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► **To cite this version:**

Marina Chauvet, Arthur Monjot, Anne Mone, Cécile Lepère. Single Cell Analysis Reveals a New Microsporidia-Host Association in a Freshwater Lake. *Microbial ecology*, In press, 10.1007/s00248-022-02023-7. hal-03666982

HAL Id: hal-03666982

<https://hal.science/hal-03666982v1>

Submitted on 13 May 2022

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1 **Single cell analysis reveals a new Microsporidia-host**
2 **association in a freshwater lake**

3 **Marina Chauvet^{1*}, Arthur Monjot^{1*}, Anne Moné¹, Cécile Lepère^{1o}**

4 ¹ CNRS, Laboratoire Microorganismes : Génome et Environnement, Université Clermont

5 Auvergne, Clermont-Ferrand, F-63000, France.

6 * These authors contributed equally to this work

7 ^o Corresponding author: cecile.lepere@uca.fr

8
9 ORCID

10 Marina Chauvet: <https://orcid.org/0000-0002-6380-6162>

11 Arthur Monjot: <https://orcid.org/0000-0002-6978-4785>

12 Anne Moné: <https://orcid.org/0000-0002-8686-703X>

13 Cécile Lepère: <https://orcid.org/0000-0003-4767-0477>

14
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16 **Acknowledgements**

17 The authors are very grateful to Jonathan Colombet and Eugénie Carriere for their help with
18 sampling. We also gratefully acknowledge the platform CLIC (CLermont-ferrand Imagerie
19 Confocale, Université Clermont Auvergne) and especially Caroline Vachias for her assistance
20 with the confocal microscopy. The work of MC and AM was supported by two PhD fellowships
21 funded by the ‘Ministère de l’Enseignement Supérieur de la Recherche et de l’Innovation’.

23 **Abstract**

24

25 Microsporidia are a large group of obligate intracellular eukaryotic parasites. Recent studies
26 suggest that their diversity can be huge in freshwater lake ecosystems especially in the <150 µm
27 size fraction. However, little is known about their hosts and therefore their impact on the trophic
28 food web functioning. In this study, single cell analysis and fluorescence microscopy were used
29 to detect new host-parasite association within rotifer communities in lake Aydat (France). Our
30 analysis showed the existence of a potential new species belonging to the *Crispospora* genus
31 able of infecting the rotifer *Kellicottia* with a high prevalence (42.5%) suggesting that
32 Microsporidia could have a great impact on the rotifer populations regulation in lakes.

33

34 **Keywords**

35 Microsporidia, host-parasite association, single cell, TSA-FISH, lake, microbial ecology

36

37 Microsporidia are obligate intracellular eukaryotic parasites related to Fungi. They are mostly
38 known to parasitize a broad range of hosts among the animal kingdom in different environments
39 [1]. Recent metabarcoding studies revealed an unexpected diversity in lake ecosystems
40 especially in the <150 µm size fraction suggesting that Microsporidia could have an impact on
41 the planktonic communities at the base of the food web [2, 3]. However, they have rarely been
42 described as parasites of protists and microzooplankton. In marine environments, they are
43 mainly known to parasite gregarines (e.g. [4]), but they were also shown in association with
44 Paramyxea [5]. In freshwaters, only a few observations showed Microsporidia parasiting
45 ciliates with only two microsporidian species characterized by morphological and molecular
46 approaches (*Euplotespora binucleata* and *Globosporidium paramecii* parasitizing the ciliates
47 *Euplotes woodruffi* and *Paramecium aurelia*, respectively [6, 7]). Among microzooplankton,
48 Microsporidia have been observed in the rotifers *Brachionus calyciflorus* as well as in
49 *Asplanchna priodonta* and *A. brightwellii* in both river and freshwater lakes [8, 9] however
50 these observations (no molecular data) remain sporadic and for most at low prevalence.
51 Moreover, with co-occurrence network and microscopic observations, a more recent study
52 suggested that Microsporidia could infect a broader range of rotifers [3]. We therefore used, in
53 this study, single cell analysis as well as Tyramide Signal Amplification – Fluorescent *In Situ*
54 Hybridization (TSA-FISH) to detect new host-parasite association within microzooplankton
55 communities in a lake ecosystem (lake Aydat, Massif Central, France) and especially (i)
56 confirm the hypothesis raised about the broader range of rotifers parasitized by Microsporidia
57 and (ii) characterize by molecular information and microscopic observation both partners.

58 Zooplankton was sampled with vertical tows of 20 µm-mesh plankton net at the central point
59 of the lake (depth = 14 m) on the 18/05/2021 (see Supplementary Material). 17 rotifers,
60 identified morphologically as probably belonging to the genera *Kellicottia* (n = 13) and
61 *Keratella* (n = 4), were isolated by micromanipulation and individually placed in a 96 wells

62 plate before Whole Genome Amplification (WGA) (see Supplementary Material). The Single
63 Amplified Genomes (SAGs) were then screened for the presence of Microsporidia by the
64 amplification of the V1-V3 region of the microsporidian gene coding for the SSU rRNA
65 according to Chauvet *et al.* [3]. The sequences obtained after cloning and sequencing were
66 compared to those available in public databases and included in a phylogenetic tree (see
67 Supplementary Material). This analysis showed that on the 17 isolated rotifers, 6 *Kellicottia*
68 had a positive PCR amplification signal with Microsporidia primers however no Microsporidia
69 amplification was recorded in *Keratella*. All Microsporidia sequences were affiliated to the
70 genus *Crispospora* (Fig. 1) which is affiliated to the clade IV2; Arlequin group (defined in
71 Dubuffet *et al.* [2]), and well represented (~ 50% of the total OTUs) in metabarcoding studies
72 conducted in lake Aydat [2, 3]. With 97% identity with *Crispospora chironomi* (the only known
73 species of the genus so far) these sequences could represent a new species of the *Crispospora*
74 genus (Fig. S2a). Indeed, although thresholds for species delineation may vary, an identity close
75 to 100% is suggested for taxa separation at the species level for Microsporidia [2, 11].
76 *Crispospora chironomi* is known to parasite chironomids eggs [12] that can be found in aquatic
77 environments especially when they are eutrophic and anthropized [13].
78 On the Microsporidia positive SAGs, rotifers were characterized by amplification of the COI
79 using the 30F and 885R primers set according to Zhang *et al.* [14] with some modifications (see
80 Supplementary Material). The rotifer affiliation analysis revealed that this potential new species
81 of *Crispospora* is able to parasite the genus *Kellicottia* (Fig. S1). Even though *Kellicottia*
82 sequences grouped into two clades in the phylogenetic analysis, species determination is
83 challenging due to high intraspecific variation of identity percent which can be higher than 20%
84 for rotifers (Fig. S2b) [15].
85 To visualize and quantify Microsporidia-rotifer associations, TSA-FISH was performed on the
86 rest of the plankton net sample, using a Microsporidia specific probe (USP01) according to

87 Chauvet *et al.* [3] (see Supplementary Material). Positive signals were obtained in *Kellicottia*
88 rotifers with a surprisingly high prevalence (42.5%, total rotifers observed = 73) (Fig. 2).
89 Microsporidia were so far recorded in a small number of rotifers [8, 9]. *Microsporidium* sp. and
90 *Microsporidium asperspora* have been observed in *Brachionus calyciflorus*, and
91 *Microsporidium polygona* in *Asplanchna priodonta* and *A. brightwellii* in freshwater
92 ecosystems most of the time at low prevalence (*e.g.* *Brachionus calyciflorus* by *Microsporidium*
93 sp. on 1.2% of the host population, [9]). However, these descriptions were only morphological
94 and neither the site of infection nor the microsporidian spore or intracellular form were
95 described. Here, we identified an infection which could be in the rotifer's stomach or
96 pseudocoelom. The intracellular forms of the Microsporidia were on average 1.53 μm long,
97 most being oval, some spherical. These findings were consistent with the described shape of
98 the only known *Crispospora* species (*i.e.* *Crispospora chironomi*), with two shapes identified:
99 short oval and spherical, with a size of 1.5 x 2.5 μm and 1.5 x 2 μm , respectively [12].
100 This finding is highly relevant since Microsporidia, along with hyphomycetes and oomycetes
101 [17, 18] could be an important regulator of these populations in lakes. Rotifers are indeed an
102 important component of plankton in aquatic ecosystems playing fundamental role in both
103 microbial loop and classical food web and as a link in energy flow between primary producers
104 (algae, cyanobacteria) and higher trophic levels (zooplankton, shellfish, fish) [16]. *Kellicottia*
105 can reach 600 ind./l in lake Aydat and is the most abundant rotifers along with *Keratella* [10].
106 Microsporidia sequences retrieved during this study had an identity >99% with an highly
107 abundant OTU identified in the same lake in a previous study where the temporal dynamic of
108 the Microsporidia diversity was analyzed over one year [3] (Fig. S2a). This *Crispospora* OTU
109 and the only OTU affiliated to *Kellicottia* presented the same pattern of temporal variations
110 (data not shown). This co-variation and the high prevalence of this association suggest an
111 impact on the dynamic of *Kellicottia* in the lake however this needs to be confirmed for example

112 by the culture of both partners which will also allow the characterization of the developmental
113 cycle of this potential new species of Microsporidia.

114

115 **References**

116

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179

180 **Statements and Declarations**

181

182 **Funding**

183 The authors declare that no funds, grants, or other support were received during the
184 preparation of this manuscript.

185

186 **Competing Interests**

187 The authors declare they have no competing interests.

188

189 **Availability of data and material**

190 All data and material are available in this publication and additional information online
191 (supplementary material and supplementary figures). Sequences are available on GenBank,
192 accession numbers: from ON026091 to ON026096 for the microsporidian sequences and from
193 ON033621 to ON033624 for the rotifers.

194 **Figure Legends**

195

196 **Fig. 1** Maximum Likelihood phylogeny of microsporidian sequences.

197 We included in this phylogeny several sequences from undescribed microsporidian species
198 (found through BLAST search) and a sequence found extensively in a previous study (in italic,
199 [3]), in addition to described species, all belonging to the Arlequin group (clade IV2).
200 Sequences from this study are given in bold. Outgroup species are representative species of
201 other groups belonging to clade IV2. Numbers next to branches indicate the ML bootstrap
202 support (100 replicates).

203

204 **Fig. 2** Microsporidia (targeted by TSA-FISH with the USP01 probe, [3]) infecting a rotifer
205 (genus *Kellicottia*).

206 Under UV showing nucleus after DAPI staining (a), under blue light showing Microsporidia in
207 green fluorescence (b), overlay (c). Magnification on microsporidian cells inside a rotifer with
208 a confocal microscope (UV (d), blue light (e), overlay (f)).

209 Scale bars, 20 μm

210



