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Marina Chauvet, Didier Debroas, Anne Moné, Aurore Dubuffet, Cécile Lepère

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1 **Temporal variations of Microsporidia diversity and discovery of new**
2 **host-parasite interactions in a lake ecosystem**

3

4 **Running title: Microsporidia diversity and new hosts in lakes**

5

6 *Marina Chauvet¹, Didier Debroas, Anne Moné, Aurore Dubuffet* & Cécile Lepère**

7

8 CNRS, Laboratoire Microorganismes : Génome et Environnement, Université Clermont
9 Auvergne, Clermont-Ferrand, F-63000, France.

10 ¹corresponding author: marina.chauvet@uca.fr

11 * These authors contributed equally to this work

12

13 ***Originality-Significance Statement***

14

15 In this study, we highlighted for the first time temporal variations of microsporidian diversity in a
16 lake ecosystem over one year. Through two complementary approaches, co-occurrence network and
17 *in situ* hybridization (with a newly designed probe specific to Microsporidia), we identified new
18 hosts of Microsporidia among community at the base of the food webs.

19

20 *Abstract*

21

22 Microsporidia are a large group of obligate intracellular eukaryotic parasites related to Fungi.
23 Recent studies suggest that their diversity has been greatly underestimated and little is known about
24 their hosts other than metazoans, and thus about their impact on the communities at the base of the
25 food web. In this work, we therefore studied the diversity of Microsporidia over one year and
26 identified potential new hosts in small-sized fractions (<150 µm) in a lake ecosystem using
27 a metabarcoding approach coupled with co-occurrence networks and TSA-FISH. Our
28 analysis shows a great Microsporidia diversity (1 472 OTUs), with an important part of this
29 diversity being unknown. Temporal variations of this diversity have been observed which
30 might follow temporal variations of their potential hosts such as protists and
31 microzooplankton. New hosts among them were identified as well as associations with
32 phytoplankton. Indeed, repeated infections were observed in *Kellicottia* (rotifers) with a
33 prevalence of 38% (infected individuals). Microsporidia inside a *Stentor* (ciliate) were also
34 observed. Finally, potential infections of the diatom *Asterionella* were identified (prevalence
35 <0.1%). The microsporidian host spectrum could be therefore even more important than
36 previously described, and their role in the functioning of lake ecosystems is undoubtedly
37 largely unknown.

38

39 ***Introduction***

40

41 Microsporidia are a diverse phylum of unicellular eukaryotes related to Fungi (Karpov *et al.*, 2014).
42 They are obligate intracellular parasites known to date to parasitize a broad range of hosts among
43 the animal kingdom as well as some protists (Murareanu *et al.*, 2021). Many species parasitizing
44 metazoan hosts have been described, with 16 metazoan phyla described as microsporidian hosts
45 (Murareanu *et al.*, 2021). Microsporidia include the canonical Microsporidia, which share common
46 features (including the presence of a polar tube, highly reduced mitochondria (mitosomes) and a
47 compact genome), and microsporidia-like organisms (Bass *et al.*, 2018; Wadi and Reinke, 2020;
48 Park and Poulin, 2021). These parasites infect hosts in all environments, but 50% of the known
49 genera infect aquatic hosts (Stentiford *et al.*, 2013). The environmental diversity of Microsporidia is
50 nevertheless clearly underestimated since these species have been described mostly after the search
51 of infectious agents in organisms of interest (edible fish and crustaceans, auxiliary insects, domestic
52 insects (silkworms, bees)), pests (crop pests, disease-carrying insects), bioindicators (daphnids,
53 amphipods), and humans. Moreover, Microsporidia are generally absent from studies focusing on
54 the diversity of protists carried out by high-throughput sequencing, due to the use of primers not
55 adapted to the microsporidian phylum (Hadziavdic *et al.*, 2014; Hugerth *et al.*, 2014). In aquatic
56 ecosystems, high-throughput sequencing studies yet suggest the presence of numerous parasites
57 with many sequences affiliated especially to alveolates and Fungi (*e.g.* Debroas *et al.*, 2017).
58 Recent studies using microsporidian-specific primers suggest that a large amount of the
59 environmental microsporidian diversity remains to be described (Ardila-Garcia *et al.*, 2013;
60 Williams *et al.*, 2018; Dubuffet *et al.*, 2021). Moreover, although Microsporidia have a very wide
61 host spectrum, they have rarely been described as parasites of protists and microzooplankton. In
62 marine ecosystems, they are notably described as hyperparasites of gregarines (*e.g.* Mikhailov *et al.*,
63 2016). In freshwater ecosystems, infections by Microsporidia have been registered in ciliates (Lutz

64 and Splendore, 1908; Krüger, 1956; Görtz, 1987; Fokin *et al.*, 2008; Yakovleva *et al.*, 2020),
65 gregarines (Rühl and Korn, 1979; Codreanu-Bălcescu and Codreanu, 1976), unicellular cnidarians
66 (Morris and Freeman, 2010) and rotifers (Gorbunov and Kosova, 2001; Wolska and Mazurkiewicz-
67 Zapalowicz, 2013). Most of these observations are based on light microscopy and/or transmission
68 electron microscopy therefore allowing only morphological descriptions. By now, only two
69 microsporidian species parasitizing protists in brackish and river ecosystems are characterized by
70 morphological and molecular approaches (*i.e.* 18S rDNA sequences): *Euplotespora binucleata* and
71 *Globosporidium paramecii* parasitizing the ciliates *Euplotes woodruffi* and *Paramecium aurelia*,
72 respectively (Fokin *et al.*, 2008; Yakovleva *et al.*, 2020). Thus, the global diversity of Microsporidia
73 in aquatic ecosystems is largely unknown, especially in small sized hosts (*i.e.* <150µm).

74

75 A first metabarcoding analysis revealed an important diversity of Microsporidia, of which about
76 50% couldn't be affiliated to a genus and about 25% not to a family (Dubuffet *et al.*, 2021). We
77 hypothesized that some of these Microsporidia may infect understudied hosts, such as protists or
78 microzooplankton. To test this hypothesis, we studied in parallel the diversity of Microsporidia and
79 other eukaryotes, as well as their variations over a year in a lake ecosystem, using a targeted
80 metabarcoding approach, focusing on fractions containing small sized hosts. Potential new host-
81 microsporidian interactions were searched through co-occurrence network analysis based on
82 metabarcoding data, as well as through a TSA-FISH approach.

83

84 **Results**

85 *Microsporidian diversity*

86

87 A total of 1 472 OTUs were obtained from 2874995 18S rRNA gene sequencing reads (1080350
88 reads after normalization). Three samples were removed because of their low reads number (<12
89 400) and poor representation of the diversity: 20/02/19-0.8/10-RNA, 06/05/19-10/150-RNA and
90 29/07/19-10/150-DNA, resulting in a dataset of 85 samples.

91 On average, 33 823 reads were obtained per sample and the rarefaction curves showed that a
92 plateau was reached for most of the samples (Supplementary Information 3).

93 Microsporidia α -diversity varied among samples, the highest diversity being recorded in the 0.8-10
94 μm size fraction (Supplementary Information 4). Despite a high number of OTUs, the
95 microsporidian community was clearly dominated by a low number of dominant OTUs, since 14
96 OTUs represented more than 50% of the total reads.

97 OTUs obtained in this study belonged to the canonical Microsporidia, and were affiliated at least to
98 a clade (which roughly correspond to the class rank, Vossbrinck *et al.*, 2014) after the first
99 taxonomic affiliation (*i.e.* implemented in PANAM2 pipeline). Among the 10 clades defined in
100 Dubuffet *et al.* (2021) for canonical Microsporidia (from clade I to clade IX, clade IV considered as
101 a 'super-clade' and being divided in two clades), our OTUs were distributed in five clades: I, IV1,
102 IV2, V, VIII. The microsporidian community was clearly dominated by clades IV2 and I, which
103 represented respectively 80.6% (77.4% reads) and 16.6% (20.6% reads) of the OTUs. The
104 remaining OTUs were classified into clades IV1: 2% (1.2% reads), V: 0.4% (0.1% reads) and VIII:
105 0.3% (0.7% reads) (Fig.1, Supplementary Information 5).

106 Within the five clades, eight groups (which mostly correspond to families, Dubuffet *et al.*, 2021)
107 were identified following the analysis on PANAM2. Two of them accounted for 74% of the OTUs
108 (83% of the reads): Aquatic outgroup (clade I, 16.6% OTUs, 20.6% reads) and Arlequin group

109 (clade IV2, 57.3% OTUs, 62.2% reads) (Fig.1, Supplementary Information 5). Among OTUs
110 assigned to a genus, *Crispospora* (50.0% OTUs, 53.8% reads) and *Pseudoberwaldia* (7.5% OTUs,
111 11.1% reads) were the most represented (Fig.1, Supplementary Information 5).
112 However, 39% of the OTUs were not assigned to a genus and 22% not even to a group following
113 the analysis on PANAM2 (Supplementary Information 5). We therefore performed phylogenetic
114 analyses on these OTUs, focusing on the most abundant (>500 reads) (Supplementary Information
115 6). These analyses placed these OTUs into five new groups previously identified in Dubuffet *et al.*
116 (2021). Three of these new groups (Van8, Van10 and BPAR5) (Fig.1) represent a significant portion
117 of the observed diversity. The Van8 group, for example, represented 9.5% of the reads, and up to
118 90% of the reads in one sample (29/07/19-0.8/10-RNA). We furthermore identified several
119 subgroups (proxies for genera) within both the known and new groups (Supplementary Information
120 6).

121

122

123 *Variations of the microsporidian diversity*

124

125 No significant differences were observed in the composition of the DNA and RNA samples
126 (Supplementary Information 7). 95% of OTUs were shared by both types of samples. Only 48 and
127 32 OTUs were specific to DNA and RNA samples, respectively (these OTUs representing at most
128 0.08% of total reads). The mean rRNA:rDNA ratio computed for each OTU was 7.3, which
129 suggests that much of the diversity discovered was active (*i.e.* rRNA:rDNA ratio >1).

130 Variations in the relative abundances of some taxa were however recorded between size fractions as
131 well as across time (Fig.2). For the size fractions, no major differences in the community
132 composition were recorded at the group level. However, for the same sampling dates, some
133 genera/subgroups showed considerable differences between the two size fractions (Fig.2). Within

134 the Van8 group, for example, the subgroup1 was almost exclusively found in the large size fraction,
135 sometimes with high abundance (e.g. 50% of the reads in the sample 01/07/19). Subgroups 2 and 3,
136 on the other hand, were mostly found in the small size fraction (Fig.2).
137 Regarding variations across time, the composition of microsporidian communities greatly changed
138 between two close sampling dates, from the clade to the genus/subgroup level. In the small size
139 fraction, for example, the sample 01/07/19 was greatly dominated by the genus *Crispospora*, while
140 the sample 29/07/19 was composed at 80% of the ‘Van8 group ; subgroup 3’ (Fig.2). Some
141 genera/subgroups seemed abundant for a particular period (e.g. ‘Van8 group ; subgroup 3’ between
142 late July and November) or even at a single sampling date (‘Arlequin group ; Cr1 subgroup’:
143 02/12/2019), while others showed a saw-toothed abundance over the year (e.g. ‘Van8 group ;
144 subgroup 1’) (Fig.2). At the OTUs level, considerable variations across time could also be observed.
145 Indeed, more than 50% of the reads were represented by two or three OTUs in several samples,
146 with temporal variations in these dominant OTUs (Supplementary Information 8).

147

148

149 *Eukaryotic diversity*

150

151 In the eukaryotic dataset, four samples were removed because of their low reads number (<12 400)
152 and poor representation of the diversity: 18/04/19-10/150-RNA, 14/06/19-10/150-DNA, 29/07/19-
153 0.8/10-DNA and 29/07/19-10/150-DNA, resulting in a dataset of 84 samples. A total of 631 OTUs
154 were obtained from 3158730 18S rRNA gene sequencing reads (1044624 reads after
155 normalization).

156 In terms of OTUs number, the main taxonomic groups were the Stramenopiles (238 OTUs),
157 Alveolata (141) and Viridiplantae (59). If we consider the read abundance, the dominant groups
158 were the Alveolata (33.3% of the reads), Stramenopiles (31%) and Metazoa (10.5%). The

159 eukaryotic community was clearly dominated by the ciliates (Alveolata) which represented almost
160 30% of the reads (Fig.3). The Cryptophyta (Cryptophyceae), Rotifera (Metazoa) and Chlorophyta
161 (Viridiplantae) were also abundant with 9.7%, 6.3% and 5.4% of the reads, respectively.
162 Considerable differences in the eukaryotic community were identified between the two different
163 size fractions. For example, rotifers and arthropods were almost exclusively found in the large size
164 fraction. Diatoms and chytrids were also more abundant in the 10-150 μm fraction. On the opposite,
165 some phyla were mostly found in the small size fraction as Cryptophyta, Chlorophyta, Cercozoa
166 and Chrysophyceae (Fig.3). Variations in the eukaryotic communities were also identified across
167 time. Ciliates for example occur in all samples but were particularly abundant during spring and
168 summer, whereas the Cryptophyta were mostly found during winter and spring. Other phyla were
169 identified with variations throughout the sampling period but without any pattern, such as the
170 Chrysophyceae, the arthropods and the rotifers. The latter were, however, sometimes really
171 abundant as in the sample 19/11/18 (large size fraction) representing 61.5% of the reads (Fig.3).

172

173

174 *Co-occurrence networks*

175

176 In order to suggest potential host-parasite relationships, eukaryotic and microsporidian OTUs with a
177 total abundance higher than 0.05% were used to build a co-occurrence network. 1 640 associations
178 were identified of which 1 324 (80.7%) were positive, in a simple connected network (only one
179 component). We focused our analysis on positive edges only, as Microsporidia are obligate
180 intracellular parasites, this requiring the simultaneous presence of both partners (Supplementary
181 Information 9). Therefore, only positive edges between Microsporidia and eukaryotes were further
182 considered, resulting in 88 microsporidian and 98 eukaryotic OTUs with 206 positive associations
183 represented in the Sankey diagram (Fig.4).

184 Microsporidia co-occurred mainly with Ciliophora, Rotifera and Arthropoda, these three phyla
185 being included in almost 50% of the associations. The two most abundant genera of Microsporidia,
186 *Crispospora* and *Pseudoberwaldia*, were notably found to co-occur mainly with Ciliophora (46%
187 and 42% of the associations, respectively). To a lesser extent, associations were also found with
188 other eukaryotes including Chytridiomycota, Dinoflagellata, Bacillariophyta (diatoms),
189 Chlorophyta and Chrysophyceae (Fig.4). Among the new groups defined in Dubuffet *et al.* (2021),
190 the Van8 group (clade IV2) showed 16 associations with 11 eukaryotic taxa (about one third of
191 these associations being with Chrysophyceae) and the Van10 group (clade IV2) showed 15
192 associations with 8 eukaryotic taxa (mostly with Chlorophyta and Rotifera) (Fig.4) (see
193 Supplementary Information 10 for a detailed table of the number of associations).

194

195

196 *TSA-FISH*

197

198 TSA-FISH was used to confirm potential host-parasite associations between Microsporidia and
199 planktonic eukaryotes (<150 µm) highlighted by the co-occurrence network. All samples (10-150
200 µm size fraction) were screened with the USP01 probe, targeting all the Microsporidia recovered in
201 the current analysis. Positive signals were observed inside rotifers identified as belonging to the
202 genus *Kellicottia* by morphological observations (Fig.5). This association was observed at four
203 different dates (27/05/19, 06/06/19, 14/06/19 and 17/06/19) and the prevalence of potentially
204 parasitized *Kellicottia* reached 50% (27/05/19) with a mean value of 38%.

205 TSA-FISH also allowed the observation of positive signals on cells attached to diatoms (genus
206 *Asterionella*) (Fig.6a-c) at five different dates (18/04/19, 08/10/19, 04/11/19, 02/12/19, 07/01/20).
207 However due to a high abundance of *Asterionella* at these dates, the prevalence was always <0.1%.

208 Moreover, a single observation (17/06/19 in the large size fraction) shows positive signals inside a
209 ciliate identified as belonging to the genus *Stentor* by morphological observations (Fig.6d-f).
210 Finally, other positive signals were observed, but the potentially infected hosts could not be
211 identified (Supplementary Information 11).

212

213 **Discussion**

214

215 So far, few studies have investigated the diversity of Microsporidia in environmental samples. A
216 cloning-sequencing study (Ardila-Garcia *et al.*, 2013) and a metabarcoding study (Williams *et al.*,
217 2018) presented a first description of this diversity, mostly in terrestrial and marine environments.
218 However, due to a low number of reads, a limited comparison of Microsporidia communities
219 between different sites was made, and variation of the microsporidian diversity through time was
220 not evaluated. In a more recent metabarcoding analysis, an important microsporidian diversity was
221 revealed in lacustrine environments, with more than 1 500 OTUs distributed across seven of the ten
222 clades defined for canonical Microsporidia (Dubuffet *et al.*, 2021) and several new groups (and
223 probable new families and/or genera) were discovered. The diversity of Microsporidia in lake Aydat
224 studied here, over one year, was of the same order of magnitude with 1 472 microsporidian OTUs
225 identified. Besides, except two clades weakly represented in Dubuffet *et al.* (2021), the same clades
226 (I, IV1, IV2, V, VIII) were recovered. Similarly to the previous study, most of the OTUs were
227 affiliated to clade IV2 and clade I, and most of the groups identified were also found in Dubuffet *et*
228 *al.* (2021) with the exception of OTUs belonging to the Enterocytozoonidae, which appear almost
229 only in February in our study. Among OTUs assigned to a genus, *Crispospora* and
230 *Pseudoberwaldia* were the most represented. These two genera are described as parasites of aquatic
231 hosts (daphnia for *Pseudoberwaldia* (Vávra *et al.*, 2019)) or hosts having at least one aquatic
232 development stage (chironomids for *Crispospora* (Tokarev *et al.*, 2010)). Chironomids eggs and
233 larvae are highly abundant in lake environments, especially when they are eutrophic and
234 anthropized (Langdon *et al.*, 2006) such as lake Aydat, which could explain the abundance of
235 Microsporidia assigned to *Crispospora*.

236 The new groups previously identified in Dubuffet *et al.* (2021) and also found in this study
237 represented a non-negligible part of the microsporidian diversity as they correspond to 10% of the

238 OTUs included in our phylogenetic analyses, suggesting that a significant proportion of
239 microsporidian groups/families and subgroups/genera remain to be studied. This unknown part of
240 the Microsporidia diversity could correspond to parasites of small hosts (protists,
241 microzooplankton), which currently represent only a small proportion of the microsporidian hosts
242 described.

243

244 Between the two size fractions (0.8-10 and 10-150 μm) studied, moderate differences were
245 observed at the finest taxonomic levels (genus/subgroup). Indeed, ‘Van8 group ; subgroup 2’, ‘Van8
246 group ; subgroup 3’ and ‘*Orthosomella*-like group ; O4 subgroup’ were found almost exclusively in
247 the small size fraction and ‘Van8 group ; subgroup 1’ in the large size fraction. Such differences
248 might be explained by the spore sizes, or the type of host parasitized. Indeed, the former could be
249 $<10 \mu\text{m}$ in size and/or infect only small hosts (assuming that Microsporidia could parasitize host
250 $<10 \mu\text{m}$), and the latter could be large (some spores having a size $>10 \mu\text{m}$, (Vávra and Larsson,
251 2014)) and/or infect hosts larger than $10 \mu\text{m}$. But as no species has been morphologically described
252 in these subgroups, we know nothing about the size of their spores. However, we know the size of
253 some of the Microsporidia found in this study. *Crispospora chironomi* for example has spores of 2.5
254 $\times 1.5 \mu\text{m}$ size which can be released in $20\text{-}30 \mu\text{m}$ thick wall capsules (Tokarev *et al.*, 2010). This is
255 thus not surprising to find this genus in the two size fractions. *Pseudoberwaldia* are released as free-
256 spores from infected daphnia (spore size $4.4 \times 2.6 \mu\text{m}$, (Vávra *et al.*, 2019)). Presence of this genus
257 in $10\text{-}150 \mu\text{m}$ fraction thus raises the hypothesis that these Microsporidia were associated with other
258 eukaryotes (such as protists or microzooplankton), either being passively ingested, or infecting
259 them. This could also support the hypothesis of a secondary host as suspected for *Pseudoberwaldia*
260 species (Vávra *et al.*, 2019).

261

262 More striking differences were observed across time (Fig.2). These variations in diversity identified
263 over time were, for some, similar to those identified by Dubuffet *et al.* (2021). For example,
264 abundances of *Conglomerata* and *Pseudoberwaldia* increased in June in our study, as observed in
265 the same lake between April to June 2016.

266 Such variations might be directly linked to environmental variables which could influence infection
267 cycles or transmission efficiency of the parasite, as already shown for various parasites found in
268 aquatic ecosystems (Cable *et al.*, 2017). These environmental variables could also affect indirectly
269 the parasite by influencing their host populations density and distribution, as Microsporidia have
270 generally a narrow host specificity (Murareanu *et al.*, 2021). The relationship between the different
271 environmental variables (temperature, oxygen and photosynthetic pigment concentration (green
272 algae, Cyanobacteria, diatoms, Cryptophyta)) and the distribution of the different microsporidian
273 subgroups/genera was tested, and only oxygen and diatoms abundance were found to be significant
274 (Supplementary Information 12 and 2). *In vitro* analyses showed that various parameters, including
275 local changes in pH, salt concentration, UV radiation and temperature, could promote or inhibit the
276 devagination of the polar tube and spore discharge, and therefore impact infection rates (Weiss *et*
277 *al.*, 2014). However, it is not known whether such variables may influence directly Microsporidia in
278 their environment, thus influencing their distribution. Regarding the potential hosts, such as
279 zooplankton or protists, some can show considerable temporal variations in lake ecosystems (Lair,
280 1990; Lepère *et al.*, 2006) which could more likely explain the microsporidian variations observed
281 here. In this study, we also identified important temporal variations among eukaryotic phyla such as
282 the ciliates, rotifers and various phytoplanktonic phyla (Fig.3).

283

284 Identifying the hosts of these Microsporidia then seems to be an essential step in the understanding
285 of the microsporidian community variations over time. One approach to unravel these host-
286 Microsporidia potential interactions is to use co-occurrence network analyses, which arguably

287 provides the most exhaustive method for attempting to holistically assess community interactions
288 (Fath *et al.*, 2007). Using statistical tools, co-occurrence networks allow the highlighting of
289 potential associations between microorganisms (Chaffron *et al.*, 2010; Lima-Mendez *et al.*, 2015),
290 although this type of analyses do not ensure discovery of biotic interactions (Blanchet *et al.*, 2020).
291 Furthermore, potential associations predicted bioinformatically are informative and useful to reduce
292 the number of hypotheses that might be tested but will always require an experimental validation of
293 the interaction (Carr *et al.*, 2019). SPIEC-EASI method builds microbial networks avoiding
294 spurious associations resulting from the application of correlation measures and has been used to
295 predict previously unknown microbial associations (Kurtz *et al.*, 2015).
296 Using this method, diverse and various potential associations between Microsporidia and
297 eukaryotes have been revealed (Fig.4, Supplementary Information 9). 50% of these potential
298 associations involved eukaryotic phyla already identified as Microsporidia hosts: Ciliophora,
299 Apicomplexa, Arthropoda and Rotifera (Murareanu *et al.*, 2021). Among these, several co-
300 occurrences identified genera already described as hosts for Microsporidia: 10 co-occurrences with
301 the ciliate genera *Vorticella*, *Frontania* and *Stentor* (Görtz, 1987; Fokin *et al.*, 2008), four
302 associations with the rotifer genus *Brachionus* (Gorbunov and Kosova, 2001; Wolska and
303 Mazurkiewicz-Zapalowicz, 2013) and 10 co-occurrences with arthropods belonging to the order
304 Cyclopoida (Stentiford *et al.*, 2013). Co-occurrences were also observed with other phyla which
305 have so far never been described as hosts of Microsporidia: Chlorophyta, Chytridiomycota,
306 Chrysophyceae, Dinoflagellata, Bacillariophyta, Cryptomycota, Perkinsozoa, Streptophyta,
307 Craspedida, Synurophyceae, Bicosoecida and Cryptophyta. Surprisingly, some of these co-
308 occurrences involve phytoplankton organisms, whereas, to date infection by Microsporidia in
309 phytoplankton has never been recorded. However, all these potential associations identified through
310 the co-occurrence network do not reflect a temporal information. Unfortunately, the sample size is
311 indeed too small here to consider subnetworks for each sampling point.

312 Although the method we used attempts to minimise spurious associations, we must bear in mind
313 that the associations identified may not be pure parasitism. Indeed, an association could be
314 identified, for example, between a microsporidian species and the host of its parasitic host
315 (hyperparasitism) or be due to a common host (Microsporidia being sometimes opportunistic of
316 another infection (Didier, 2005)). An association could also reflect a simple feeding relationship
317 (ingestion of a Microsporidia spore by another eukaryote). Also, actual interactions may not have
318 been identified because of the weakness of the signal in the data (Blanchet *et al.*, 2020).

319

320 Actual associations can be observed with the use of fluorescence microscopy. Indeed, TSA-FISH
321 has shown its effectiveness in the detection of host-parasite associations in the environment
322 (Mangot *et al.*, 2009; Chambouvet *et al.*, 2019). But to date, this method has never been applied to
323 detect new Microsporidia-host associations. Using a newly designed specific probe for
324 Microsporidia, positive signals were repeatedly obtained in rotifers (genus *Kellicottia*), especially in
325 May and June samples (Fig.5). These signals may rather correspond to developing stages of
326 Microsporidia rather than ingested spores, as spores are usually not stained by FISH methods due to
327 their impermeability (Panek *et al.*, 2018). Microsporidia-rotifers associations highlighted by the co-
328 occurrence network were thus confirmed by these observations. Indeed, seven of the 25 co-
329 occurrences with rotifers were with the Brachionidae, a rotifer family which includes the genus
330 *Kellicottia*. *Kellicottia* rotifers are abundant in freshwater ecosystems, including lake Aydat where
331 they can reach more than 600 individuals per litre in summer (Lair and Oulad Ali, 1990). Rotifers
332 account for a large proportion of freshwater zooplankton and act as intermediaries between primary
333 producers (algae, cyanobacteria) and higher trophic levels (zooplankton, shellfish, fish) (Rico-
334 Martínez *et al.*, 2016). This newly identified host-parasite association is thus of great importance
335 considering the high prevalence rate observed (38% on average).

336 Although observed only once, the association between Microsporidia and the *Stentor* ciliate
337 (Fig.6d-f) also confirms the four co-occurrences identified with this genus. A case of microsporidian
338 parasitism has already been described in *Stentor roeseli* and *S. polymorphus* (Görtz, 1987).
339 For the first time to our knowledge, Microsporidia have also been observed in association with
340 phytoplankton (*i.e.* the diatom *Asterionella*) even if these observations remained observed at a low
341 prevalence rate (Fig.6a-c). This association was also highlighted by the co-occurrence network with
342 seven associations between Microsporidia and Fragilariaceae, the family to which the genus
343 *Asterionella* belong. These surprising observations, if confirmed through electron microscopy (to
344 determine whether these Microsporidia perform a complete life cycle in these hosts), could further
345 expand the already diverse host range of Microsporidia. Such observations of physical associations
346 with diatoms have already been described by Jones *et al.* (2011) with parasites formerly called
347 Cryptomycota, which could actually be Microsporidia (Bass *et al.*, 2018; Adl *et al.*, 2019).
348
349 This type of associations leads to many hypotheses on the role of Microsporidia in lacustrine
350 environments. Indeed, the structure and the succession of the different planktonic populations are
351 regulated by the nutritive resources as well as by different biotic factors including parasitism. In
352 order to better understand the functioning of food webs, it appears important to appreciate the
353 diversity of parasites (including Microsporidia) and to quantify their impact on the dynamics and
354 diversity of microzooplankton and phytoplankton, which play a major role at the basis of aquatic
355 food webs. Microsporidia, which now seem to be very abundant and diverse in lacustrine
356 ecosystems, could thus play an important role in the regulation of planktonic populations. It will
357 therefore be essential to confirm, identify and characterize these host-parasite associations more
358 precisely. The use of electron microscopy would allow a more detailed observation of the
359 associations. Also, the isolation and sequencing of a host-parasite pair could lead to the precise
360 identification of both organisms.

361

362 **Conclusion**

363

364 In conclusion, our metabarcoding approach with microsporidian universal primers coupled with a
365 network co-occurrence analyse and fluorescence microscopy unveiled a huge microsporidian
366 diversity in a lake ecosystem associated with important variations across a year, and provided a hint
367 on new microsporidian hosts. We notably identified repeated infections in *Kellicottia* (rotifer), and
368 potential infections of phytoplankton. It now seems very important to study the diversity of
369 Microsporidia on a larger scale, screening more environments and over a longer period of time.
370 Also, it will be essential to investigate more precisely the hosts of these Microsporidia in order to
371 understand their role in the ecosystems functioning.

372 *Experimental procedures*

373 *Study site and sampling procedure*

374

375 The study was conducted in lake Aydat (Massif Central, France, 45°39' 50" N, 2° 59' 04" E). It is a
376 dimictic and eutrophic lake with a maximum depth of 15 m, situated at an altitude of 825 m and a
377 recreative lake in summer. From 19/11/18 to 07/01/20 water samples were collected in the euphotic
378 zone of the lake at a permanent station located at the deepest zone of the water column using an
379 integrated sampler IWS III (HYDRO-BIOS, Altenholz, Germany) (see Supplementary Information
380 1 for a global view of the experimental design). Sampling was performed monthly, except from
381 27th May to 20th June 2019 when it was done twice a week. Temperature and dissolved oxygen
382 were measured along the water column using an oxycal-SL 197 multiparameter probe (WWT,
383 Limonest, France). Phytoplankton biomass was estimated using a submersible fluorescence
384 photometer (Fluoroprobe; BBE-Moldaenke, Kiel, Germany) (see Supplementary Information 2 for
385 temperature, oxygen and phytoplankton biomass data).

386 14 litres of water were filtered successively through 150 µm and 10 µm pore-size filters in order to
387 concentrate Microsporidia spores (<10 µm fraction, microsporidian spores being mainly <10 µm in
388 length (Murareanu *et al.*, 2021)) and planktonic species susceptible to host Microsporidia (10-150
389 µm fraction). Part of the microbial biomass of each size-fraction was collected on 0.8-µm-pore-size
390 polycarbonate filters (Millipore, Burlington, MA) at a very low vacuum to prevent cell damage
391 (<15 kPa) and stored at -80°C until nucleic acids extraction (dry-stored filters for DNA extraction,
392 in RLT buffer (RNeasy Mini kit, Qiagen, Hilden, Germany) with 1% 2-mercaptoethanol for RNA
393 extraction). Two filters were produced and subsequently processed for each sample (Supplementary
394 Information 1). The other part of the microbial biomass obtained was fixed with paraformaldehyde
395 (final concentration, 2%) and incubated at 4°C for 24 h. Fixed cells were then collected on 0.8-µm-
396 pore-size polycarbonate filters (Millipore; pressure <15 kPa). Filters were preserved by dehydration

397 in an ethanol series (50, 80, and 100% ethanol for three min each) and stored at -20°C until TSA-
398 FISH staining.

399

400

401 *Nucleic acids extraction, amplification and sequencing*

402

403 Filters prepared for DNA extraction were processed as described in Dubuffet *et al.* (2021), while
404 filters prepared for RNA extraction were processed with the RNeasy Mini kit with some
405 modifications. Samples underwent a first grinding of three cycles, 30 Hz 30 s (Bead Beater Retsch,
406 Haan, Germany), the supernatant was kept. 400 µl of RLT buffer with 1% 2-mercaptoéthanol and
407 0.1 g of 0.1 mm glass beads (Sigma-Aldrich, Saint-Louis, MO) were added and a second grinding
408 was carried out under the same conditions, the supernatant was pooled with the first one. Protocol
409 was then carried out according to the manufacturer's recommendations, with a triple treatment with
410 DNase I. RNA was finally eluted in 30 µl of RNase free water. Quality and quantity of RNA was
411 analysed with the RNA screen Tape kit and the 2200 Tape station (Agilent Technologies, Santa
412 Clara, CA) and absence of DNA was checked by PCR. cDNA was obtained by reverse transcription
413 with SuperScript III and randoms primers (Invitrogen, Waltham, MA) according to the
414 manufacturer's recommendations.

415 Amplification of the V1-V3 region of the microsporidian gene coding for the 18S rRNA was
416 performed using the universal microsporidian V1F (CACCAGGTTGATTCTGCCTGAC) and
417 530R (CCGCGGCTGCTGGCAC) primers set (Baker *et al.*, 1994; Weiss *et al.*, 1994). Each PCR
418 was performed in a total volume of 30 µL containing 6 µL of 5x Green GoTaq Flexi buffer, 2.4 µL
419 of 25 mM MgCl₂, 0.18 µL of GoTaqG2 Flexi DNA Polymerase (Promega, Madison, WI, 5 U/µL),
420 0.6 µL of 10 mM each dNTP, 0.3 µL of 50 mg/mL BSA and 0.6 µL of each 10 µM primer. The
421 amplification conditions consisted of initial denaturation at 95°C for 5 min followed by 35 cycles

422 for 1 min at 95°C, 1 min 30 s at 62°C and 1 min 30 s at 72°C, and a final elongation of 7 min at
423 72°C.

424 Amplification of the V4 region of total eukaryotes (excluding Microsporidia) was performed using
425 the universal primer 515F (GTGYCAGCMGCCGCGGTA) (Caporaso *et al.*, 2011) and the
426 eukaryotic primer 951R (TTGGYRAATGCTTTCGC) (Mangot *et al.*, 2013). Each PCR was
427 performed in the mix described above. The amplification conditions consisted of initial denaturation
428 at 95°C for 5 min followed by 33 cycles for 1 min at 95°C, 1 min at 55°C and 1 min 30 s at 72°C,
429 and a final elongation of 10 min at 72°C.

430 The amplicons were purified and concentrated using the QIAquick Gel Extraction kit (Qiagen).
431 Samples were then sequenced on a MiSeq Illumina instrument (2x250 bp) (Biofidal, Vaulx-en-
432 Velin, France).

433

434

435 *Amplicons analysis and taxonomic affiliation*

436

437 The PANAM2 (Phylogenetic Analysis of Next-generation AMPlicons v2 - [https://github.com/meb-](https://github.com/meb-team/PANAM2)
438 [team/PANAM2](https://github.com/meb-team/PANAM2)) pipeline (Taib *et al.*, 2013) was used to process the sequencing data. The clean-up
439 procedures consisted of eliminating sequences with ambiguous 'N' bases, those below 200 bp and
440 chimeras. The sequences were then assembled and clustered into Operational Taxonomic Units
441 (OTUs) with a similarity threshold of 99% for Microsporidia (Dubuffet *et al.*, 2021) and 95% for
442 other eukaryotes (Mangot *et al.*, 2013; Debroas *et al.*, 2017). Finally, representative sequences of
443 each OTU were affiliated by similarity and phylogeny using a custom curated reference database
444 (Freshwater-database: <https://github.com/panammeb/Freshwater-database>). This database contains
445 eukaryote references sequences extracted from the SSU Ref SILVA database (Pruesse *et al.*, 2007),
446 typical freshwater lineages identified in previous studies (*e.g.* Debroas *et al.*, 2015) and 528

447 sequences of 265 microsporidian species phylogenetically classified into clades, groups and genera
448 (Dubuffet *et al.*, 2021).

449 For Microsporidia, affiliations to groups and genera were considered as valid when the identity
450 scores were above 85% and 94.5%, respectively (Dubuffet *et al.*, 2021). As clustering threshold
451 used (especially for Microsporidia, *i.e.* 99%) may generate OTUs resulting from sequencing errors,
452 we only kept for the final analysis OTUs representing more than 0.005% (Bokulich *et al.*, 2013) of
453 the total reads and OTUs present in at least two samples. Data were normalized using the SRS
454 (Scaling with Ranked Subsampling) method (Beule and Karlovsky, 2020).

455

456

457 *Phylogenetic analysis of OTUs non-affiliated beyond the clade*

458

459 All microsporidian OTUs obtained in this analysis were assigned at least to a clade, 78% to a group
460 and 61% to a genus. We therefore performed phylogenetic analyses on OTUs non-affiliated beyond
461 the clade (including those with identity scores below 85%) or beyond the group (including those
462 with identity scores below 94.5%) in order to determine whether new groups (which roughly
463 correspond to families) or new subgroups (similar to genera) could be highlighted. We focused our
464 analyses on abundant OTUs (>500 reads, 619 OTUs), which represent 91% of the total reads. We
465 included in these analyses several sequences from undescribed microsporidian species (found
466 through BLAST search and/or bibliographic review), in addition to species from the database
467 previously used. After an alignment using the MAFFT algorithm (Katoh *et al.*, 2005), ML trees
468 were obtained using a General Time-Reversible (GTR) model of evolution with gamma-distributed
469 rate heterogeneity (G) and a proportion of invariable sites (I) (GTR + G + I), with 200 bootstrap
470 replications and partial deletion (85%), using MEGA 7 (Kumar *et al.*, 2016).

471

472

473 *Diversity analyses*

474

475 The rRNA:rDNA ratio was calculated for each OTU by dividing the number of RNA reads by the
476 number of DNA reads. Diversity analyses were performed using R (R Development Core Team,
477 2012). Community richness and diversity indices (Richness observed, Chao1, Shannon and
478 Simpson) were used to infer the taxa richness of Microsporidia. These estimators were computed
479 with the phyloseq package (McMurdie and Holmes, 2013) as well as the non-metric
480 multidimensional scaling (NMDS) ordination based on a Bray-Curtis dissimilarity matrix (Bray and
481 Curtis, 1957; Paliy and Shankar, 2016). To investigate the potential relationships between the
482 microsporidian community composition and biotic and abiotic factors, canonical correspondence
483 analysis (CCA) was used (ter Braak, 1986) with the vegan package (Oksanen *et al.*, 2020).

484

485

486 *Co-occurrence network*

487

488 Using statistical tools, co-occurrence networks are used to detect *in situ* associations between
489 micro-organisms (Chaffron *et al.*, 2010, Lima-Mendez *et al.*, 2015). We therefore used this
490 approach to depict potential host-parasite associations between Microsporidia and other eukaryotes.
491 We focused the analysis on the large size fraction (10-150 μm , 40 samples) which might contain
492 Microsporidia associated to potential new hosts among zooplankton and protists. DNA and RNA
493 samples were used in order to have a sufficient number of samples. OTUs with a total abundance
494 higher than 0.05% (206 microsporidian and 148 eukaryotic OTUs) were grouped and analysed to
495 build a co-occurrence network with SPIEC-EASI (SParse Inverse Covariance Estimation for
496 Ecological Association Inference (Kurtz *et al.*, 2015)). Raw data were used (before normalization),

497 as the SpiecEasi function includes a standardisation step (centered log-ratio transformation) to
498 alleviate compositionality bias (Aitchison, 1981). The co-occurrence network was inferred using the
499 neighborhood selection (Meinshausen and Bühlmann, 2006). We used the SPIEC-EASI parameters
500 determined as those generating the sparser graphs by Benoiston (2019). Thus, the scaling factor
501 (λ_{\min}) was set at 0.001, the number of tested λ (n_{λ}) was set as 20 and the
502 number of StARS subsamples (rep_{num}) was set to 20. In this network, OTUs are represented by
503 nodes and a positive association between OTUs are represented by edges between them (only
504 positive associations were kept in the output graph). Positive edges identified were extracted to
505 visualize only associations between microsporidian genera/subgroups and eukaryotic phyla with a
506 Sankey diagram using the networkD3 package on R (R Development Core Team, 2012; Allaire *et*
507 *al.*, 2017).

508

509

510 *TSA-FISH (Tyramide Signal Amplification-Fluorescent In Situ Hybridization)*

511

512 To visualize potential microsporidian-hosts associations, TSA-FISH was performed, using a probe
513 designed to target all the microsporidian OTUs identified in this metabarcoding analysis (USP01
514 probe, 5' CCAGGTTGATTCTGCCTGAC 3'). Hybridization conditions were determined
515 beforehand on two microsporidian species cultured in the laboratory: *Anncaliia algerae* and
516 *Encephalitozoon cuniculi*. The former was considered as a non-target species, as it had one
517 mismatch with the USP01 probe, while the later was a target species (no mismatches). At 45°C and
518 10% deionized formamide, *in situ* hybridization signals were obtained on the target species and not
519 on the non-target species (not shown), and these hybridization conditions were thus retained.

520 TSA-FISH was performed on each sample (22 sampling dates) of the large size fraction, in
521 duplicate, as described in Lepère *et al.* (2016) with the following modifications: hybridization

522 buffer (10% deionized formamide), hybridization at 45°C overnight, washing steps at 47°C with
523 washing buffer (450 mM NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl [pH 7.5]). The
524 filters were mounted in a mixture containing antifading reagent AF1 (Citifluor; Electron
525 Microscopy Sciences, Hatfield, PA), Vectashield fluorescent mounting medium (Vector
526 laboratories, Burlingame, CA) and 4',6-diamidino-2-phenylindole (DAPI).
527 Hybridized cells were visualized under two fluorescence microscopes: Leica DM IRB (Leica
528 Microsystems, Wetzlar, Germany) and Zeiss Axio Imager 2 (Carl Zeiss, Oberkochen, Germany).

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Competing Interests

The authors declare they have no competing interests.

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Figure Legends

536 Fig.1. Taxonomic affiliation of the microsporidian OTUs (DNA and RNA samples).

537 Inner circle represents clades (IV2, I, IV1, VIII and V). Deeper taxonomic ranks (groups and
538 genera/subgroups) are presented in outer circles. Group names are given in capital letters and
539 genera names in italic. Groups and subgroups defined during phylogenetic analyses are given in
540 bold. Hatched patterns represent OTUs unaffiliated beyond the group or clade. Genera/subgroups
541 listed are ranked from most to least abundant.

542 See Supplementary Information 5 for the taxonomic affiliation of the microsporidian OTUs
543 following the analysis on PANAM2 (before the phylogenetic analyses).

544

545 Fig.2. Relative abundances of the most abundant (>0.5% of the total reads) microsporidian
546 genera/subgroups across DNA samples.

547 The white zone on the top of each bar represents the cumulative proportion of other
548 genera/subgroups with low abundances.

549 Label = sampling date (DD/MM/YY), from 19/11/2018 (left) to 07/01/2020 (right) with a monthly
550 sampling and additional sampling every 3 to 5 days in May and June 2019

551 See Supplementary Information 13 for relative abundances of the less abundant (<0.5% of the total
552 reads) microsporidian genera/subgroups across samples.

553

554 Fig.3. Relative abundances of the most abundant (>0.5% of the total reads) eukaryotic phyla across
555 DNA samples.

556 The white zone on the top of each bar represents the cumulative proportion of other phyla with low
557 abundances.

558 Label = sampling date (DD/MM/YY), from 19/11/2018 (left) to 07/01/2020 (right) with a monthly
559 sampling and additional sampling every 3 to 5 days in May and June 2019

560 See Supplementary Information 14 for relative abundances of the less abundant (<0.5% of the total
561 reads) eukaryotic phyla across samples.

562

563 Fig.4. Sankey diagram showing the edges between microsporidian OTUs (left) and eukaryotic
564 OTUs (right) in the co-occurrence network.

565 Microsporidian OTUs are grouped by genus/subgroup, eukaryotic OTUs are grouped by phylum or
566 class. 88 microsporidian OTUs and 98 eukaryotic OTUs were involved in the network with 213
567 positive associations (edges). One line represents one edge from the co-occurrence graph. Co-
568 occurrences involving eukaryotic OTUs not affiliated to a phylum are not shown.

569

570 Fig.5. Microsporidia (targeted by TSA-FISH with the USP01 probe) infecting a rotifer (genus
571 *Kellicottia*).

572 Under UV showing nucleus after DAPI staining (a), under blue light showing Microsporidia in
573 green fluorescence (b), overlay (c). Magnification on microsporidian cells inside the rotifer (UV (d),
574 blue light (e), overlay(f)).

575 Scale bars, 10 μ m

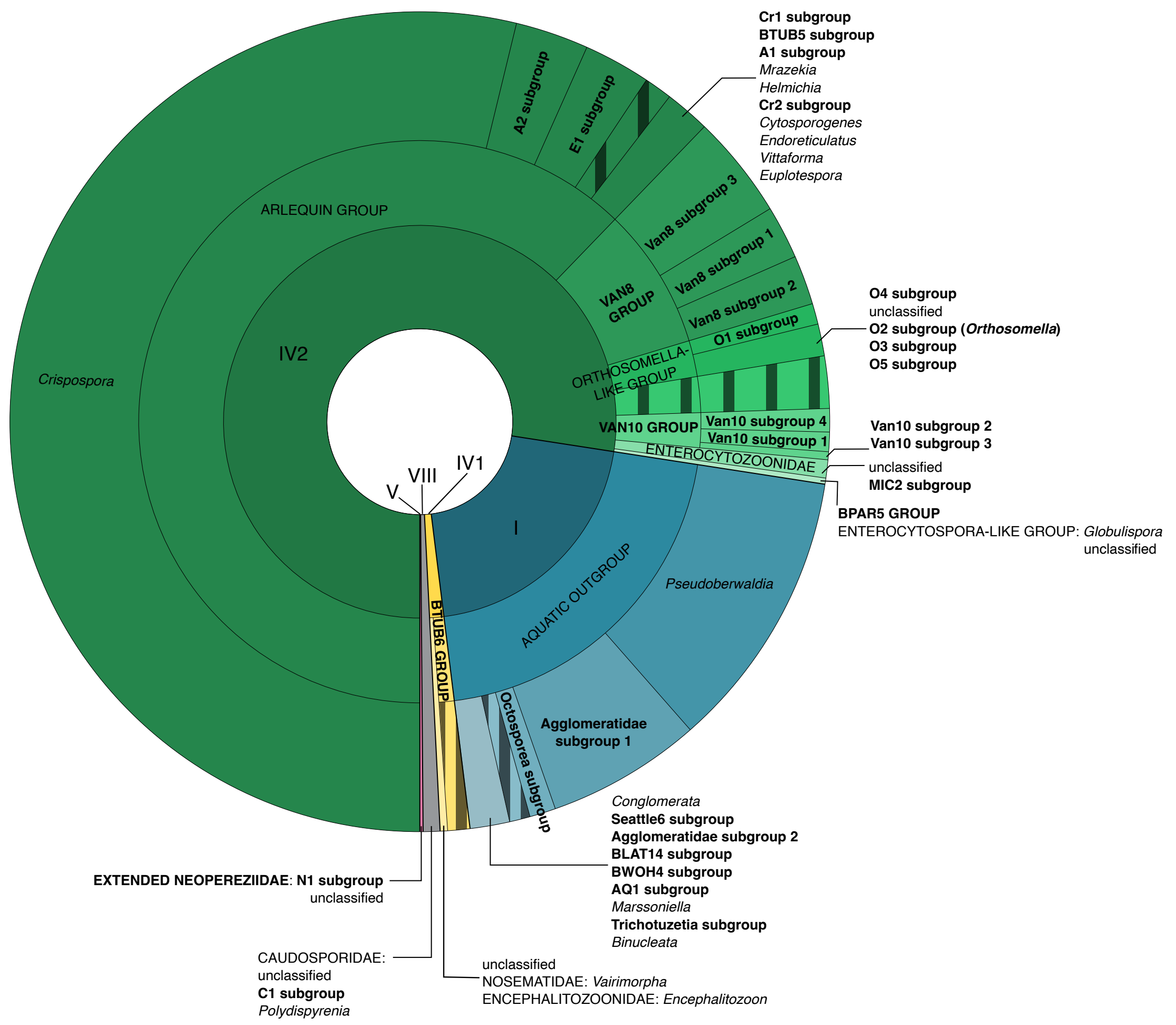
576

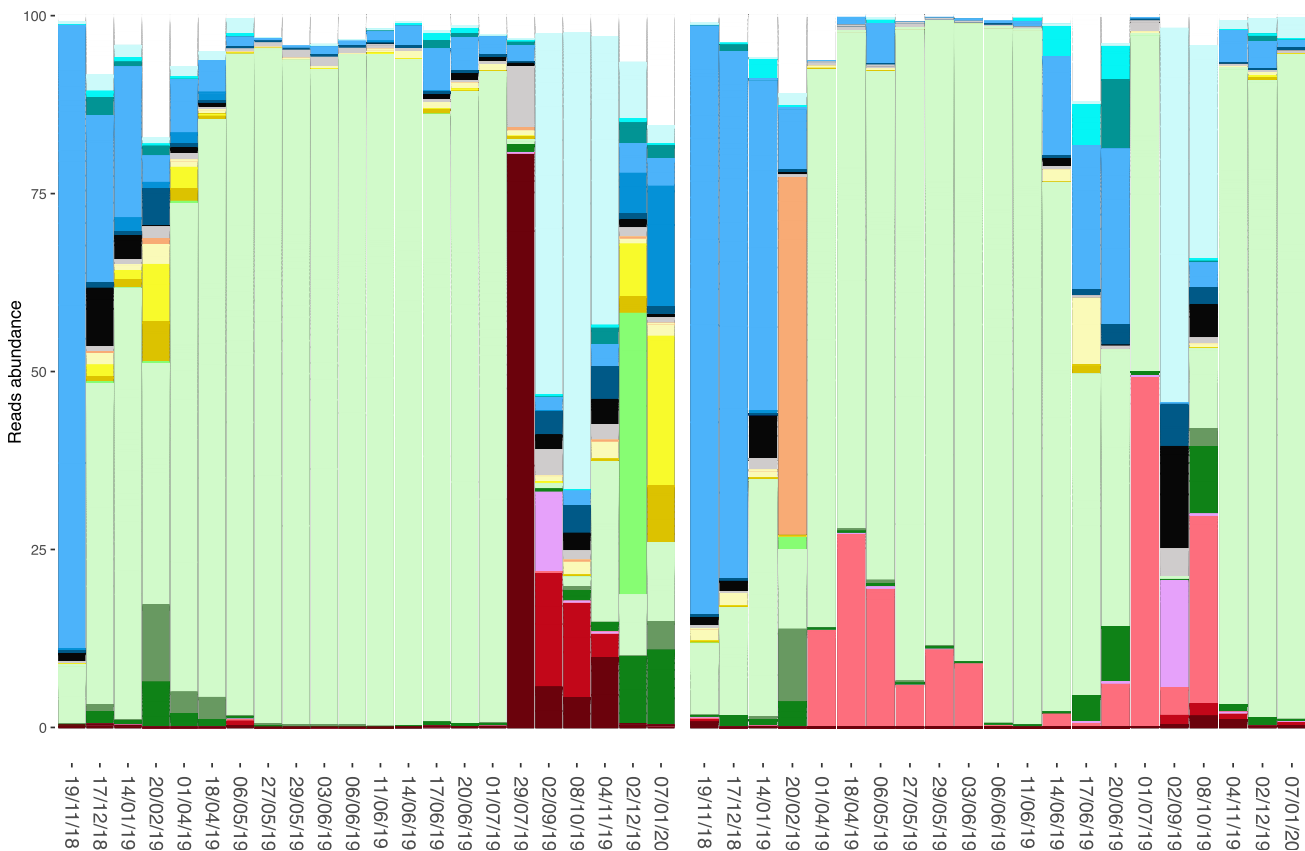
577 Fig.6. Microsporidia (targeted by TSA-FISH with the USP01 probe) attached to a diatom (genus
578 *Asterionella*) (a, b, c) and infecting a ciliate (genus *Stentor*) (d, e, f).

579 Under UV showing nucleus after DAPI staining (a, d), under blue light showing Microsporidia in
580 green fluorescence (b, e), overlay (c, f).

581 Scale bars, 10 μ m

582



0.8-10 μm 10-150 μm 

Other genera/subgroups with total abundance <0.5%

Clade IV1

BTUB6 group ; unclassified

Clade I

Aquatic outgroup ; Agglomeratidae subgroup 1

Aquatic outgroup ; *Conglomerata*

Aquatic outgroup ; Octosporea subgroup

Aquatic outgroup ; *Pseudoberwaldia*

Aquatic outgroup ; Seattle6 subgroup

Aquatic outgroup ; unclassified

Clade IV2

unclassified

Enterocytozoonidae ; unclassified

Orthosomella-like group ; O1 subgroup

Orthosomella-like group ; O4 subgroup

Orthosomella-like group ; unclassified

Arlequin group ; Cr1 subgroup

Arlequin group ; *Crispospora*

Arlequin group ; E1 subgroup

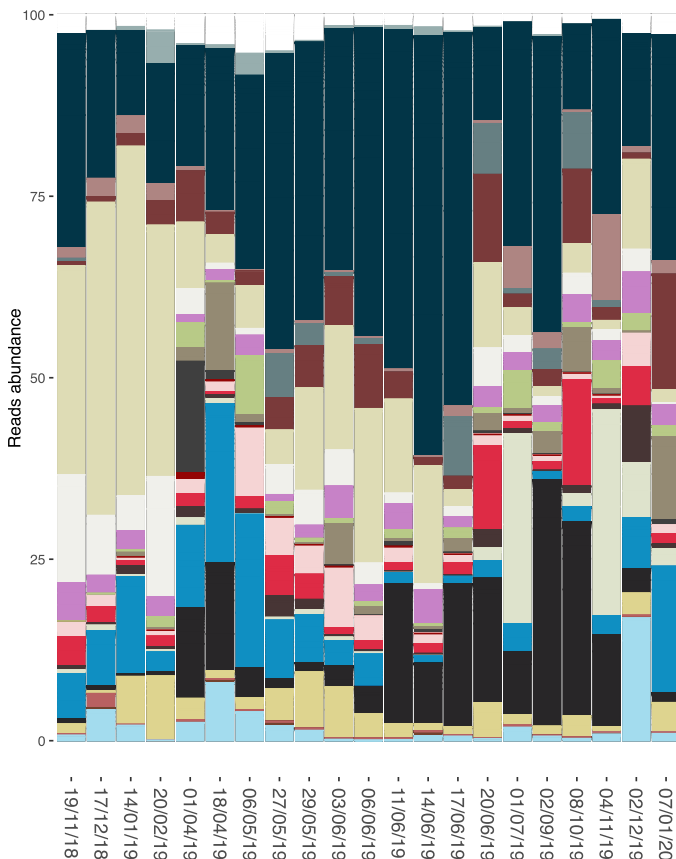
Arlequin group ; unclassified

Van10 group ; subgroup 4

Van8 group ; subgroup 1

Van8 group ; subgroup 2

Van8 group ; subgroup 3

0.8-10 μm 10-150 μm 