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

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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-Szychalska 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 Stempelia 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 sympatriically 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 Cran-gonyx sp. have been analysed for microsporidian infections (Table 1; for further infor-
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 ATY 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 keruthi* | Ireland     | Country Clare, Ballyvaghan, Polldubh Cave | 10/21/2017 | cave           |                                               |
| *Niphargellus arndtii* | Czech Republic | Málkov, spring near Málkov              | 4/13/2018  | spring         |                                               |
|                     | Germany      | Bavaria, Münchenberg, 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         |                                               |
|                     | 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 Jungfernnquelle   | 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         | *Dictyocoela duebenum* (99.45% similar to MH753359) |
|                     | 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, Trinkwasserequelle Hilpischmühle | 8/14/2018 | spring         |                                               |
|                     | Germany      | Saarland, Saarhölzbach, Schankbir            | 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 Gangloff, Tèse              | 3/15/2018  | spring         |                                               |
### Taxonomy

| 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 |                                                                          |
|                                        | Luxembourg     | Minette, Esch sur Alzette, Minière Lange gronn            | 8/3/2018  | spring       | Microsporidium sp. (93.1% similarity to FJ755996)                                    |
| Niphargus aquilex-complex, lineage J  | France         | Loir et Cher, 35 Pleine-Fougères                           | n.a.      | n.a.         |                                                                          |
| Niphargus aquilex-complex, lineage K  | Belgium        | Wallonia, Péruezel, 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 Kroedorfer Forst           | 22.07.2016| spring       |                                                                          |
| Niphargus glenniei                    | United Kingdom | Devon, Ashburton, Pridshamsleigh Cavern                    | 9/7/2016  | cave         |                                                                          |
| Niphargus irlandicus                  | Ireland        | County Clare, Ballyvaghan, Ailwee Cave                     | 10/22/2017| cave         |                                                                          |
| Niphargus kochianus-complex (not A-D)  | the Netherlands | Stokhem, Dorpstratwell                                    | 5/20/2017 | well         |                                                                          |
| Niphargus puteanus                    | Germany        | Baden-Württemberg, Schlattstall, Wasserhäuser Schwarze Laut | 6/13/2017 | spring       |                                                                          |
| Niphargus schellenbergi               | Belgium        | Wallonia, Felenne, Source abrevvoir 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       | Dictyocela dauemum (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, Valfricourt, Lavoir de Valfricourt         | 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  | _Dictyocoela duebenum_ (99.45% similar to MH753359) |
|               | Germany        | Thuringia, Bad Klosterlausnitz, Holzborn           | 3/15/2018  | 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 Mühnequelle  | 8/13/2017  | spring  |                                               |
|               | Germany        | Hesse, Martinhagen, Quelle der Knieppanlage Martinhagen | 5/9/2018   | spring  |                                               |
|               | Germany        | Hesse, Wärme-Quelle                                | 5/9/2018   | spring  |                                               |
|               | Germany        | North Rhine-Westphalia, Brilon, Obere Mühnequelle  | 8/13/2017  | spring  |                                               |
|               | Germany        | Rhineland-Palatinate, Trier ST Euren, Quelle überm Talbildchen | 10/13/2017 | spring  |                                               |
|               | Germany        | Sauerland, Mettdach, Quelle über Mettdach           | 1/28/2017  | spring  | _Dictyocoela duebenum_ (99.45% similar to MH753359) |
|               | Germany        | Bavaria, Neuschlechach, Aurachquelle               | 8/23/2017  | spring  |                                               |
|               | Luxembourg     | Gutland, Diekirch, Quelle Diekirch                 | 2/11/2018  | spring  | _Dictyocoela duebenum_ (99.45% similar to MH753359) |
|               | Luxembourg     | Ösling, Urselt, Quelle am aale Koepchen            | 5/20/2018  | spring  |                                               |
|               | Luxembourg     | Gutland, Girsterklau, 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...
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 pittelkoe; 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 microsporidi-ans 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, *Paramyxidae* 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 *Dictyocoela* 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.

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**References**

Bacela-Spychalska K, Wattier RA, Genton C, Rigaud T (2012) Microsporidian disease of the invasive amphipod *Dikerogammarus villosus* and the potential for its transfer to local invertebrate fauna. Biological Invasions 14: 1831–1842. https://doi.org/10.1007/s10530-012-0193-1

Bacela-Spychalska K, Wróblewski P, Mamos T, et al. (2018) Europe-wide reassessment of *Dictyocoela* (Microsporida) infecting native and invasive amphipods (Crustacea): molecular versus ultrastructural traits. Scientific Reports 8: 8945: 1–16. https://doi.org/10.1038/s41598-018-26879-3

Bazikalova, AY (1945) Amphipods of Lake Baikal. Proceedings of Baikal Limnological *Station* 11: 1–440.

Bradbury PC (1994) Parasitic protozoa of molluscs and crustacea. In: Parasitic protozoa (pp. 139–264). Academic Press.

Bradbury PC (2005) *Gymnodinioides pitelkae* n. sp. (Ciliophora, Apostomatina) from the littoral amphipod, *Marinogammarus obtusatus*, a trophont with remnants of the tomite’s infraciliature. European Journal of Protistology 41: 85–92. https://doi.org/10.1016/J.EJOP.2004.10.001

Bulnheim (1971) Über den Wirtskreis der Mikrosporidie *Stenpellia mülleri*. Archiv für Protistenkunde 113: 137–145.

Bulnheim H-P (1975) Microsporidian infections of amphipods with special reference to host-parasite relationships: A review. Marine Fisheries Review 37: 39–45
Chantangsi C, Lynn DH, Rueckert S, et al. (2013) *Fusiforma themisticola* n. gen., n. sp., a new genus and species of apostome ciliate infecting the hyperiid amphipod *Themisto libellula* in the Canadian Beaufort Sea (Arctic Ocean), and establishment of the Pseudocolliniidae (Ciliophora, Apostomatia). Protist 164(6): 793–810.

Dimova M, Madyarova E, Gurkov A, et al. (2018) Genetic diversity of Microsporidia in the circulatory system of endemic amphipods from different locations and depths of ancient Lake Baikal. PeerJ 6: e5329. https://doi.org/10.7717/peerj.5329

Dunn A, Smith J (2001) Microsporidian life cycles and diversity: the relationship between virulence and transmission. Microbes and Infection 3: 381–388.

Ginsburger-Vogel T, Desportes I (1979) Structure and biology of *Marteilia* sp. in the amphipod *Orchestia gammarellus*. Marine Fisheries Review 41: 3–7.

Fišer C, Trontelj P, Luštrik R, Sket B (2009) Toward a unified taxonomy of *Niphargus* (Crustacea: Amphipoda): a review of morphological variability. Zootaxa 2061(1): 1–22.

Flot J-F, Wörheide G, Dattagupta S (2010) Unsuspected diversity of *Niphargus* amphipods in the chemoautotrophic cave ecosystem of Frasassi, central Italy. BMC Evolutionary Biology 10: 171.

Ginet R (1988) Rejet du taxon *Niphargus minutus* (Gervais, 1835) et suppression de *Niphargus moniezii* Wrzeniowski, 1890 (Crustacés Amphipodes). Publications de la Société Linnéenne de Lyon 57(5): 147–149.

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

Grabner DS (2017) Hidden diversity: parasites of stream arthropods. Freshwater Biology 62: 52–64.

Gudmundsdóttir R, Kornobis E, Kristjánsson BK, Pálsson S (2018) Genetic analysis of ciliates living on the groundwater amphipod *Crangonyx islandicus* (Amphipoda: Crangonyctidae). Acta Zoologica 99: 188–198. https://doi.org/10.1111/azo.12204

Haine ER, Brondani E, Hume KD, Perrot-Minnot MJ, Gaillard M, Rigaud T (2004) Co-existence of three microsporidia parasites in populations of the freshwater amphipod *Gammarus roeseli*: evidence for vertical transmission and positive effect on reproduction. International Journal for Parasitology 34: 1137–1146. https://doi.org/10.1016/j.ijpara.2004.06.006

Ironside JE, Smith JE, Hatcher MJ, Sharpe RG, Rollinson D, Dunn AM (2003) Two species of feminizing microsporidian parasite coexist in populations of *Gammarus duebeni*. Journal of Evolutionary Biology 16: 467–473.

Karpov SA, Mamkaeva MA, Aleoshin VV, Nasonova E, Lilje O, Gleason FH (2014) Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. Frontiers in Microbiology 5: 1–11.

Krebes L, Blank M, Frankowski J, Bastrop R (2010) Molecular characterisation of the Microsporidia of the amphipod *Gammarus duebeni* across its natural range revealed hidden diversity, wide-ranging prevalence and potential for co-evolution. Infection, Genetics and Evolution 10: 1027–1038. https://doi.org/10.1016/j.meegid.2010.06.011
Madyarova EV, Adelshin RV, Dimova MD, Axenov-Gribanov DV, Lubyaga YA, Timofeyev MA (2015) Microsporidian parasites found in the hemolymph of four Baikalian endemic amphipods. PLoS One 10: e0130311. https://doi.org/10.1371/journal.pone.0130311

McInerney CE, Maurice L, Robertson AL, Knight LR, Arnseidt J, Venditti C, Dooley JSG, Mathers T, Matthijs S, Eriksson K, Proudlove GS, Hänfling B (2014) The ancient Britons: groundwater fauna survived extreme climate change over tens of millions of years across NW Europe. Molecular Ecology 23(5): 1153–1166.

Poisson R (1924) Sur quelques Microsporidies parasites d'Arthropodes. Comptes rendus de l’Académie des Sciences 178: 664–666.

Quiles A, Bacela-Spychalska K, Teixeira M, Lambin N, Grabowski M, Rigaud T, Wattier RA (2019) Microsporidian infections in the Amphipoda species complex Gammarus roeselii over its geographic range: evidence for both host-parasite co-diversification and recent host-shifts. Parasites & Vectors 12(1): 327. https://doi.org/10.1186/s13071-019-3571-z

Ratnasingham S, Hebert PD (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7(3): 355–364.

Rivarola-Duarte L, Otto C, Jühling F, Schreiber S, Bedulina D, Jakob L, Gurkov A, Axenov-Gribanov D, Sahyoun AH, Lucassen M, Hackermüller J, Hoffmann S, Sartoris F, Pörtner H-O, Timofeyev M, Luckenbach T, Stadler PF (2014) A first glimpse at the genome of the Baikalian amphipod Eulimnogammarus verrucosus. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 322(3): 177–189.

Short S, Guler Y, Yang G, Kille P, Ford AT (2012) Paramyxean-microsporidian co-infection in amphipods: is the consensus that Microsporidia can feminise their hosts presumptive? International Journal for Parasitology 42: 683–691. https://doi.org/10.1016/j.ijpara.2012.04.014

Smith JE (2009) The ecology and evolution of microsporidian parasites. Parasitology 136: 1901–1914.

Stentiford GD, Feist SW, Stone DM, Bateman KS, Dunn AM (2013) Microsporidia: diverse, dynamic, and emergent pathogens in aquatic systems. Trends in Parasitology 29: 567–578. https://doi.org/10.1016/j.pt.2013.08.005

Stentiford GD, Dunn AM (2014) Microsporidia in aquatic invertebrates. In: Weiss LM, Becnel JJ (Eds) Microsporidia: Pathogens of Opportunity, First Edit. John Wiley & Sons, Inc., Iowa, pp. 579–603.

Stentiford GD, Ramilo A, Abollo E, Kerr R, Bateman KS, Feist SW, Bass D, Villalba A (2017) Hyperspora aquatica n. gn., n. sp. (Microsporidia), hyperparasitic in Marteilia cochillia (Paramyxida), is closely related to crustacean-infecting microsporidian taxa. Parasitology 144: 186–199. https://doi.org/10.1017/S0031182016001633

Terry RS, Smith JE, Bouchon D, Rigaud T, Duncanson P, Sharpe RG, Dunn AM (1999) Ultrastructural characterisation and molecular taxonomic identification of Nosema granulosis n. sp., a transovarially transmitted feminising (TTF) Microsporidium. Journal of Eukaryotic Microbiology 46: 492–499.

Terry RS, Smith JE, Sharpe RG, Rigaud T, Littlewood DTJ, Ironside JE, Rollinson D, Bouchon D, MacNeil C, Dick JTA, Dunn AM (2004) Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora. Proceed-
Verovnik R, Sket B, Trontelj P (2005) The colonization of Europe by the freshwater crustacean *Asellus aquaticus* (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity. Molecular Ecology 14(14): 4355–4369.

Weigand AM, Kremers J, Grabner DS (2016) Shared microsporidian profiles between an obligate (*Niphargus*) and facultative subterranean amphipod population (*Gammarus*) at sympatry provide indications for underground transmission pathways. Limnologica 58: 7–10. https://doi.org/10.1016/j.limno.2016.01.005

Wilkinson TJ, Rock J, Whiteley NM, Ovcharenko MO, Ironside JE (2011) Genetic diversity of the feminising microsporidian parasite *Dictyocoela*: new insights into host-specificity, sex and phylogeography. International Journal for Parasitology 41: 959–966. https://doi.org/10.1016/j.ijpara.2011.04.002

**Supplementary material I**

**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 type, 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.

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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.  
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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  
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