
	Germany	Rhineland-Palatinate, Trier ST Euren, Quelle überm Talbildchen	10/13/2017	spring	*Dictyocoela duebenum* (99.72% similar to JQ673483)
	Germany	Bavaria, Kulmbach, Quelle am Steinernen Gässchen	3/14/2018	spring	
	Germany	Thuringia, Bad Klosterlausnitz, Holzborn	3/15/2018	spring	*Dictyocoela duebenum* (99.45% similar to MH753359)
	Germany	North Rhine-Westphalia, Behlingen, spring near Behlingen	8/10/2017	spring	*Dictyocoela duebenum* (99.48% similar to MH753359)
	Germany	North Rhine-Westphalia, Behlingen, spring near Behlingen	8/10/2017	spring	*Dictyocoela duebenum* (99.56% similar to MG063275)
	Germany	North Rhine-Westphalia, Brilon, Obere Möhnequelle	8/13/2017	spring	
	Germany	Hesse, Martinhagen, Quelle der Kneippanlage Martinhagen	5/9/2018	spring	
	Germany	Hesse, Warme-Quelle	5/9/2018	spring	
	Germany	North Rhine-Westphalia, Brilon, Obere Möhnequelle	8/13/2017	spring	
	Germany	Rhineland-Palatinate, Trier ST Euren, Quelle überm Talbildchen	10/13/2017	spring	
	Germany	Saarland, Mettlach, Quelle über Mettlach	1/28/2017	spring	
	Germany	Bavaria, Neuschleichach, Aurachquelle	8/23/2017	spring	*Dictyocoela duebenum* (99.45% similar to MH753359)
	Luxembourg	Gutland, Diekirch, Quelle Diekirch	2/11/2018	spring	
	Luxembourg	Ösling, Urspelt, Quelle am aale Koepchen	5/20/2018	spring	*Dictyocoela duebenum* (99.45% similar to MH753359)
	Luxembourg	Gutland, Girsterklaus, Source de Girsterklaus	1/30/2018	spring	
	Luxembourg	Gutland, Osweiler, Wiesenquelle 2 Fromburg	1/31/2018	spring	
Niphargus cf. *schellenbergi*	France	Meurthe-et-Moselle, Xirocourt, Fontaine Jevoncourt	4/6/2018	spring	
	France	Haute-Saône, Le Thillot, Tunnel du Col des Croix	7/3/2016	tunnel	
	France	Saône-et-Loire, Le Creusot, Le Creusot, tunnel	2/2/2018	tunnel	
Niphargus sp.	Germany	Bavaria, Steinamwasser, Höhle Ohne Namen	8/24/2017	cave	
	Luxembourg	Gutland, Osweiler, Tümpelquelle Fromburg	1/31/2018	spring	
	France	Meurthe-et-Moselle, Chaligny, Lavoir de Chaligny	4/5/2018	spring	

The detection of microsporidians with the primers V1 / Mic-uni3R (targeting a section of about 450 bp of the small subunit (SSU) rRNA gene) was done as described in Weigand et al. (2016). Additionally, selected microsporidian-positive samples were amplified with the primers HG4f and 580r (amplifying a product of about 500 bp) as suggested by Bacela-Spychalska et al. (2018) to obtain additional sequence information from the internal transcribed spacer (ITS) and the large subunit (LSU) of the rDNA gene. The intention was mainly to unambiguously match the isolates of *Dictyocoela* spp. to the respective GenBank entries. PCR products were purified with a Micro Elute Cycle Pure Kit according to manufacturer's instructions (Omega Bio-Tek) and sequenced (Eurofins Genomic Services).

Results

Host diversity and cryptic species

The COI marker was used for DNA barcoding of 57 specimens, the 28S locus analysed for 38 specimens – with a total of 32 specimens being investigated for both markers (Suppl. Table S1). Six specimens were identified by morphology only, as PCR amplification and/or DNA sequencing were not successful. The total groundwater amphipod dataset screened for microsporidian infections comprised 58 *Niphargus* specimens, 9 *Niphargellus* specimens, *Crangonyx* sp. and *Microniphargus leruthi* (Table 1). With 26 specimens *Niphargus* (cf.) *schellenbergi* was the most frequent taxon. Furthermore, the *N. aquilex* morphospecies was revealed to be represented by ten cryptic species in our dataset, which already comprised taxonomic annotations (*N. aquilex* A, B and F sensu McInerney et al. 2014) or were newly named in this study (i.e. *N. aquilex*-complex lineages G to M) using the terminology as introduced by McInerney et al. (2014). The COI sequences can be retrieved from Suppl. material 2.

Microsporidian diversity

A literature review was performed on known microsporidian infections in niphargid amphipods, and our own results added (Table 2).

No microsporidians were detected in the single *M. leruthi* and *Crangonyx* sp. In total, 13 niphargids were tested positive for microsporidians by PCR (19.1%, Table 1). Most of the isolates (9, 13.2%) were identified as *Dictyocoela duebenum* (according to Bacela-Spychalska et al. 2018). This microsporidium was found mainly in *N. schellenbergi* (7 out of 9) as well as in *Niphargus aquilex* lineages B and F. It was found almost exclusively in spring habitats. The sequences of the remaining four microsporidians were clearly different and only one host individual was found infected each (1.4%). One isolate from *Niphargellus arndti* was similar to *Nosema* sp. (97.2% to KM977840) previously isolated from *Eulimnogammarus verrucosus* (Madyarova

Table 2. Overview of microsporidian infections in groundwater amphipods of the family Niphargidae.

Host	Microsporidium	Reference
Niphargellus arndti	*Hyperspora aquatica* (99.8% similar to KX364284)	this study
	Microsporidium sp. (97.5% to KX137915)*	this study
	Nosema sp. (97.2% to KM977840)	this study
Niphargus aquilex B	*Dictyocoela duebenum* (99.5% to MH753359)	this study
Niphargus aquilex F	*Dictyocoela duebenum* (99.5% to MH753359)	this study
Niphargus aquilex G	*Microsporidium* sp. (93.1% to FJ755996)*	this study
Niphargus ilidzensis	*Pleistophora mülleri* (probable syn. *Stempellia mülleri*, *Microsporidium giraudi*, *Thelohania mülleri*, *T. giraudi*, *Pleistophora blochmanni*, *Glugea mülleri*)	Bulnheim (1971)
Niphargus schellenbergi	*Dictyocoela duebenum* (99.5% to MH753359; 99.7% to JQ673483; 99.6% to MG063275)	this study
	Microsporidium sp. BPAR3 (KT633993)*	Weigand et al. (2016)
	Microsporidium sp. I (KT633992)*	Weigand et al. (2016)
	Nosema granulosis	Weigand et al. (2016)
	Orthosomella sp.	Weigand et al. (2016)
Niphargus stygius species group	*Microsporidium vandeli* (probable syn. *Microsporidium niphargi*, *Mrazekia niphargi*, *Bacillidium niphargi*, *Thelohania vandeli*)	Poisson (1924)

**Microsporidium* sp. is a transitory genus for genetically identified microsporidian isolates without a link to a morphological description. Therefore, GenBank accession numbers for the isolate or the respective best match are given in these cases.

et al. 2015) and 96.8% to *Nosema granulosis* (MK719384) isolated from *Gammarus roeselii* (Quiles et al. 2019). An isolate obtained from *N. aquilex* lineage G showed a similarity of 93.1% to a microsporidian sequence from the amphipod *Crypturopus tuberculatus* collected in Lake Baikal (FJ755996). Two additional microsporidian isolates were sequenced from *Niphargellus arndti*; one was 97.5% similar to a microsporidian detected in caddisfly larvae (KX137915, Grabner et al. 2017), the other was 99.8% similar to the hyperparasitic microsporidian *Hyperspora aquatica* (KX364284, Stentiford et al. 2017).

Sequencing of the PCR product obtained with the HG4f-580r primers from two *N. schellenbergi*-specimens resulted in two non-overlapping fragments that were between 95.4% (*Pseudocollinia beringensis*; HQ591477) to 98.5% (*Gymnodinioides pitelkae*; EU503534) genetic similarity to sequences of apostome ciliates from krill and marine amphipods. The SSU rDNA sequences can be retrieved from Suppl. material 3.

Discussion

Due to a generally low supply of nutrients and often species-poor local communities, groundwater(-dependent) ecosystems are ecologically particularly sensitive. Therefore, transmission pathways might be ecologically more relevant and effects of parasites might have a stronger regulatory role in these environments. In the present study, five different microsporidian isolates could be obtained from 68 tested niphargid individuals, which correspond to about 0.07 microsporidian species per host individual. This is much lower compared to the study of Weigand et al. (2016) who found four microsporidian species in 21 tested *N. schellenbergi*, therefore a rate

of 0.19 parasite species per host individual. Also, the overall prevalence was much higher in the latter (>80%), compared to the present study (19%). This difference might be explained by the close connection of the investigated *Niphargus*-population to a surface population of *Gammarus fossarum* lineage 13 with a microsporidian prevalence of 90% in the study of Weigand et al. (2016). Transmission from this highly infected surface population might have been the cause for the comparatively high prevalence in *Niphargus* spp. found in their study. Nevertheless, it has to be noted that the majority of niphargid specimens from the present study had been sampled from spring habitats, and as such, also co-exist with epigean arthropods, including *Gammarus fossarum* (lineage 13). Alternatively, in particular *Niphargus schellenbergi* might be susceptible for microsporidian infections. Further indication might be seen in our study results as well, as out of the 26 specimens identified as *Niphargus* (cf.) *schellenbergi* seven were infected, corresponding to a rate of 0.27 parasite species per host individual.

The most abundant microsporidium found in the present study was *Dictyocoela duebenum*, a common species occurring in a variety of amphipods (Terry et al. 2004, Grabner et al. 2015, Wilkinson et al. 2011, Bacela-Spychalska et al. 2018). This species is generally transmitted vertically (from mother via eggs directly to the offspring) and can feminize the host population (Ironside et al. 2003). But there is also evidence for phases of horizontal transmission (masses of spores are released after host death and infect other individuals when they ingest the spores) that will cause increased host mortality (Wilkinson et al. 2011). Therefore, *D. duebenum* might be transmitted to *Niphargus* populations when they come in contact with other infected amphipods and persist in the population by vertical transmission. As we tested only few host individuals per site (1-3), we cannot draw conclusions about the actual absence of microsporidians at sites with specimens that were tested negative for microsporidians. Nevertheless, some *Niphargus* populations seem to be free of *D. duebenum*, as this species was not found in the study of Weigand et al. (2016).

In the study by Weigand et al. (2016), *Nosema granulosis* was detected in *N. schellenbergi*. This microsporidium was originally described from *Gammarus duebeni* (Terry et al. 1999) and is the only species of this genus recorded from different species of amphipods (Terry et al. 2004). The *Nosema* isolate from *Niphargellus arndti* detected in the present study shows a sequence divergence of 3.2% to the closest *Nosema granulosis* sequence in GenBank and might be in fact a new *Nosema* species. It is most closely related to a *Nosema* sp. isolate found in the freshwater amphipod *Eulimnogammarus verrucosus* (97.2% to KM977840). This amphipod is endemic to Lake Baikal (Russia) where it inhabits the upper and sub-littoral zones, being commonly sampled in high numbers from water depths between 0.1-15 m (Bazikalova 1945, Rivarola-Duarte et al. 2014). Similar to *D. duebenum*, *Nosema* species might be transmitted to *Niphargus*-populations from other amphipods in phases of horizontal transmission. As the *Nosema* sp. detected here in *Niphargellus arndti* was not revealed by any other genetic study on amphipod microsporidians so far, it might be a niphargid-specific species.

The microsporidian isolate from *N. aquilex* (lineage G) showed only a low similarity (93.1%) to a previously characterized microsporidian isolate from amphipods. Therefore, it should be considered as a new sequence record. Also the isolate from *Niphargellus arndti* with 97.5% similarity to a microsporidian isolate from caddisfly larvae is probably a species that has not been sequenced and described yet.

A puzzling finding is the microsporidium from *Niphargellus arndti* that was genetically 99.8% similar to *Hyperspora aquatica*, a microsporidian hyperparasite of *Marteilia cochillia* (Paramyxida) from cockles (Stentiford et al. 2017). To date, Paramyxea of amphipods were only described from marine species (Ginsburger-Vogel and Desportes 1979, Short et al. 2012), but the sequence of *H. aquatica* shows a close relationship also to other microsporidians of freshwater amphipods. Genetically most similar is a *Microsporidium* sp. (98.9% to HM800853) from a marine parasitic copepod, and Stentiford et al. (2017) discussed a possible involvement of copepods in the life cycle of *H. aquatica*. Based on these assumptions, we can speculate that a related freshwater species utilizes (only) amphipods as hosts.

In this context, it should be mentioned that whole-body homogenates of the hosts were used for DNA extraction, including gut content and organisms associated with the amphipod, e.g. epibiotic ciliates. While *Dictoyocoela* and *Nosema* spp. are well characterized parasites of amphipods, we cannot be sure about the location of the other three microsporidian isolates detected in the present study. Therefore, the possibility exists that the microsporidium from the present study with high sequence similarity to *H. aquatica* is actually infecting protists associated with the amphipods (see also discussion in Stentiford et al. 2017) or originates from groundwater copepods ingested by niphargids.

An unexpected finding was the detection of a sequence most similar to an apostome ciliate in two individuals of *N. schellenbergi* from a single site (North Rhine-Westphalia, spring near Behlingen). Apostome ciliates are exuvitrophic or parasitoids of invertebrates, mainly crustaceans, and were described previously from marine and freshwater amphipods (e.g. Bradbury 2005, Chantangsi et al. 2013, Gudmundsdóttir et al. 2018, Lynn and Strüder-Kypke 2019). Apostome ciliates were also detected in groundwater habitats. For example, *Collinia neophargi* was described from *Crangonyx subterraneus* (syn. *Neoniphargus moniezi,* Ginet 1988) (Bradbury 1994). Gudmundsdóttir et al. (2018) isolated five different sequences of apostome ciliates from the groundwater amphipods *Crangonyx islandicus* and *Crymostygius thingvallensis* from Iceland. Interestingly, the ciliate sequence from the present study was most similar (98.5%) to an apostome ciliate from the marine amphipod *Gymnodinioides pitelkae* (Bradbury 2005), probably due to the lack of related sequence information from freshwater species.

Conclusion

In the present study, *Niphargus schellenbergi* was the most frequent taxon, but also demonstrated a proportionally high infection rate. A total of five different microsporidian species were discovered, with *Dictyocoela duebenum* being the most frequent and

found in different niphargid isolates, but preferably in *N. schellenbergi*. This shows that different populations of groundwater amphipods can be impacted by this feminizing microsporidium. Other single findings of microsporidians give an indication of the diversity but a larger sample size and ultrastructural studies would be desirable to link the genetic data to previous morphological descriptions. We want to conclude that more studies on microsporidians (and other parasites) in groundwater species are needed to improve our understanding on their effect on the host populations and sensitive aquatic communities.

Acknowledgements

This publication is based upon work from COST Action DNAqua-Net (CA15219), supported by the COST (European Cooperation in Science and Technology) programme. We thank John Boulton, Sandra Cervantes, Robert Dondelinger, Christine Harbusch, Lee Knight, Christa Locke, Florian Malard, Patrice Notteghem, Joep Orbons, Adam Pyka, Rainer Sennewald, Vid Svara and Verena Weber for assistance during sampling. AW was financed by a grant of the German Research Foundation (WE 6055/1-1).

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Supplementary material 1

Overview table of all groundwater amphipod specimens screened for microsporidians

Authors: Daniel Grabner, Dieter Weber, Alexander M. Weigand

Data type: specimen metadata information

Explanation note: The table lists specimen ID, taxonomy, collection information, habitat ype, microsporidium infection, GenBank Accession numbers for the parasite, identification results via morphology and DNA (COI, 28S), BOLD BINs and comments to the identification method for each specimen analysed.

Link: https://doi.org/10.3897/subtbiol.33.48633.suppl1

Supplementary material 2

COI sequences of groundwater amphipods screened for microsporidians

Authors: Daniel Grabner, Dieter Weber, Alexander M. Weigand

Data type: sequences

Explanation note: Cytochrome C oxidase subunit 1 (COI) sequences for groundwater amphipods screened for microsporidians.

Link: https://doi.org/10.3897/subtbiol.33.48633.suppl2

Supplementary material 3

SSU rDNA sequences for microsporidian parasites of groundwater amphipods

Authors: Daniel Grabner, Dieter Weber, Alexander M. Weigand

Data type: sequences

Explanation note: SSU rDNA sequences for microsporidians of groundwater amphipods

Link: https://doi.org/10.3897/subtbiol.33.48633.suppl3