bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1 # Outwitting planarian's antibacterial defence mechanisms: 2 *Rickettsiales* bacterial trans-infection from *Paramecium* 3 *multimicronucleatum* to planarians

4

5 Letizia Modeo[1*], Alessandra Salvetti[2*], Leonardo Rossi[2], Michele Castelli[3], Franziska Szokoli[4],
6 Sascha Krenek[4,5], Elena Sabaneyeva[6], Graziano Di Giuseppe[1], Sergei I. Fokin[1,7], Franco Verni[1],
7 Giulio Petroni[1]

8

9 [1]Department of Biology, University of Pisa, Pisa, Italy
10 [2]Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
11 [3]Centro Romeo ed Enrica Invernizzi Ricerca Pediatrica, Department of Biosciences, University of
12 Milan, Milan, Italy
13 [4]Institute of Hydrobiology, Dresden University of Technology, Dresden, Germany
14 [5]Department of River Ecology, Helmholtz Center for Environmental Research - UFZ, Magdeburg,
15 Germany
16 [6]Department of Cytology and Histology, Faculty of Biology, Saint Petersburg State University,
17 Saint Petersburg, Russia
18 [7]Department of Invertebrate Zoology, Saint Petersburg State University, Saint Petersburg, Russia

19

20 *Corresponding authors
21 letizia.modeo@unipi.it (LM); alessandra.salvetti@unipi.it (AS)

22

23 RUNNING PAGE HEAD: *Rickettsiales* macronuclear endosymbiont of a ciliate can transiently
24 enter planarian tissues

25

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

26 ORCID ID
27 Letizia Modeo: 0000-0003-2291-7218
28 Alessandra Salvetti: 0000-0002-9154-9773
29 Leonardo Rossi: 0000-0002-1143-8695
30 Michele Castelli: 0000-0003-2177-5772
31 Sascha Krenek: 0000-0002-5062-4140
32 Elena Sabaneyeva: 0000-0002-7939-8263
33 Graziano Di Giuseppe: 0000-0002-9999-7650
34 Sergei Fokin: 0000-0002-7329-5837
35 Franco Verni: 0000-0003-3537-5024
36 Giulio Petroni: 0000-0001-9572-9897

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

37 # Abstract

38 Most of the microorganisms belonging to genera responsible for vector-borne diseases (VBD) have
39 hematophagous arthropods as vector/reservoir. Recently, many new species of microorganisms
40 phylogenetically related to agents of VBD were found in a variety of aquatic eukaryotic hosts, in
41 particular, numerous new bacterial species related to the genus *Rickettsia* (*Alphaproteobacteria*,
42 *Rickettsiales*) were discovered in protist ciliates and other unicellular eukaryotes. Although their
43 pathogenicity for humans and terrestrial animals is not known, these bacteria might act as
44 etiological agents of possible VBD of aquatic organisms, with protist as vectors. In the present
45 study, we characterized a novel strain of the *Rickettsia*-Like Organism (RLO) endosymbiont
46 "*Candidatus* (*Ca.*) Trichorickettsia mobilis" in the macronucleus of the ciliate *Paramecium*
47 *multimicronucleatu*m through Fluorescence *In Situ* Hybridization (FISH) and molecular analyses.
48 Ultrastructural investigations on the presence of flagella confirmed previous studies on the same
49 bacterial species. The potential trans-infection per *os* of this bacterium to planarians (*Dugesia*
50 *japonica*), a widely used model system able to eliminate a wide range of bacteria pathogenic to
51 humans and other Metazoa, was further verified. Ciliate mass cultures were set up, and trans-
52 infection experiments were performed by adding homogenized paramecia to food of antibiotic-
53 treated planarians, performed. Treated and non-treated (i.e. control) planarians were investigated at
54 day 1, 3, and 7 after feeding for endosymbiont presence by means of PCR and ultrastructural
55 analyses. Obtained results were fully concordant and suggest that this RLO endosymbiont can be
56 transferred from ciliates to metazoans, being detected up to day 7 in treated planarian enterocytes
57 inside and, possibly, outside phagosomes.

58

59 KEY WORDS Planarians, molecular and ultrastructural investigations, *Rickettsiaceae,* RLO
60 endosymbiont, trans-infection experiments, vector-borne disease

61

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

62 # Introduction

63 Bacteria of order *Rickettsiales* (*Alphaproteobacteria*) live in an obligate association with a wide range
64 of eukaryotes [1-8], and, for their vast majority, are localized intracellularly, although the case of an
65 extracellular *Rickettsiales* bacterium was recently documented [9]. *Rickettsiales* are widely studied
66 for their involvement in medical and veterinary fields. Indeed, many of them (e.g. *Rickettsia* spp.,
67 *Anaplasma* spp., *Ehrlichia* spp., and *Orientia tsutsugamushi*) are vectored by lice, ticks and mites,
68 and cause mild to severe disease [1, 10], such as epidemic typhus, Rocky Mountain spotted fever [11-
69 13], anaplasmosis, ehrlichiosis [14, 15], heartwater [16], and scrub typhus [17].

70 In the last two decades, many new genera and species of *Rickettsiales* were found as symbionts
71 in a variety of other non-vector eukaryotic hosts, both from terrestrial and aquatic environments
72 [reviewed in 3, 4, 7]. In particular, numerous such novel bacterial species were retrieved in aquatic
73 protists [18-22], including, notably, parasitic (*Ichthyophthirius multifiliis*) [23, 24] and various free-
74 living ciliates (e.g. *Paramecium* spp., *Euplotes* spp., *Diophrys oligothrix*, *Pseudomicrothorax dubius*,
75 *Spirostomum minus*, and *Colpidium striatum*) [25-33].

76 Such findings have clearly indicated the ability of those "aquatic" *Rickettsiales* to perform
77 horizontal transmission including host species shift, although mostly from indirect evidence, i.e.
78 closely related bacteria in hosts as different as ciliates, hydra [34], corals [35], ring worms [36],
79 ascidians [37, 38], as well as incongruent host and symbionts phylogenies [3, 39, 40]. In few cases,
80 direct evidence in experimental interspecific transfers between unicellular hosts was produced [19,
81 29].

82 In most cases the relationships between *Rickettsiales* associated with aquatic eukaryotes and
83 their hosts were not clarified in detail, as in general no evident effect on host biology was observed
84 [7], with the exception of "*Candidatus* (*Ca*.) Xenohaliotis californiensis", which is considered the
85 cause of the withering syndrome in its abalone hosts [41, 42]. It has been hypothesized by different
86 authors that bacteria, possibly including *Rickettsiales*, harboured by aquatic protists might constitute
87 etiological agents of possible Vector Borne Diseases (VBD) of aquatic animals [55, 61]. Indeed,

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

88 protists could be the potential vectors for some of the numerous cases of epidemics caused by
89 *Rickettsia*-Like Organisms (RLOs; i.e. intracellular bacteria morphologically similar to *Rickettsia*)
90 determining an increasing number of massive deaths in intensive aquaculture facilities during the last
91 years [62], including molluscs [41, 43, 44], crustaceans [45- 47) and fishes [48-50]. Although many
92 RLOs were actually shown to be phylogenetically unrelated to *Rickettsiales* (e.g.
93 *Gammaproteobacteria*) [51, 52, 46], at least in one case available data indicate a probable connection
94 between a truly *Rickettsiales* bacterium and fish disease. In detail, a "*Ca*. Midichloria mitochondrii"-
95 related bacterium was linked to the red-mark syndrome in rainbow trout [53-54]. Most importantly,
96 although a transmission route was not directly proven, the same bacterium was found in association
97 with the ciliate *I. multifiliis* [24], which is indeed a fish parasite. Interestingly, even other *Rickettsiales*
98 symbionts ("*Ca*. Megaira" genus) can be found in the same ciliate host species, which might suggest
99 a potential transmission route for these symbionts as well [23, 24].

100 Taking into account such premises, in order to investigate the hypothesis that protists can act
101 as natural reservoir for potentially pathogenic bacteria [25, 55-61], we experimentally tested for the
102 first time the possibility that endosymbionts of ciliates could be transferred to aquatic Metazoa. The
103 *Rickettsiales* "*Ca.* Trichorickettsia" was chosen as candidate for trans-infection experiments as it
104 shows a broad ciliate host range, infecting different cell compartment (i.e. cytoplasm and nucleus) of
105 *Paramecium multimicronucleatum*, *Paramecium nephridiatum*, *Paramecium calkinsi* and *Euplotes*
106 *aediculatus* [28, 63]. Additionally, in *P. multimicronucleatum* and *P. calkinsi*, "*Ca.* Trichorickettsia"
107 appears covered by long flagella and is able to actively move [28, 63]. Thus, for our research purpose,
108 we selected the newly isolated *P. multimicronucleatum* strain US_Bl 16I1 as donor host in the trans-
109 infection experiments. We characterized its macronuclear bacterial symbiont, assigning it to "*Ca.*
110 Trichorickettsia mobilis subspecies hyperinfectiva" (*Rickettsiaceae*, *Rickettsiales*), previously
111 discovered and described in the cytoplasm of *Paramecium calkinsi* [63]. As recipient, we selected the
112 freshwater planarian *Dugesia japonica*. Freshwater planarians are benthic organisms, living in mud
113 and under rocks in ponds and streams. They are zoophages or fleshing-eating animals, but ingest also

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

114 detritus, fungi, and bacteria [64]. Thus, they may encounter in their environment a large variety of
115 microbes [65], including ciliates possibly hosting endosymbionts. Planarians have always been
116 considered an important model for studying stem cells and regeneration [66], but recently they
117 became also important for studying the natural immunity system of Metazoa [67-72]. Based on all
118 these considerations, they appeared a suitable model for such experiments.

119 Bacterial endosymbiont trans-infection from *P. multimicronucleatum* to planarians was
120 investigated by checking for the presence of "*Ca*. Trichorickettsia mobilis" in tissues of ciliate-treated
121 planarians by means of PCR and Transmission Electron Microscopy (TEM). The findings obtained
122 by means of the two investigation approaches allowed us to test the hypothesis that this endosymbiont
123 can transfer from ciliate protists to Metazoa.
124

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

126 # Materials and methods
127

128 ## Ciliate host isolation, culturing, and identification

129 *Paramecium multimicronucleatum* monoclonal strain US_Bl 16I1 was established from a cell
130 isolated from a freshwater sample collected from the Yellowwood Lake (39°11’29,0’’N,
131 86°20’31,4’’W), IN, USA and cultivated in San Benedetto mineral water (San Benedetto S. p. A.,
132 Italy)
133 The strain was maintained in the laboratory in an incubator at a temperature of 19 ± 1 °C and
134 on a 12:12 light/dark cycle (irradiance by means of NATURAL L36W/76 and FLORA L36W/77
135 neon tubes, OSRAM, Berlin, Germany). Instead of using bacteria as common food source for
136 paramecia [73], cells were fed on monoclonal cultures of flagellated green algae, i.e. *Chlorogonium*
137 sp. (freshwater) or, alternatively, *Dunaliella tertiolecta* (brackish water, 1 ‰ of salinity) to minimize
138 bacterial load in cell cultures. They were fed two to three times per week to obtain a mass culture (1.5
139 L) suitable for trans-infection experiments. Living ciliates were observed with a Leitz (Weitzlar,
140 Germany) microscope equipped with differential interference contrast (DIC) at a magnification of
141 100-1250x for general morphology and swimming behaviour according to [74, 75]. DIC observation
142 of ciliates was also aimed at checking for macronuclear endosymbiont motility. Feulgen stained
143 ciliates were observed to obtain information on the nuclear apparatus. Microscope images were
144 captured with a digital camera (Canon PowerShot S45). Cell measurements were performed using
145 ImageJ (https://imagej.nih.gov/ij/). Morphological identification was conducted according to
146 literature data [76].
147

148 ## Characterization of ciliate and endosymbiont

149 Characterization of the ciliate host and its bacterial endosymbiont was performed through the “full
150 cycle rRNA approach” [77], i.e. through the nuclear 18S rRNA gene (generally considered the
151 preferred marker to study molecular taxonomy and phylogeny of eukaryotic organisms - just as an

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

152 example see [78]), ITS region and partial 28S gene (ciliate), and 16S rRNA gene (endosymbiont)
153 sequencing, combined with Fluorescence *In Situ* Hybridization (FISH) experiments. Additionally,
154 mitochondrial cytochrome c oxidase subunit I (COI) gene was sequenced as well, to display
155 haplogroup variation in *P. multimicronucleatum* [79].
156 *Molecular characterization*. For molecular characterization of both ciliate host and bacterial
157 endosymbiont, total DNA was extracted from approximately 50 *Paramecium* cells as described in
158 [30, 31]. All PCR reactions were carried out with the Takara ExTaq (Takara, Kusatsu, Japan) reaction
159 kit. COI gene was amplified using the degenerated forward primer F338dT and reverse primer
160 R1184dT [80], while for 18S rRNA gene amplification the forward 18S F9 Euk [81] and the reverse
161 18S R1513 Hypo primers [82] were used. The ITS region (including partial 18S rRNA gene, ITS1,
162 5.8S rRNA gene, ITS2, and partial 28S rRNA gene) sequence was obtained by PCR with the forward
163 18S F919 and the reverse 28S R671 primers (modified according to [83]). 16S rRNA gene of the
164 bacterial symbiont was amplified with the *Alphaproteobacteria*-specific forward primer 16S Alpha
165 F19b and the universal bacterial reverse primer 16S R1522a [84]. Purified PCR products by
166 NucleoSpin gel and PCR cleanup kit, Macherey-Nagel GmbH Düren, Germany) were directly
167 sequenced at GATC Biotech AG (Constance, Germany). Internal primers were used to sequence 16S
168 rRNA gene [84], 18S rRNA gene [85] and ITS regions [83]. The latter two sequences were then
169 joined together, exploiting the partial overlap on the 18S rRNA gene sequences. For the COI gene
170 sequencing, amplification primers were employed from both directions.
171 *FISH experiments.* Preliminary FISH experiments were performed using the eubacterial universal
172 probe EUB338 [86] labelled with fluorescein-isothiocyanate (FITC) and the specifically designed
173 probe Rick_697 (5'-TGTTCCTCCTAATATCTAAGAA-3') labelled with Cy3 to verify the presence
174 of endosymbiotic bacteria belonging to the family *Rickettsiaceae* [28]. Based on the obtained results,
175 i.e. the presence of a single positive signal in the ciliate macronucleus and the newly characterized
176 16S rRNA gene sequence corresponding to "*Ca*. Trichorickettsia mobilis subspecies hyperinfectiva",
177 a second FISH experiment was carried out using a species-specific probe, i.e. the probe

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

178 Trichorick_142 (5'-GTTTCCAAATGTTATTCCATAC-3') [28] in combination with the eubacterial
179 universal probe EUB338 [86]. The experiments followed the protocol by [87] employing a
180 hybridization buffer containing no formamide, according to the recommendations for the used probes.
181 Slides were mounted with SlowFade Gold Antifade with DAPI (Invitrogen, Carlsbad, USA) and
182 viewed with a Zeiss AxioPlan fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped
183 with an HBO 100W/2 mercuric vapor lamp. Digital images were captured by means of a dedicated
184 software (ACT2U, version 1.0).
185

186 Planarian culturing

187 Planarians used in this work belonged to the species *Dugesia japonica*, asexual strain GI [88].
188 Animals were kept in artificial water ($CaCl_2$ 2.5mM; $MgSO_4$ 0.4mM; $NaHCO_3$ 0.8mM; KCl 77μM;)
189 at 18°C in dim light conditions and fed with chicken liver (purchased from local food stores) prepared
190 according to [89], once a week. Non-regenerating specimens, within 5-8 mm of length, were used for
191 all experimental procedures, after being starved for about two weeks.
192

193 Transmission electron microscopy (TEM)

194 TEM preparations were obtained both for *P. multimicronucleatum* cells and planarians to study the
195 ultrastructure of the ciliate-associated endosymbiont "*Ca.* Trichorickettsia mobilis subspecies
196 hyperinfectiva" and to verify the success of trans-infection experiments (i.e. to check the animals for
197 the presence of trans-infected ciliate endosymbionts in their tissues) respectively.
198 TEM preparations of *P. multimicronucleatum* cells were obtained according to [90]. Briefly,
199 cells were fixed in a 1:1 mixture of 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4),
200 and 2% (w/v) OsO_4 in distilled water, ethanol and acetone dehydrated, and embedded in Epon-
201 Araldite mixture. Ultrathin sections were stained with 4% (w/v) uranyl acetate followed by 0.2%
202 (w/v) lead citrate.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

203 TEM preparations of planarian specimens were obtained as previously described [91, 92],
204 with minor modifications. Therefore, planarians were fixed with 3 % glutaraldehyde in 0.1 M
205 cacodylate buffer, and post-fixed with 2 % osmium tetroxide for 2 h. After ethanol dehydration,
206 samples were embedded in Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate
207 and lead citrate and observed with a Jeol 100 SX transmission electron microscope.

208 *Negative staining.* For negative staining, several *P. multimicronucleatum* cells of the strain
209 US_Bl 16I1 were washed in distilled water and squashed by means of a syringe; a drop of the resulting
210 suspension was placed on a grid. Bacteria were allowed to precipitate for 2–3 min, then a drop of 1%
211 uranyl acetate in distilled water was added for no longer than 1 min. The liquid was then absorbed
212 with filter paper, the grid was air-dried, and observed under TEM.

213

214 Trans-infection experiments

215 Two trans-infection experiments with the same protocol were sequentially carried out along a period
216 of four months. Each experiment was conducted by treating a fixed number of planarians with ciliate-
217 enriched food, i.e. liver paste mixed with *P. multimicronucleatum* homogenate; from now on these
218 animals will be referred to as “treated planarians”. As for experimental control, planarians fed on
219 plain liver paste were used; these animals will be referred to as “control planarians”. Each trans-
220 infection experiment was performed according to the following protocol:

221 1. 96 planarians were selected by eye from culture (see above), washed in fresh culturing water and
222 collected in a Petri dish with 50 μg/ml gentamicin sulfate (Sigma, Saint Luis, MO, USA) dissolved
223 in their culturing water (see above). This preliminary antibiotic treatment was performed to
224 minimize potential endogenous bacteria contaminants without endangering the animals [93].
225 Planarians were left in antibiotic treatment for 24 h under regular culturing conditions concerning
226 temperature and light (see above) and kept under visual control during that period to verify their
227 viability during the antibiotic treatment and by the time of trans-infection. Then, planarians were
228 washed 6 times in their fresh culturing water and split into two equal groups of 48 individuals each

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

229 and accommodated in two different Petri dishes for the trans-infection procedure by means of
230 feeding.
231 2. 1.5 L of *P. multimicronucleatum* mass culture (cell concentration: ~ 4 x 10^5 cell/L) was filtered
232 with a nylon filter (pore size: 100 µm). Cells were washed twice in sterile San Benedetto mineral
233 water to minimize potential bacterial contaminants, concentrated and harvested by means of
234 centrifugation (400 x *g* per 10 min), so to reduce the medium volume to 2 ml. Then, cells were
235 mechanically homogenized for 20 min by repeated passages through a syringe (needle: 22GA,
236 0.70 mm in diameter). Cell homogenate was centrifuged (10,000 x *g* per 10 min), and the resulting
237 pellet was resuspended in 50 µl of planarian food (homogenized liver paste) by direct
238 resuspension.
239 3. A group of 48 planarians was fed on *P. multimicronucleatum*-enriched liver paste (treated
240 planarians), while the other group was in parallel fed on plain liver paste (control planarians). For
241 feeding, food was sown on the bottom of the Petri dish. Animals were allowed to reach it and
242 comfortably feed for a period of 2h under regular culturing conditions (see above). Attention was
243 paid to planarian feeding behaviour during this period. As no differences were noted concerning
244 feeding behaviour between treated and control planarians, and feeding procedure was exerted by
245 all planarians as expected according to regular planarian culturing, we proceeded with the next
246 steps. Two washing steps were then carried out removing the medium and adding fresh planarian
247 culturing water. Finally, the two groups of animals were left in their fresh culturing water and in
248 regular cultivation conditions in the two Petri dishes until the collection of specimens for the next
249 TEM and PCR analyses at the three timepoints (see below) of the experiments. Animals were kept
250 under visual control throughout the experiment to regularly check their viability.
251 4. At day 1, 3, and 7 after feeding (experimental timepoints), from each of the two Petri dishes
252 containing treated and control planarians a group consisting of 16 animals were sampled: 4 animals
253 were fixed and processed for TEM, and 12 were rapidly frozen and stored at -80°C and dedicated
254 to DNA extraction (4 animals per each sample).

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

255 ## PCR verification of trans-infection success

256 Treated and control planarians were processed for genomic DNA extraction, purification, and PCR
257 amplification with endosymbiont-specific primers. For each experimental condition (treated and
258 control planarians) and each experimental timepoint (1, 3, and 7 days after feeding), genomic DNA
259 was extracted from frozen samples by using the Wizard Genomics DNA purification kit (Promega,
260 Madison, WI, USA). One microliter of purified DNA was analysed by gel electrophoresis to check
261 for integrity. DNA was quantified using a Nanodrop spectrophotometer. For each experimental
262 timepoint of both experimental conditions (i.e. treated and control planarians), similar amounts of
263 genomic DNA were used for amplification using the ampli-Taq-gold DNA polymerase (Applied
264 Biosystems, Foster City, CA, USA) and for a nested PCR assay. The first primer pair used, specific
265 for "*Ca.* Trichorickettsia mobilis", was RickFla_ F69 5'-GTTAACTTAGGGCTTGCTC-3' and
266 Rick_R1455 5'-CCGTGGTTGGCTGCCT-3' [28]; PCR conditions were: 3 minutes at 94°C; 5 cycles
267 each consisting of 30 s at 94°C, 30 s at 58°C, 2 minutes at 72°C; 10 cycles each consisting of 30 s at
268 94°C, 30 s at 54°C, 2 minutes at 72°C; 25 cycles each consisting of 30 s at 94°C, 30 s at 50°C, 2
269 minutes at 72°C; ending with 7 minutes at 72°C. The second, nested primer pair used was
270 RickFla_F87 5'-CTCTAGGTTAATCAGTAGCAA-3' and Rick_R1270 5'-
271 TTTTAGGGATTTGCTCCACG-3' [28]. For nested PCR assay, one microliter of PCR product of
272 the first PCR assay was used as a template; PCR conditions were as above.

273 For each experimental condition and each timepoint of the two trans-infection experiments,
274 the DNA amplification was performed in duplicate. Samples were considered positive if a single band
275 of the expected size was recorded after nested amplification. Sequencing of amplicons was carried
276 out to confirm the presence of "*Ca.* Trichorickettsia mobilis" using the primer RickFla_F87 (see
277 above) and Sanger sequencing (BMR Genomics, Padova, Italy).

278 Samples obtained from control planarians were used as PCR negative control. As positive
279 control, genomic DNA purified from *P. multimicronucleatum* monoclonal strain US_Bl 16I1 was
280 processed.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

281 # Results

282 ## Host morphological and molecular identification

283 Ciliate strain US_Bl 16I1 (Figs 1a, 1b; Supplementary Material; S1 and S2 Figs) was confirmed in
284 morphological inspections as *Paramecium multimicronucleatum* Powers and Mitchell, 1910 [94]
285 considering features of key-characters such as cell size, number and features of micronuclei (mi), and
286 number and features of contractile vacuoles (CV) (S1 and S2 Figs), as described in previous literature
287 [76, 95-98]. The molecular analysis of the combined (partial) 18S rRNA-ITS1-5S rRNA gene-ITS2-
288 (partial) 28S rRNA gene sequence (2792 bp, GenBank accession number: MK595741) confirmed the
289 species assignation by morphological identification with a 100% sequence identity with the sequences
290 of other *P. multimicronucleatum* strains already present in GenBank presenting either 18S rRNA gene
291 portion only (AB252006 and AF255361), or the ITS portion (AY833383, KF287719, JF741240 and
292 JF741241). COI gene sequence identity of strain US_Bl 16I1 (760 bp, accession number: MK806287)
293 is highest with another *P. multimicronucleatum* haplotype (FJ905144.1; 96.3%).

294

295 ## Endosymbiont identification and features

296 As in *Paramecium* three different subspecies of RLOs "*Ca.* Trichorickettsia mobilis" have been
297 described [63], 16S rRNA gene amplification was performed to identify endosymbionts associated
298 with *P. multimicronuclatum* US_Bl 16I1 strain (sequence length 1,563 bp, accession number:
299 MK598854), which allowed to assign it to "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva", an
300 endosymbiont described in the cytoplasm of *Paramecium calkinsi* (99.8 % identity of the novel
301 sequence with type strain MF039744.1) [63]. FISH experiments (Figs 1c-1e) performed by using
302 eubacterial universal probe EUB338 (Fig 1c) and the species-specific probe Trichorick_142 (Fig 1d)
303 confirmed this result but disclosed a different, novel bacterial localization, i.e. the ciliate
304 macronucleus (roughly with a presence of about 100 endosymbionts). Additionally, the full
305 overlapping of eubacterial universal probe EUB338 and "*Ca*. Trichorickettsia"-specific probe signals

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

306 indicated that this symbiont constituted the total set of intracellular bacteria in host *P.*
307 *multimicronucleatum* US_Bl 16I1 cells (Fig 1e, merge).

308 In TEM-processed ciliate cells, the endosymbionts were confirmed to be hosted in the
309 macronucleus; they showed a two-membrane cell wall typical for Gram-negative bacteria and
310 appeared rod-shaped, with rounded to narrower ends (Fig 2). They were surrounded by a clear halo
311 and not encircled by any symbiosomal vacuole, i.e. they appeared in direct contact with host nuclear
312 material. Their size was ~ 1.2--2.1 x 0.5--0.6 μm, and they were scattered throughout the
313 macronucleus of the ciliate (Figs 2a-2d). Sometimes in their cytoplasm several electron-lucid "holes"
314 (diameter: ~ 0.2 μm), were observed (Fig 2a), as already described in the same endosymbiont in *P.*
315 *calkinsi* [63]. Bacterial cytoplasm was electrondense and rich in ribosomes (Fig 2b); the presence of
316 additional cytoplasmic components (e.g. electron-dense particles with a special arrangement) was not
317 disclosed. Endosymbionts displayed thin (diameter: ~ 0.009 μm) and short flagella distributed all
318 around the cell (Figs 2a and 2c-2d) which sometimes formed a putative tail emerging from a cell end
319 (Fig 2d). The presence of longer flagella was evident after negative staining procedure (Fig 3): besides
320 some short flagella occurring all around the cell, at least a few longer and thicker flagella (~ 2 x 0.012
321 μm) arose from one of cell ends.

322 Some active bacterial motility, likely obtained by means of their flagella, was recorded inside
323 the macronucleus of intact *P. multimicronucleatum* cells during observation under DIC microscope
324 at 1,000x. Endosymbionts were seen to move through the chromatin bodies covering short distances
325 (Supplementary Movie SM1). After ciliate cell squashing the released bacteria were still capable of
326 movement (data not shown).

327

328 **Fig 1. Results of FISH experiments on a *P. multimicronucleatum* US_Bl 16I1 cell.** (**a**) bright field
329 microscopy (Ma, macronucleus). (**b**) after DAPI staining. (**c**) treated with the almost universal
330 bacterial probe EUB338 double labelled with fluorescein (green) (probe labelling both at 5' and 3'
331 ends). (**d**) treated with species-specific probe Trichorick_142 labelled with Cy3 (red) targeting "*Ca.*

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

332 Trichorickettsia mobilis". (**e**) merge of **c** and **d**. The number of endosymbionts targeted by the species-
333 specific probe in the macronucleus reached ~ 100 (**d**, **e**). Scale bars stand for 10 µm.
334
335 **Fig 2. TEM pictures showing the morphological-ultrastructural details of "*Ca.* Trickorickettsia**
336 **mobilis subsp. hyperinfectiva" in the macronucleus of *P. multimicronucleatum* US_Bl 16I1.** (**a**)
337 longitudinally and transversely-sectioned endosymbionts inside macronucleus (Ma) with a double
338 membrane and surrounded by a clear halo; electron-lucid roundish areas occur in the cytoplasm of
339 some endosymbionts; (**b**) numerous bacterial ribosomes are visible; (**c, d**) bacterial flagella are short,
340 located either around (arrow) the endosymbiont cell or at a cell pole, where they can form a putative
341 tail (arrowhead). Scale bars stand for 0.5 µm.
342
343 **Fig 3. Negative contrast at TEM of a specimen of "*Ca.* Trickorickettsia mobilis subsp.**
344 **hyperinfectiva".** Some short flagella are present and at least two longer flagella (arrow) arise from
345 one of cell ends. Scale bar stands for 1 µm.
346
347
Trans-infection experiments

348 The success of each of the two independent trans-infection experiments performed was investigated
349 by means of DNA extraction/PCR experiments and TEM observation on treated (i.e. fed on ciliate
350 homogenate added to liver paste) and control (i.e. untreated, fed on plain liver paste) planarians at the
351 different experimental timepoints, according to the described experimental procedure. By verifying
352 the possible presence of *P. multimicronucleatum* endosymbiont "*Ca.* Trichorickettsia mobilis" in
353 extracted as well as TEM processed animals, the two investigation methods produced completely
354 overlapping results on planarians used in the two trans-infection experiments and with full
355 concordance on trans-infection success. Thus, for each of the two different investigation methods,
356 results of the two trans-infection experiments are concurrently reported in the following sections.
357 Similarly, figures showing the results of the trans-infection experiment at the different experimental

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

358 timepoints (Figs 4 and 5, S3 Fig) are meant to be representative for both performed experiments,
359 irrespective of the specific experiment they refer to.

360 *PCR experiments*. The DNA of "*Ca.* Trichorickettsia mobilis" was detected at all
361 experimental timepoints (1, 3, and 7 days after feeding) in genomic DNA preparations obtained from
362 all investigated planarians fed on *P. multimicronucleatum* lysate-enriched liver paste. The size of the
363 nested amplicon (1360 bp) obtained from treated planarian samples matched the size of the amplicon
364 obtained in DNA purified from ciliate monoclonal strain US_Bl 16I1 (positive control). The
365 sequencing of the obtained amplicons confirmed the specificity for "*Ca*. Trichorickettsia mobilis"
366 (100% sequence identity with MF039744.1).

367 No amplification product was obtained in genomic DNA samples from control planarians fed
368 on plain liver paste (negative control).

369 *TEM observation*. Ultrastructural observation was conducted on TEM-processed specimens
370 per each timepoints of the experiments, for both experimental groups, i.e. treated and control
371 planarians.

372 In tissues collected from the investigated planarians fed on *Paramecium* US_Bl 16I1 lysate-
373 enriched liver paste, the presence of bacteria with morphology and sizes (see below for details) fitting
374 with those of ciliate endosymbionts within intestinal cells was detected at all timepoints of the two
375 experiments (Figs 4 and 5).

376 One day after feeding, several bacteria were recognizable in planarian enterocyte phagosomes.
377 Some of them were still intact (Figs 4a and 4b), even performing cell division. Others appeared
378 degraded, being subjected to digestion (data not shown). In several cases, membrane of bacteria-
379 including phagosomes was damaged and interrupted (Figs 4a and 4b). Bacteria free in the cytoplasm
380 of planarian intestinal cells, i.e. in direct contact with their cytoplasm, were detected as well; they
381 showed a two-membrane cell wall and a surrounding clear halo (Figs 4c-4f), with electron-lucid
382 "holes" sometimes visible inside their cytoplasm (Fig 4e). Flagella (diameter: ~ 0.009 μm) could also
383 be detected all around bacteria (Figs 4b-4d and 4f). Additionally, extruded trichocysts (extrusive

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

384 organelles typical of *Paramecium*), presumably included in *Paramecium* homogenate and ingested
385 by treated planarians, were easily recognizable inside intestinal cell phagosomes (Fig 4g).
386 A similar scenario was observed 7 days after feeding, when most of the bacteria occurred
387 enclosed inside planarian enterocyte phagosomes (Figs 5a and 5b); some appeared degraded (not
388 shown), while many others did not show degraded conditions and could survive in the phagosomes
389 (Figs 5a and 5b). In few cases, bacterial-like circular-ovoid shapes were also observed in the
390 cytoplasm of intestinal cells, outside from phagosomes (Fig 5c).
391 No bacteria were ever observed in tissues other than intestine in treated planarians; similarly,
392 no bacteria were observed in tissues of investigated TEM-processed control animals (S3 Fig).
393
394 **Fig 4. TEM pictures of the intestine of *D. japonica* treated with liver paste enriched with the**
395 **homogenate of *P. multimicronucleatum* US_Bl 16I1 cells and fixed at day 1 after feeding.** (**a, b**)
396 (**b,** enlargement of a particular of **a**) a flagellated bacterium (B) inside a phagosome whose membrane
397 is interrupted (arrowhead); L, lumen of planarian intestine; (**c, d**) (**d**, enlargement of a particular of **c**)
398 a longitudinally sectioned bacterium (B) free in the cytoplasm of an intestinal cell of a treated
399 planaria; (**e**) another free bacterium, showing cytoplasmic electron-lucid "holes"; (**f**) a recovered
400 flagellated bacterium in cross section. (**g**) an intact extruded trichocyst (T) (extrusive organelle) of
401 *Paramecium* detected in planarian intestine. Arrows, flagella. Scale bars stand for 1 μm (**a-e**, **g**), and
402 0.5 μm (**f**).
403
404 **Fig 5. TEM pictures of the intestine of *D. japonica* fixed at day 7 after feeding with liver paste**
405 **enriched with the homogenate of *P. multimicronucleatum* US_Bl 16I1 cells.** (**a, b**) bacteria
406 recognizable inside phagosomes and (**c**) bacterial shapes in the cytoplasm of an intestinal cell. L, lipid
407 droplet; arrow, bacteria; arrowhead, mitochondria; double arrowhead, flagella. Scale bars stand for 1
408 μm.
409

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

410 # Discussion

411 ## Trans-infection experiments

412 The alphaproteobacterium "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva", previously
413 described in the cytoplasm of *P. calkinsi* [63], was retrieved in this study also in the macronucleus of
414 the ciliate *P. multimicronucleatum* strain US_Bl 16I1 s. "*Ca.* Trichorickettsia mobilis" up to now has
415 been exclusively retrieved as an endosymbiont of ciliates belonging to the genera *Paramecium* and
416 *Euplotes* [28, 63]. At present, three subspecies of this RLO endosymbiont have been identified on
417 molecular basis [63]; a comparison among them in the light of the present findings, which suggest a
418 certain morphological plasticity of this bacterium, is presented in Supplementary Material.

419 The aim of the present paper was to verify the potential trans-infection of the *Rickettsia*-related
420 macronuclear endosymbiont "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva" of the ciliate *P.*
421 *multimicronucleatum* strain US_Bl 16I1 to the metazoan model planarian *D. japonica*. There are
422 several studies reporting on the host/symbiont relationships of different *Paramecium* species with
423 different *Rickettsiales*; *P. multimicronucleatum* lies in this ciliate selection, and is a rather a common
424 species, sharing the freshwater habitat with planarians. Thus, it was chosen as donor in trans-infection
425 experimental context as, in our opinion, it can be considered a suitable candidate as putative
426 environmental vector for RLOs. Additionally, the biology of this ciliate host is well-known, and the
427 strain US_Bl 16I1 could be comfortably cultivable under laboratory conditions using flagellates
428 instead of bacteria as food source.

429 According to the present findings, the trans-infection experiments were successful, i.e. they
430 showed the capability of "*Ca.* Trichorickettsia mobilis" to enter the tissues of planarians. Indeed, in
431 the intestine of planarians, previously antibiotic-treated to avoid bacterial contamination and fed on
432 liver paste enriched with pellet of ciliate homogenate (including "*Ca*. Trichorickettsia" symbionts),
433 we could observe up to day 7 after feeding the presence of flagellated bacteria with a morphology
434 and a size fully resembling those of the RLO endosymbiont of *P. multimicronucleatum* US_Bl 16I1.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

435 In our TEM experiments, besides undigested bacteria enclosed in phagosomes, in few cases circular-
436 ovoid shapes, resembling "*Ca*. Trichorickettsia" bacteria, were observed in the cytoplasm of planarian
437 intestine cells, free from phagosomal membrane (Figs 4 and 5).

438 On the contrary with respect to treated animals, in TEM preparations of controls (i.e.
439 antibiotic-treated planarians fed on plain liver paste) no bacteria were found (S3 Fig).

440 These results were confirmed and supported by PCR analysis and sequencing of obtained
441 amplicons: the DNA of "*Ca.* Trichorickettsia mobilis" was recovered in treated planarians up to day
442 7 after feeding while in control animals no RLO DNA was amplified and detected. On the other side,
443 no morphological or behavioural alterations were observed in the planarians

444 Our findings are in line with those by [99]. These authors studied the phylogenetic identities
445 of digestion-resistant bacteria that could survive starvation and form relatively stable associations
446 with some marine and freshwater ciliate species, and demonstrated that the classes
447 *Alphaproteobacteria* (which includes the order *Rickettsiales*) and *Gammaproteobacteria* are
448 prevalent as digestion-resistant bacteria; from this study a putative significant role of secretion
449 systems in promoting marine protist-bacteria associations resulted as well.

450 In our experiments, after being ingested by planarians, the bacteria were observed enclosed
451 inside phagosomes of intestinal cells. This also occurred in previous experiments investigating the
452 resistance of planarians to infection by bacterial strains pathogenic for humans and other metazoans
453 [67]. In that research, planarians could eliminate most of the phagocytised bacterial strains within 3-
454 6 days post-feeding thanks to 18 resistance genes, such as *MORN2*, so that the authors suggested that
455 planarians can be considered a model to identify innate resistance mechanisms. Thus, the evidence
456 we obtained that the "*Ca*. Trichorickettsia" endosymbionts of *P. multimicronucleatum* US_Bl 16I1
457 are still detectable in planarian intestine enterocytes inside and outside phagosomes up to 7 days after
458 feeding could possibly indicate the capability of "*Ca.* Trichorickettsia mobilis subsp. hyperinfectiva"
459 to avoid typical defence mechanisms exploited by planarians.

460 TEM observations indicated that in some cases *P. multimicronucleatum* RLO could possibly

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

461 occur outside digestive vacuoles, whose membrane often appeared fractured. Thus, we can
462 hypothesize that this bacterium can transitionally enter and perhaps survive within planarian tissues,
463 although present results do not allow yet to drive unambiguous conclusions on this regard.
464 Interestingly, similarly to some *Gammaproteobacteria* such as *Rheinheimera* sp. strain EpRS3
465 (*Chromatiaceae*), capable of escaping from phagosomes of the ciliate *Euplotes aediculatus* when fed
466 on the bacterium plus its culture medium [100], *Rickettsiaceae* are already known for their ability to
467 escape the host vacuolar membrane, residing freely in the host cytoplasm, where they may exploit
468 host cytoskeleton for movement [101-103, 12, 17].

469 Another interesting parallelism might be made with the so-called "eta" particles (i.e.
470 intracellular bacteria) hosted in the cytoplasm of the ciliate *Euplotes crassus* killer cell strain M on
471 *E. crassus* sensitive cell strain 21A7 fed on strain M homogenate [104]. In that study, "eta" particles
472 conferred ciliate strain 21A7 a transitory resistance to killing effect during their permanence as free
473 entities in its cytoplasm: indeed, after being collected into the digestive vacuoles, "eta" particles were
474 pushed towards the periphery of the vacuole and those still not digested were capable of escaping into
475 ciliate cytoplasm through vacuole membrane rupture.

476 Unfortunately, just as in the studies by Chiellini et al. [100] and Verni et al. [104], we are
477 currently not able to provide here clues on the potential bacterial mechanisms involved in planarian
478 phagosome escaping.

479 To the best of our knowledge, this is the first time that a set of experimental bioassays was
480 performed to verify the transmission of a "true" and ascertained *Rickettsiales* bacterium from an
481 infected protist to an uninfected metazoan of the same aquatic environment (freshwater). On the other
482 side, in the past, efforts have been put to experimentally verify the transmission of morphologically
483 RLOs among aquatic organisms. For example, Nunan et al. [105] performed bioassays to verify the
484 transmission of the infection between two species of commercially farmed shrimps, i.e. the infected
485 *Penaeus monodon* and the specific pathogen-free *Penaeus vannamei*, with the aim of investigating
486 the suspected causative agent of severe mortality in farms where those organisms are in co-culture

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

487 (grow-out ponds). The bioassays were performed both via injection of infected shrimp homogenate
488 into uninfected shrimps and per *os* exposure of uninfected shrimps to tissue taken from infected
489 shrimps. Only injection bioassays were successful leading to an infection, while per *os* infection
490 failed. Among different possible reasons for this negative result those authors cited the potential need
491 for a vector to disseminate the disease. According to our findings, ciliates could represent suitable
492 vectors in this kind of situation. In our experiments, as distinct from Nunan et al. [105], we chose
493 only to perform the bioassay per *os* exposure of animals instead of injection into the planarian
494 intestine. Endosymbionts, separated from ciliate cells through cell rupture and centrifugation, could
495 be easily mixed with planarian food and seeded on the bottom of a Petri dishes so as to allow animals
496 to reach it and feed. As we dealt with endosymbionts, which are present in limited numbers inside
497 their ciliate host, we chose to treat the planarians with cell mass culture homogenate instead of adding
498 living ciliates to planarians food. This allowed processing of as many ciliates as possible to maximize
499 the probability of endosymbiont ingestion by the animals, thereby increasing the chance of detection
500 of successful trans-infection via PCR and TEM-based approaches.

501 We believe that our findings may offer intriguing insights when considered from several
502 points of view, such as concerning the pathologies caused by *Rickettsiales* or RLOs occurring in fish
503 farms or in the wild, which might have ciliates or other protists as putative vectors. Although there is
504 still a need for further investigations on this topic to expand its implications, we think that our study
505 can serve as basis for conceiving long-lasting experiments aiming to better understand whether "*Ca.*
506 Trichorickettsia mobilis", as well as other *Rickettsiales* symbionts of protists, can be able to survive
507 longer and potentially replicate in tissues of planarians and other aquatic Metazoa, and whether these
508 RLOs may have some impact on the recipient host health.

509

510 # Compliance with Ethical Standards

511 *Ethics Approval.* This study does not contain any studies with human participants performed by any
512 of the authors. All procedures performed in studies involving animals were in accordance with the

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

513 ethical standards of the institution or practice at which the studies were conducted.

514 *Conflict of Interest.* The authors declare that they have no conflict of interest.

515

516 # Acknowledgments

517 The authors wish to thank Francesco Paolo Frontini, Fabrizio Erra, and Federica Vantaggio for ciliate
518 culturing and *in vivo* processing; Claudio Ghezzani and Simone Gabrielli for assistance with TEM
519 material; Simone Gabrielli for help with graphic artwork, Thomas Berendonk for the opportunity to
520 collaborate on *Paramecium*. Financial support: project PRA_ 2016_58, University of Pisa to GP;
521 RFBR grant N° 18-04-00562-a to ES. All authors critically read and approved the manuscript.

522

523 # Author Contributions

524 Conceptualization: GP, FV, AS, LM, LR.

525 Formal analysis: LM, AS, LR, FS, MC.

526 Funding acquisition: GP, ES.

527 Investigation: LM, AS, LR, FS.

528 Project administration: GP.

529 Supervision: GP, FV.

530 Visualization: LM, AS.

531 Writing – original draft: LM, AS, LR, MC.

532 Writing – review & editing: FV, GP, FS, ES, GDG, SIF, SK.

533

534 # References

535 1. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera
536 in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some
537 species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*,

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

538 descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as
539 subjective synonyms of *Ehrlichia phagocytophila*. Int J Syst Evol Microbiol. 2001; 51: 2145-2165.
540 doi: 10.1099/00207713-51-6-2145.

541 2. Taylor MJ, Bandi C, Hoerauf A. *Wolbachia* endosymbionts of filarial nematodes. Adv. Parasitol.
542 2005; 60: 245-284. doi: 10.1016/S0065-308X(05)60004-8.

543 3. Perlman SJ, Hunter MS, Zchori-Fein E. The emerging diversity of *Rickettsia*. Proc R Soc B. 2006;
544 273: 2097-2106. doi: 10.1098/rspb.2006.3541.

545 4. Weinert LA. Werren JH, Aebi A, Stone GN, Jiggins FM. Evolution and diversity of *Rickettsia*
546 bacteria. BMC Biol. 2009; 7:6. doi: 10.1186/1741-7007-7-6.

547 5. Gillespie JJ, Nordberg EK, Azad AF, Sobral BWS. Phylogeny and comparative genomics: The
548 shifting landscape in the genomics era. In: Palmer GH, Azad AF, editors. Intracellular pathogens II
549 *Rickettiales*. Washington: ASM Press; 2012. pp. 84-141.

550 6. Dumler JS, Walker DH. *Rickettsiales*. In: Whitman WB, Rainey F, Kämpfer P, Trujillo M, Chun
551 J, De Vos P, Hedlund B, Dedyshed S, editors. Bergey's Manual of systematics of Archaea and
552 Bacteria. John Wiley & Sons, Ltd. 2015. Available from:
553 https://doi.org/10.1002/9781118960608.obm00074.

554 7. Castelli M, Sassera D, Petroni G. Biodiversity of “non-model” *Rickettsiales* and their association
555 with aquatic organisms. In: Thomas, S editor. *Rickettsiales* - Biology, Molecular Biology,
556 Epidemiology, and Vaccine Development. Cham, Switzerland: Springer International Publishing;
557 2016. pp. 59-91.

558 8. Chan LL, Mak JW, Ambu S, Chong PY. Identification and ultrastructural characterization of
559 *Acanthamoeba* bacterial endocytobionts belonging to the *Alphaproteobacteria* class. PLoS ONE.
560 2018. 13: e0204732. Available from: https://doi.org/10.1371/journal.pone.0204732.

561 9. Castelli M, Sabaneyeva E, Lanzoni O, Lebedeva N, Floriano A M, Gaiarsa S, et al. *Deianiraea*,
562 an extracellular bacterium associated with the ciliate *Paramecium*, suggests an alternative scenario

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

563 for the evolution of *Rickettsiales*. 2019a. ISME J. Available from: https://doi.org/10.1038/s41396-
564 019-0433-9.
565 10. Dumler JS, Walker DH. *Rickettsiales*. In: Whitman WB, Rainey F, Kämpfer P, Trujillo M, Chun
566 J, De Vos P, Hedlund B, Dedyshed S, editors. Bergey's Manual of systematics of Archaea and
567 Bacteria. John Wiley & Sons, Ltd. 2015. Available from:
568 https://doi.org/10.1002/9781118960608.obm00074.
569 11. Raoult V, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. Clin
570 Microbiol Rev. 1997; 10: 694-719.
571 12. Walker DH, Ismail N. Emerging and re-emerging rickettsioses: endothelial cell infection and
572 early disease events. Nat Rev Microbiol. 2008; 6: 375-386. doi: 10.1038/nrmicro1866.
573 13. McQuiston JH, Paddock CD. Public health: Rickettsial infections and epidemiology. In:
574 Intracellular Pathogens II: *Rickettsiales*. American Society of Microbiology. 2012; pp 40-83.
575 14. Dumler JS, Madigan JE, Pusterla N, Bakken JS. Ehrlichioses in humans: epidemiology, clinical
576 presentation, diagnosis, and treatment. Clin Infect Dis. 2007; 45: S45-S51. doi: 10.1086/518146.
577 15. Rikihisa Y. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of
578 host cells. Nat Rev Microbiol. 2010; 8: 328-339. doi: 10.1038/nrmicro2318.
579 16. Allsopp BA. Heartwater – *Ehrlichia ruminantium* infection: Rev Sci Tech Off Int Epiz. 2015;
580 34: 557-568.
581 17. Ge Y and Rikihisa Y. Subversion of host cell signaling by *Orientia tsutsugamushi*. Microbes and
582 Infect. 2011; 13: 638-648. doi: 10.1016/j.micinf.2011.03.003.
583 18. Kawafune K, Hongoh Y, Hamaji T, Sakamoto T, Kurata T, Hirooka et al. Two different rickettsial
584 bacteria invading *Volvox carteri*. PloS ONE. 2015; 10: e0116192. Available from:
585 https://doi.org/10.1371/journal.pone.0116192.
586 19. Schulz F, Martijn J, Wascher F, Lagkouvardos I, Kostanjšek R, Ettema TJG, et al. A *Rickettsiales*
587 symbiont of amoebae with ancient features. Environ Microbiol. 2016; 18: 2326-2342. doi:
588 10.1111/1462-2920.12881.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

589 20. Yang A, Narechania A, Kim E. Rickettsial endosymbiont in the "early-diverging" streptophyte
590 green alga *Mesostigma viride*. J Phycol. 2016; 52: 219-229. doi: 10.1111/jpy.12385.
591 21. Hess S. Description of *Hyalodiscus flabellus* sp. nov. (Vampyrellida, Rhizaria) and identification
592 of its bacterial endosymbiont, "*Candidatus* Megaira polyxenophila" (*Rickettsiales,*
593 *Alphaproteobacteria*). Protist. 2017; 168: 109-133. doi: 10.1016/j.protis.2016.11.003.
594 22. Yurchenko T, Ševčíková T, Přibyl P, El Karkouri K, Klimeš V, Amaral R, et al. A gene transfer
595 event suggests a long-term partnership between eustigmatophyte algae and a novel lineage of
596 endosymbiotic bacteria. ISME J. 2018; 12: 2163-2175. doi: 10.1038/s41396-018-0177-y.
597 23. Sun HY, Noe J, Barber J, Coyne RS, Cassidy-Hanley D, Clark TG, et al. Endosymbiotic bacteria
598 in the parasitic ciliate *Ichthyophthirius multifiliis*. Appl Environ Microbiol. 2009; 75: 7445-7452. doi:
599 10.1128/AEM.00850-09.
600 24. Zaila KE, Doak TG, Ellerbrock H, Tung C-H, Martins ML, Kolbin D, et al. Diversity and
601 universality of endosymbiotic *Rickettsia* in the fish parasite *Ichthyophthirius multifiliis*. Front
602 Microbiol. 2017; 8: 189. doi: 10.3389/fmicb.2017.00189.
603 25. Ferrantini F, Fokin SI, Modeo L, Andreoli I, Dini F, Görtz HD, et al. "*Candidatus* Cryptoprodotis
604 polytropus," A novel *Rickettsia*-like organism in the ciliated protist *Pseudomicrothorax dubius*
605 (Ciliophora, Nassophorea). J Eukaryot Microbiol. 2009; 56: 119-129. doi: 10.1111/j.1550-
606 7408.2008.00377.x.
607 26. Boscaro V, Fokin SI, Schrallhammer M, Schweikert M, Petroni G. Revised systematics of
608 *Holospora*-like bacteria and characterization of "*Candidatus* Gortzia infectiva," a novel
609 macronuclear symbiont *of Paramecium jenningsi*. Microb Ecol. 2013; 65: 255-267. doi:
610 10.1007/s00248-012-0110-2.
611 27. Schrallhammer M, Ferrantini F, Vannini C, Galati S, Schweikert M, Görtz HD, et al. "*Candidatus*
612 Megaira polyxenophila" gen. nov., sp. nov.: considerations on evolutionary history, host range and
613 shift of early divergent rickettsiae. PLoS ONE. 2013; 8: e72581. Available from:
614 https://doi.org/10.1371/journal.pone.0072581.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

615 28. Vannini C, Boscaro V, Ferrantini F, Benken K, Mironov T, Schweikert M, et al. Flagellar
616 movement in two bacteria of the family *Rickettsiaceae*: a re-evaluation of motility in the evolutionary
617 perspective. PloS One; 2014. Available from: https://doi.org/10.1371/journal.pone.0087718.
618 29. Senra MVX, Dias RPJ, Castelli M, Silva-Neto I-D, Verni F, Soares CAG. A house for two-
619 double bacterial infection in *Euplotes woodruffi* Sq1 (Ciliophora, Euplotia) sampled in southeastern
620 Brazil. Microb Ecol. 2016; 71: 505-517. doi: 10.1007/s00248-015-0668-6.
621 30. Szokoli F, Castelli M, Sabaneyeva E, Schrallhammer M, Krenek S, Doak T, et al. Disentangling
622 the taxonomy of *Rickettsiales* and description of two novel symbionts ("*Candidatus* Bealeia
623 paramacronuclearis" and "*Candidatus* Fokinia cryptica") sharing the cytoplasm of the ciliate protist
624 *Paramecium biaurelia*. Appl Environ Microbiol. 2016a; 82: 7236-7247. doi: 10.1128/AEM.02284-
625 16.
626 31. Szokoli F, Sabaneyeva E, Castelli M, Krenek S, Schrallhammer M, Soares C, et al. "*Candidatus*
627 Fokinia solitaria", a novel "stand-alone" symbiotic lineage of *Midichloriaceae* (*Rickettsiales*). PLoS
628 One, 2016b; 11: e0145743. Available from: https://doi.org/10.1371/journal.pone.0145743.
629 32. Castelli M, Serra V, Senra MVX, Basuri CK, Soares CAG, Fokin SI, et al. The hidden world of
630 *Rickettsiales* symbionts: "*Candidatus* Spectririckettsia obscura," a novel bacterium found in Brazilian
631 and Indian *Paramecium caudatum*. Microb Ecol. 2019b; 77: 748-758. doi: 10.1007/s00248-018-
632 1243-8.
633 33. Lanzoni O, Sabaneyeva E, Modeo L, Castelli M, Lebedeva N, Verni F, et al. Diversity and
634 environmental distribution of the cosmopolitan endosymbiont "*Candidatus* Megaira". Sci Rep. 2019;
635 9: 1179. doi: 10.1038/s41598-018-37629-w.
636 34. Fraune S, Bosch TC. Long-term maintenance of species-specific bacterial microbiota in the basal
637 metazoan *Hydra*. P Natl Acad Sci USA. 2007; 104: 13146-13151. doi: 10.1073/pnas.0703375104.
638 35. Sunagawa S, DeSantis TZ, Piceno YM, Brodie EL, DeSalvo MK, Voolstra CR, et a1.
639 Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral
640 *Montastraea faveolata*. ISME J. 2009; 3: 512-521. doi: 10.1038/ismej.2008.131.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

641 36. Murakami T, Segawa T, Dial R, Takeuchi N, Kohshima S, Hongoh Y. Bacterial microbiota
642 associated with the Glacier ice worm is dominated by both worm-specific and Glacier-derived
643 facultative lineages. Microbes Environ. 2017; 32: 32-39. doi: 10.1264/jsme2.ME16158.
644 37. Dishaw LJ, Flores-Torres J, Lax S, Gemayel K, Leigh B, Melillo D, et al. The gut of
645 geographically disparate *Ciona intestinalis* harbors a core microbiota. PLoS One. 2014; 9, e93386.
646 Available from: https://doi.org/10.1371/journal.pone.0093386.
647 38. Kwan JC, Schmidt W. Bacterial endosymbiosis in a chordate host: long-term co-evolution and
648 conservation of secondary metabolism. PLoS ONE. 2013; 8: e80822. Available from:
649 https://doi.org/10.1371/journal.pone.0080822.
650 39. Epis S, Sassera D, Beninati T, Lo N, Beati L, Piesman J, et al. *Midichloria mitochondrii* is
651 widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse
652 species. Parasitology. 2008; 13: 485-494. doi: 10.1017/S0031182007004052.
653 40. Driscoll T, Gillespie JJ, Nordberg EK, Azad AF, Sobral BW. Bacterial DNA sifted from the
654 *Trichoplax adhaerens* (Animalia: Placozoa) genome project reveals a putative rickettsial
655 endosymbiont. Genome Biol. Evol 2013; 5: 621-645. doi: 10.1093/gbe/evt036.
656 41. Friedman CS, Andree KB, Beauchamp KA, Moore JD, Robbins TT, Shields JD, et al.
657 "*Candidatus* Xenohaliotis californiensis", a newly described pathogen of abalone, *Haliotis* spp.,
658 along the west coast of North America. Int J Syst Evol Microbiol. 2000; 50: 847-855. doi:
659 10.1099/00207713-50-2-847.
660 42. Crosson LM, Wight N, Van Blaricom GR, Kiryu I, Moore JD, Friedman CS. Abalone withering
661 syndrome: distribution, impacts, current diagnostic methods and new findings. Dis Aquat Organ.
662 2014; 108: 261-270. doi: 10.3354/dao02713.
663 43. Sun J, Wu X Histology, ultrastructure, and morphogenesis of a rickettsia-like organism causing
664 disease in the oyster, *Crassostrea ariakensis* Gould. J Invertebr Pathol. 2004; 86: 77-86. doi:
665 10.1016/j.jip.2004.04.004.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

666 44. Ross PM, Pande A, Jones JB, Cope J, Flowers G. First detection of gas bubble disease and
667 *Rickettsia*-like organisms in *Paphies ventricosa*, a New Zealand surf clam. J Fish Dis. 2018; 41: 187-
668 190. doi: 10.1111/jfd.12684.
669 45. Loy JK, Dewhirst FE, Weber W, Frelier PF, Garbar TL, Tasca SI, et al. Molecular phylogeny
670 and *in situ* detection of the etiologic agent of necrotizing hepatopancreatitis in shrimp. Appl Environ
671 Microbiol. 1996; 62: 3439-3445.
672 46. Longshaw M. Diseases of crayfish: A review. J Invertebr Pathol. 2011; 106: 54-70. doi:
673 10.1016/j.jip.2010.09.013.
674 47. Wang W. Bacterial diseases of crabs: A review. J Invertebr Pathol. 2011; 106: 18-26. doi:
675 10.1016/j.jip.2010.09.018.
676 48. Athanassopoulou F. Groman D, Prapas T, Sabatakou O. Pathological and epidemiological
677 observations on rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece. J Appl
678 Ichthyol. 2004; 20: 525-529. doi: 10.1111/j.1439-0426.2004.00571.x.
679 49. Corbeil S, Hyatt AD, Crane MStJ. Characterisation of an emerging rickettsia-like organism in
680 Tasmanian farmed Atlantic salmon *Salmo salar*. Dis Aquat Organ. 2005; 64: 37-44. doi:
681 10.3354/dao064037.
682 50. Timur G, Erkan M, Yardimci RE, Ercan MD, Çanak O, Ürkü Ç. Light and electron microscopic
683 study of rickettsia-like organisms causing systemic granulomas in farmed sea bass (*Dicentrarchus*
684 *labrax*). Isr J Aquacult Bamid. 2013; 65: 874-880
685 51. Fryer JL, Lannan CN, Giovannoni SJ, Wood ND. *Piscirickettsia salmonis* gen. nov., sp. nov.,
686 the causative agent of an epizootic disease in salmonid fishes. Int J Syst Bacteriol.1992; 42: 120-126.
687 doi: 10.1099/00207713-42-1-120.
688 52. Tan CK, Owens L. Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella*
689 *cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. Dis Aquat Organ. 2000; 41:
690 115-122. doi: 10.3354/dao041115.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

691 53. Lloyd SJ, La Patra SE, Snekvik KR, St-Hilaire S, Cain KD, Call DR. Strawberry disease lesions
692 in rainbow trout from southern Idaho are associated with DNA from a *Rickettsia*-like organism. Dis
693 Aquat Organ. 2008; 82: 111-118. doi: 10.3354/dao01969.
694 54. Cafiso A, Sassera D, Serra V, Bandi C, McCarthy U, Bazzocchi C. Molecular evidence for a
695 bacterium of the family Midichloriaceae (order *Rickettsiales*) in skin and organs of the rainbow trout
696 *Oncorhynchus mykiss* (Walbaum) affected by red mark syndrome. J Fish Dis. 2015; 39: 497-450. doi:
697 10.1111/jfd.12371.
698 55. Barker J, Brown MR. Trojan horses of the microbial world: Protozoa and the survival of bacterial
699 pathogens in the environment. Microbiology. 1994; 140 (Pt 6): 1253-1259. doi: 10.1099/00221287-
700 140-6-1253.
701 56. Gao LY, Harb OS, Abu Kwaik Y. Utilization of similar mechanisms by *Legionella pneumophila*
702 to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. Infect
703 Immun. 1997; 65: 4738-4746.
704 57. Görtz H-D, Brigge T. Intracellular bacteria in protozoa. Naturwissenschaften. 1998; 85: 359-368.
705 58. Harb OS, Gao LY, Abu Kwaik Y. From protozoa to mammalian cells: A new paradigm in the life
706 cycle of intracellular bacterial pathogens. Environ Microbiol. 2000; 2: 251-265. doi: 10.1046/j.1462-
707 2920.2000.00112.x.
708 59. Horn M, Wagner M. Bacterial endosymbionts of free-living amoebae. J Eukaryot Microbiol.
709 2004; 51: 509-514. doi: 10.1111/j.1550-7408.2004.tb00278.x.
710 60. Molmeret M, Horn M, Wagner M, Santic M, Abu Kwaik Y. Amoebae as training grounds for
711 intracellular bacterial pathogens. Appl Environ Microbiol. 2005; 71: 20-28. doi:
712 10.1128/AEM.71.1.20-28.2005.
713 61. Taylor-Mulneix DL, Bendor L, Linz B, Rivera I, Ryman VE, Dewan KK, et al. *Bordetella*
714 *bronchiseptica* exploits the complex life cycle of *Dictyostelium discoideum* as an amplifying
715 transmission vector. PLoS Biol. 2017; 15: e2000420. Available from:
716 https://dx.doi.org/10.1371/journal.pbio.2000420.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

717 62. Gollas-Galván T, Avila-Villa LA, Martínez-Porchas M, Hernandez-Lopez J. *Rickettsia*-like
718 organisms from cultured aquatic organisms, with emphasis on necrotizing hepatopancreatitis
719 bacterium affecting penaeid shrimp: An overview on an emergent concern. Rev Aquacult. 2014; 6:
720 256-269. doi: 10.1111/raq.12043.
721 63. Sabaneyeva E, Castelli M, Szokoli F, Benken K, Lebedeva N, Salvetti A, et al. Host and symbiont
722 intraspecific variability: the case of *Paramecium calkinsi* and "*Candidatus* Trichorickettsia mobilis".
723 Europ J Protistol. 2018; 62: 79-94. doi: 10.1016/j.ejop.2017.12.002.
724 64. González-Estévez C. Autophagy meets planarians. Autophagy. 2009; 5: 290-297. doi:
725 10.4161/auto.5.3.7665.
726 65. Petersen CH. Planarian resistance to blades and bugs. Cell Host Microbe. 2014; 16: 271-272.
727 doi: 10.1016/j.chom.2014.08.016.
728 66. Rossi L, Salvetti A, Batistoni R, Deri P, Gremigni V. Planarians, a tale of stem cells. Cell Mol
729 Life Sci. 2008; 65: 16-23. doi: 10.1007/s00018-007-7426-y.
730 67. Abnave P, Mottola G, Gimenez G, Boucherit N, Trouplin V, Torre C, et al. Screening in
731 planarians identifies MORN2 as a key component in LC3-associated phagocytosis and resistance to
732 bacterial infection. Cell Host & Microbe. 2014; 16: 338-350. doi: 10.1016/j.chom.2014.08.002.
733 68. Conti F, Abnave P, Ghigo E. Unconventional animal models: a booster for new advances in host-
734 pathogen interactions. Front Cell Infect Microbiol. 2014; 4: 142. doi: 10.3389/fcimb.2014.00142.
735 69. Torre C, Ghigo É. La planaire: un ver immortel pour élucider la réponse immunitaire de l'homme
736 [Planaria: an immortal worm to clarify human immune response]. Med Sci (Paris). 2015; 31: 20-22.
737 [Article in French]. doi: 10.1051/medsci/20153101006.
738 70. Arnold CP, Merryman MS, Harris-Arnold A, McKinney SA, Seidel CW, Loethen S, et al.
739 Pathogenic shifts in endogenous microbiota impede tissue regeneration via distinct activation of
740 TAK1/MKK/p38. eLife. 2016; 5: e16793. Available from: https://doi.org/10.7554/eLife.16793.
741 71. Torre C, Abnave P, Tsoumtsa LL, Mottola G, Lepolard C, Trouplin V, et al. *Staphylococcus*
742 *aureus* promotes Smed-PGRP-2/Smed-setd8-1 methyltransferase signalling in planarian neoblasts to

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

743 sensitize anti-bacterial gene responses during re-infection. EBioMedicine. 2017; 20: 150-160. doi:
744 10.1016/j.ebiom.2017.04.031.
745 72. Tsoumtsa LL, Torre C, Trouplin V, Coiffard B, Gimenez G, Mege JL, et al. Antimicrobial
746 capacity of the freshwater planarians against *S. aureus* is under the control of timeless. Virulence.
747 2017; 4:1-10. doi: 10.1080/21505594.2016.1276689.
748 73. Krenek S, Berendonk TU, Petzoldt T. Thermal performance curves of *Paramecium caudatum*: a
749 model selection approach. Eur J Protistol. 2011; 47: 124-137. doi: 10.1016/j.ejop.2010.12.001.
750 74. Modeo L, Fokin SI, Boscaro V, Andreoli I, Ferrantini F, Rosati G, et al. Morphology,
751 ultrastructure, and molecular phylogeny of the ciliate *Sonderia vorax* with insights into the
752 systematics of order Plagiopylida. BMC Microbiol. 2013a; 13: 40. Available from:
753 https://doi.org/10.1186/1471-2180-13-40.
754 75. Nitla V, Serra V, Fokin SI, Modeo L, Verni F, Sandeep BV, et al. Critical revision of the family
755 Plagiopylidae (Ciliophora: Plagiopylea), including the description of two novel species, *Plagiopyla*
756 *ramani* and *Plagiopyla narasimhamurthii,* and redescription of *Plagiopyla nasuta* Stein, 1860 from
757 India. Zool J Linnean Soc. 2019; 186: 1-45. doi: 10.1093/zoolinnean/zly041.
758 76. Fokin S. *Paramecium* genus: biodiversity, some morphological features and the key to the main
759 morphospecies discrimination. Protistology. 2010/2011; 6: 227-235.
760 77. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and *in situ* detection of
761 individual microbial cells without cultivation. Microbiol Rev. 1995; 59: 143-169.
762 78. Illa K, Shameem U, Serra V, Melai M, Mangam S, Basuri CK, et al. Multidisciplinary
763 investigation on the catfish parasite *Hamatopeduncularia* Yamaguti, 1953 (Monogenoidea:
764 Dactylogyridae): Description of two new species from India and phylogenetic considerations. Europ
765 Zool J. 2019; 86: 132-155. doi: 10.1080/24750263.2019.1597931.
766 79. Barth D, Krenek S, Fokin S, Berendonk T. Intraspecific genetic variation in *Paramecium* revealed
767 by mitochondrial cytochrome c oxidase I sequences. J Eukaryot Microbiol. 2006; 53: 20-25. doi:
768 10.1111/j.1550-7408.2005.00068.x.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

769 80. Strüder-Kypke MC, Lynn DH. Comparative analysis of the mitochondrial cytochrome *c* oxidase
770 subunit I COI gene in ciliates Alveolata, Ciliophora and evaluation of its suitability as a biodiversity
771 marker. Syst Biodiv. 2010; 8: 131-148. doi: 10.1080/14772000903507744.
772 81. Medlin L, Elwood HJ, Stickel S, Sogin ML. The characterization of enzymatically amplified
773 eukaryotic 16S-like rRNA-coding regions. Gene. 1988; 71: 491-499.
774 82. Petroni G, Dini F, Verni F & Rosati G A molecular approach to the tangled intrageneric
775 relationships underlying phylogeny in *Euplotes* (Ciliophora, Spirotrichea). Mol Phylogenet Evol.
776 2002; 22: 118-130. doi: 10.1006/mpev.2001.1030.
777 83. Boscaro V, Fokin SI, Verni F, Petroni G. Survey of *Paramecium duboscqui* using three markers
778 and assessment of the molecular variability in the genus *Paramecium*. Mol Phylogenet Evol. 2012;
779 65: 1004-1013. doi: 10.1016/j.ympev.2012.09.001.
780 84. Vannini C, Rosati G, Verni F, Petroni G. Identification of the bacterial endosymbionts of the
781 marine ciliate *Euplotes magnicirratus* (Ciliophora, Hypotrichia) and proposal of "*Candidatus*
782 Devosia euplotis". Int J Syst Evol Microbiol. 2004; 54: 1151-1156. doi: 10.1099/ijs.0.02759-0.
783 85. Rosati G, Modeo L, Melai M, Petroni G, Verni F. A multidisciplinary approach to describe
784 protists: a morphological, ultrastructural, and molecular study on *Peritromus kahli* Villeneuve-
785 Brachon, 1940 (Ciliophora, Heterotrichea). J Eukaryot Microbiol. 2004; 51: 49-59. doi:
786 10.1111/j.1550-7408.2004.tb00160.x.
787 86. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S
788 rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial
789 populations. Appl Environ Microbiol. 1990; 56: 1919-1925.
790 87. Fokin SI, Serra V, Ferrantini F, Modeo L, Petroni G. "*Candidatus* Hafkinia simulans" gen. nov.,
791 sp. nov., a novel *Holospora*-like bacterium from the macronucleus of the rare brackish water ciliate
792 *Frontonia salmastra* (Oligohymenophorea, Ciliophora): multidisciplinary characterization of the new
793 endosymbiont and its host. Microb. Ecol. 2019; 1-15. doi: 10.1007/s00248-018-1311-0.
794 88. Rossi L, Cassella L, Iacopetti P, Ghezzani C, Tana L, Gimenez G, et al. Insight into stem cell

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

795 regulation from sub-lethally irradiated worms. Gene. 2018, 662: 37-45. doi:
796 10.1016/j.gene.2018.04.009.

797 89. King RS, Newmark PA. *In situ* hybridization protocol for enhanced detection of gene expression
798 in the planarian *Schmidtea mediterranea*. BMC Dev Biol. 2013; 13: 8. Available from:
799 https://doi.org/10.1186/1471-213X-13-8

800 90. Modeo L, Petroni G, Lobban CS, Verni F, Vannini C. Morphological, ultrastructural, and
801 molecular characterization of *Euplotidium rosati* n. sp. (Ciliophora, Euplotida) from Guam. J
802 Eukaryot Microbiol. 2013b; 60: 25-36. doi: 10.1111/jeu.12003.

803 91. Rossi L, Bonuccelli L, Iacopetti P, Evangelista M, Ghezzani C, Tana L, et al. Prohibitin 2
804 regulates cell proliferation and mitochondrial cristae morphogenesis in planarian stem cells. Stem
805 Cell Rev. 2014; 10: 871-87. doi: 10.1007/s12015-014-9540-1.

806 92. Cassella L, Salvetti A, Iacopetti P, Ippolito C, Ghezzani C, et al. Putrescine independent wound
807 response phenotype is produced by ODC-like RNAi in planarians. Sci Rep. 2017; 7: 9736. Available
808 from: https://doi.org/10.1038/s41598-017-09567-6.

809 93. Roberts-Galbraith RH, Brubacher JL, Newmark PA. A functional genomics screen in planarians
810 reveals regulators of whole-brain regeneration. Elife. 2016. pii: e17002. Available from:
811 https://doi.org/10.7554/eLife.17002.

812 94. Powers JH, Mitchell C. A new species of *Paramecium* (*Paramecium multimicronucleata*)
813 experimentally determined. Biol Bull. 1910; 19: 324-332.

814 95. Fokin S. Morphological diversity in the micronuclei in *Paramecium*. Arch Protistenkd. 1997;
815 148: 375-387.

816 96. Fokin SI, Chivilev SM. *Paramecium*. Morphometric analysis and taxonomy. Acta Protozool.
817 2000. 39: 1-14

818 97. Wichterman R. The biology of *Paramecium.* 2nd edn. New York and London: Plenum Press.
819 1986.

820 98. Allen RD. Cytology. In: Görtz H-D, editor. *Paramecium*. Berlin, Heidelberg, New York:

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

821 Springer-Verlag; 1988. pp 4-40.

822 99. Gong J, Qing Y, Fu R, Su L, Zhang X, Zhang Q. Protist-Bacteria associations:
823 Gammaproteobacteria and Alphaproteobacteria are prevalent as digestion-resistant bacteria in
824 ciliated protozoa. Front Microbiol. 2016; 7: 498. Available from:
825 https://doi.org/10.3389/fmicb.2016.00498.

826 100. Chiellini C, Pasqualetti C, Lanzoni O, Fagorzi C, Bazzocchi C, Fani R, et al. 2019. Harmful
827 effect of *Rheinheimera* sp. EpRS3 (*Gammaproteobacteria*) against the protist *Euplotes aediculatus*
828 (Ciliophora, Spirotrichea): insights into the ecological role of antimicrobial compounds from
829 environmental bacterial strains. Front Microbiol. 2019; 10: 510. Available from:
830 https://doi.org/10.3389/fmicb.2019.00510.

831 101. Renesto P, Dehoux P, Gouin E, Touqui L, Cossart P, Raoult D. Identification and
832 characterization of a phospholipase D-superfamily gene in rickettsiae. J Infect Dis. 2003; 188: 1276-
833 1283. doi: 10.1086/379080.

834 102. Gouin E, Egile C, Dehoux P, Villiers V, Adams J, Gertler F, et al. The RickA protein of
835 *Rickettsia conorii* activates the Arp2/3 complex. Nature. 2004; 427: 457-461. doi:
836 10.1038/nature02318.

837 103. Cardwell MM and Martinez JJ. The Sca2 autotransporter protein from *Rickettsia conorii* is
838 sufficient to mediate adherence to and invasion of cultured mammalian cells. Infect Immun. 2009;
839 77: 5272-5280. doi: 10.1128/IAI.00201-09.

840 104. Verni F, Rosati G, Nobili R. Infection mechanisms of “eta” killing particles in sensitive cells of
841 the ciliate *Euplotes crassus*. Trans Amer Micros Soc. 1977; 96: 363-369.

842 105. Nunan LM, Noble B, Le Groumellec M, Lightner DV. Experimental infection of *Penaeus*
843 *vannamei* by a rickettsia-like bacterium (RLB) originating from *P. monodon*. Dis Aquat Organ. 2003;
844 54: 43-48. doi: 10.3354/dao054043.

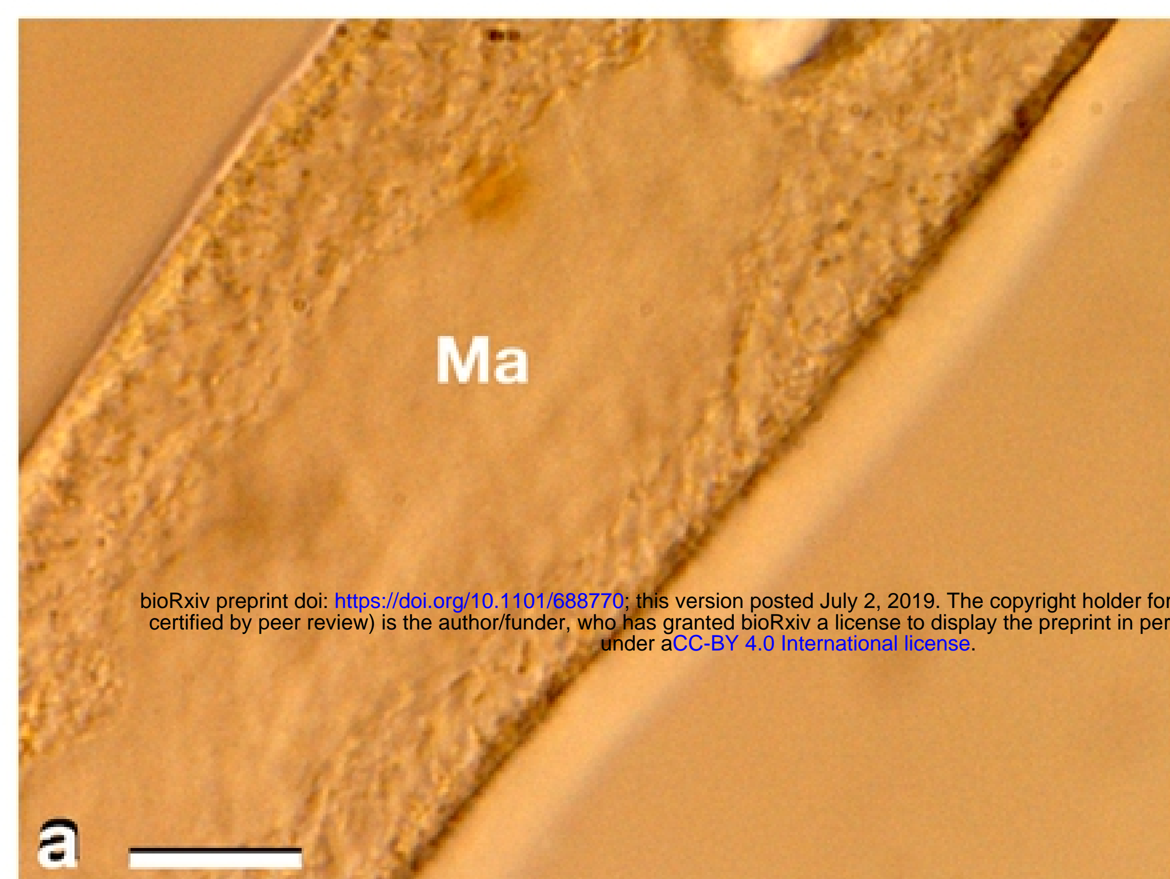
845

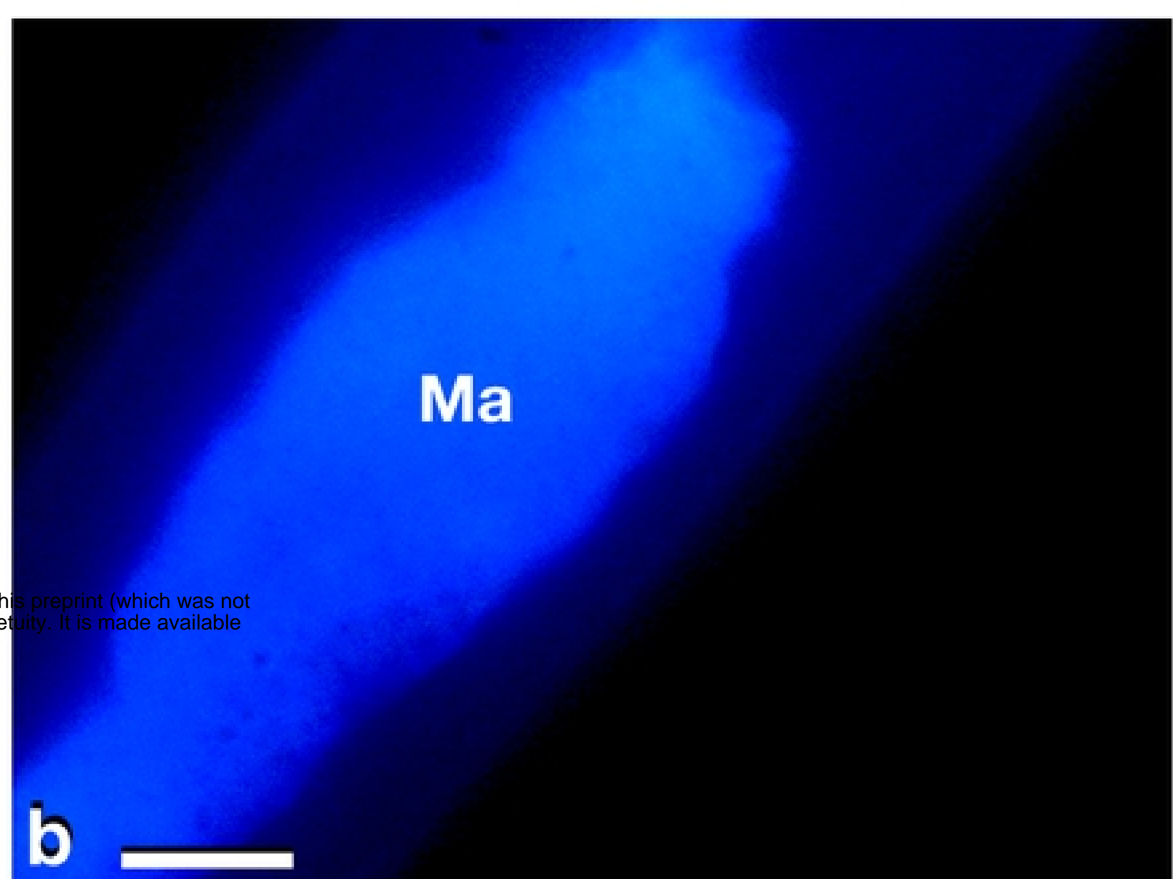
846 # S1_Supporting information

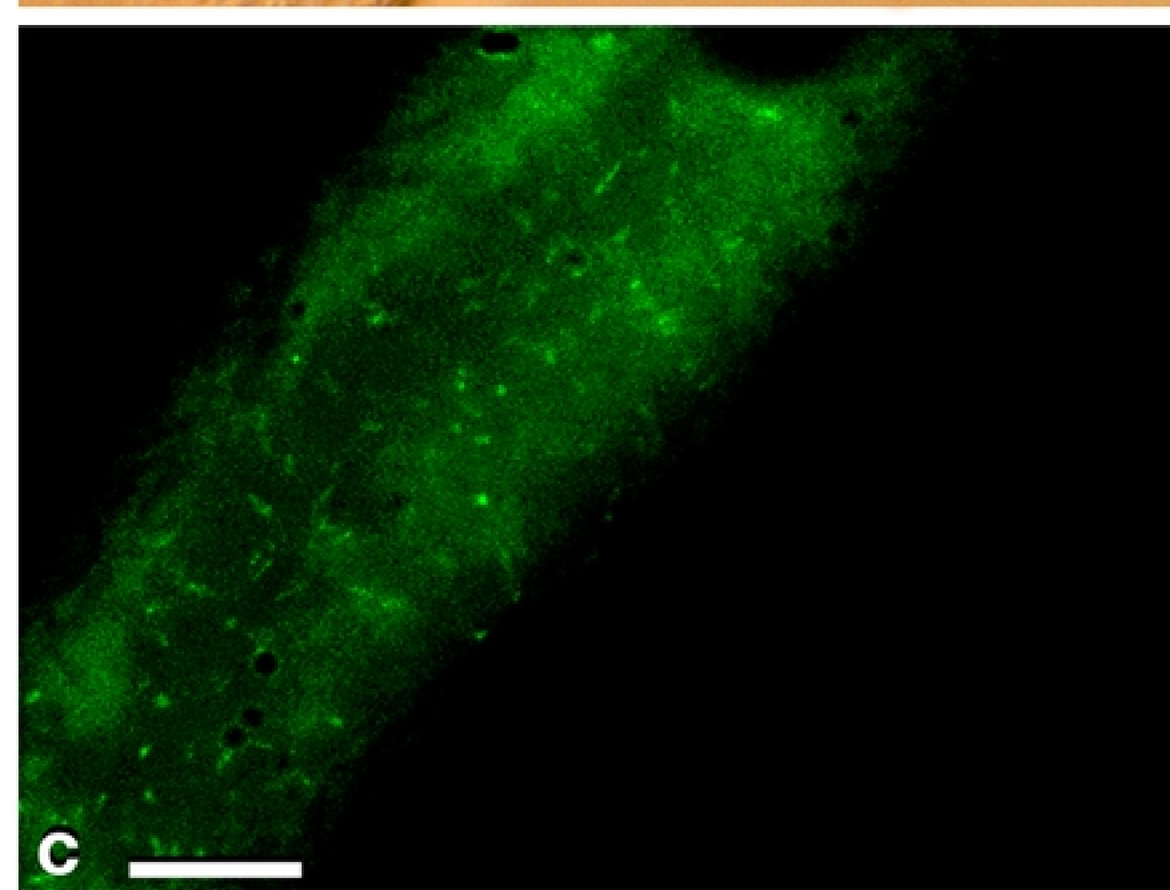
bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

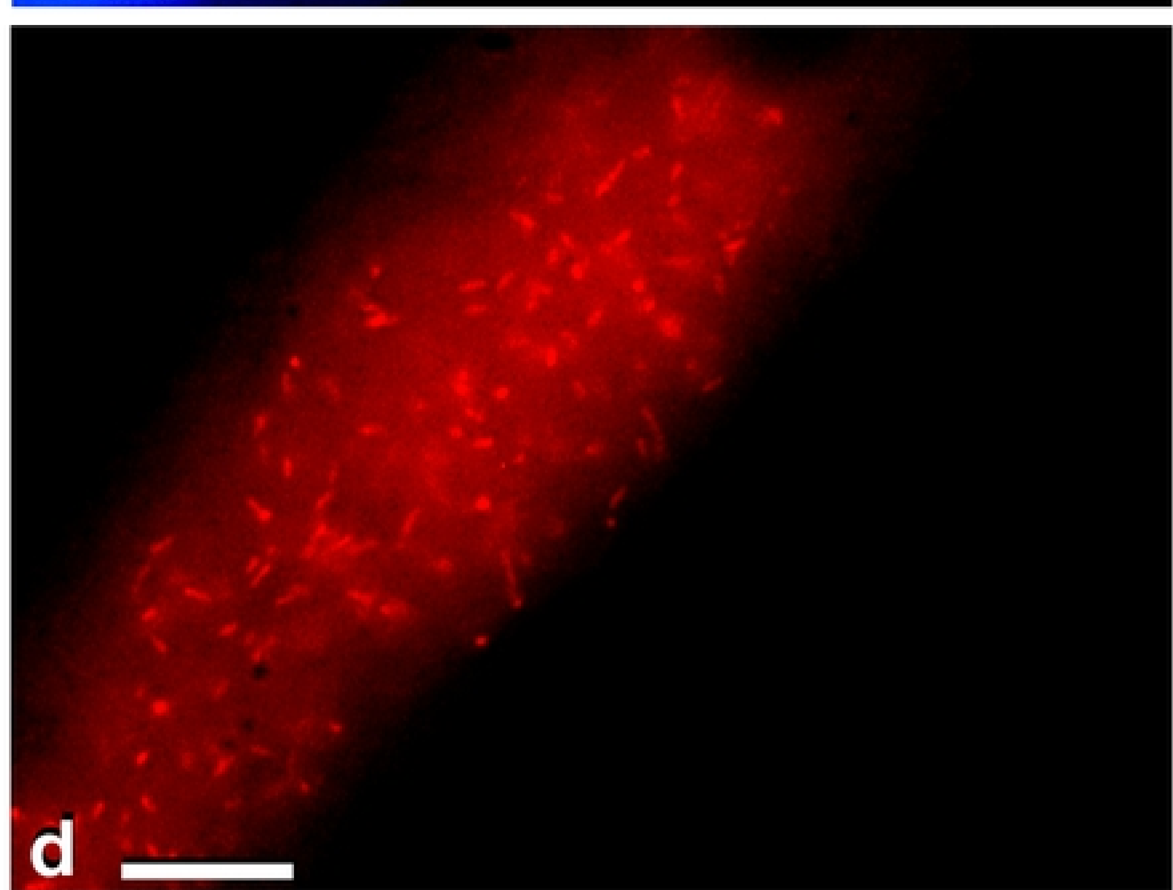
847 **S1 Fig. Light microscope observation of *P. multimicronucleatum* strain US_Bl 16I1. (a-c)** *In vivo*
848 specimens; (**d**) Feulgen stained cell. Ma, macronucleus. (**c**) the cytostome (C) at a higher
849 magnification with trichocysts (T) inserted in the cortex. (**d**) Feulgen staining highlights the Ma and
850 the three micronuclei (mi). Scale bars stand for 10 µm.
851
852 **S2 Fig. TEM pictures of *P. multimicronucleatum* strain US_Bl 16I1.** (**a, b**) cortex with trichocysts
853 inserted (T) in resting state (**a**) and about to extrude (**b**); m, mitochondria. (**c, d**) macronucleus (Ma)
854 encircled by a layer of rarefied material (asterisk) with endosymbionts (arrows) and nucleoli (n); Ph,
855 phagosomes. (**e**) Endosymbionts inside the Ma, a transversally sectioned T occurs near Ma
856 membrane. (**f**) Ma portion and one of the three micronuclei (mi). Scale bars stand for 1 µm.
857
858 **S3 Fig. TEM pictures of the intestine of *D. japonica* specimens fixed after feeding with plain**
859 **liver paste (control animals).** (**a**) day 1 after feeding; (**b**) day 7 after feeding. L, lumen of planarian
860 intestine. Scale bars stand for 1µm.

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.









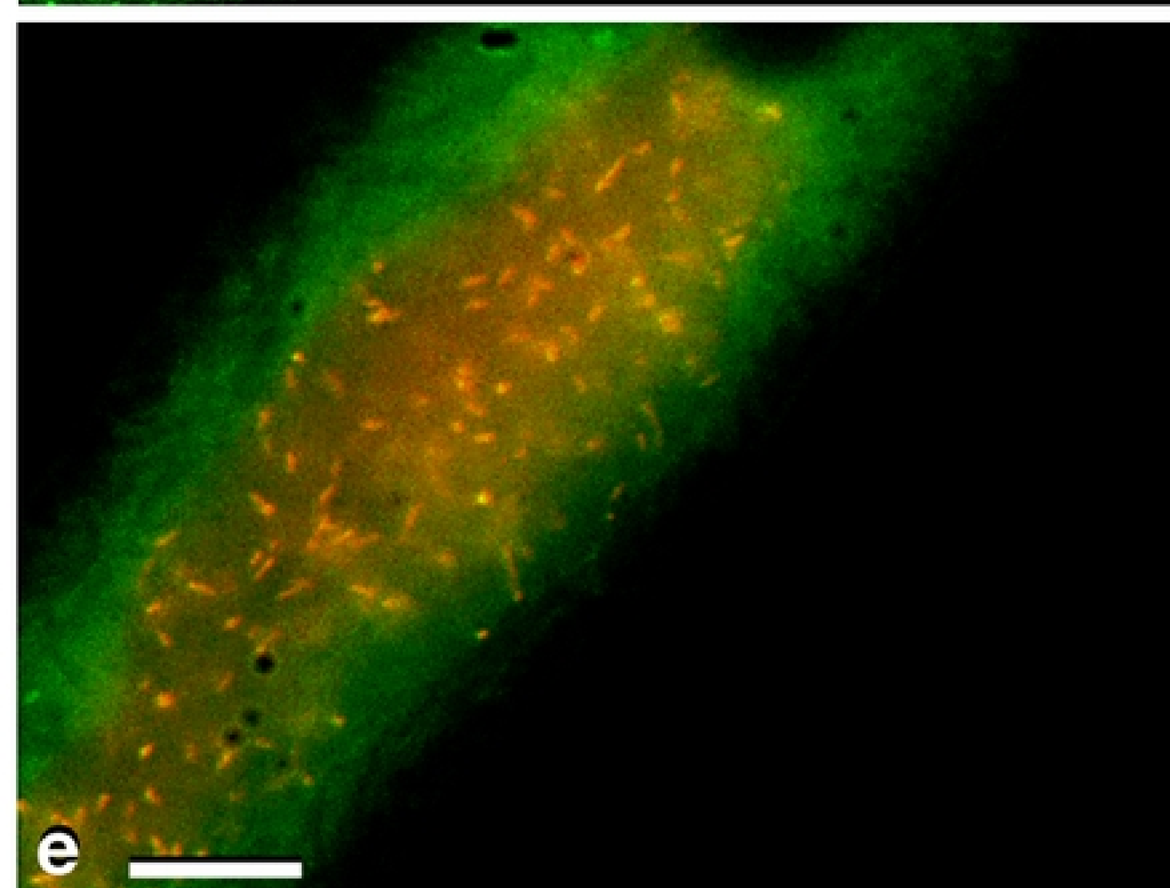
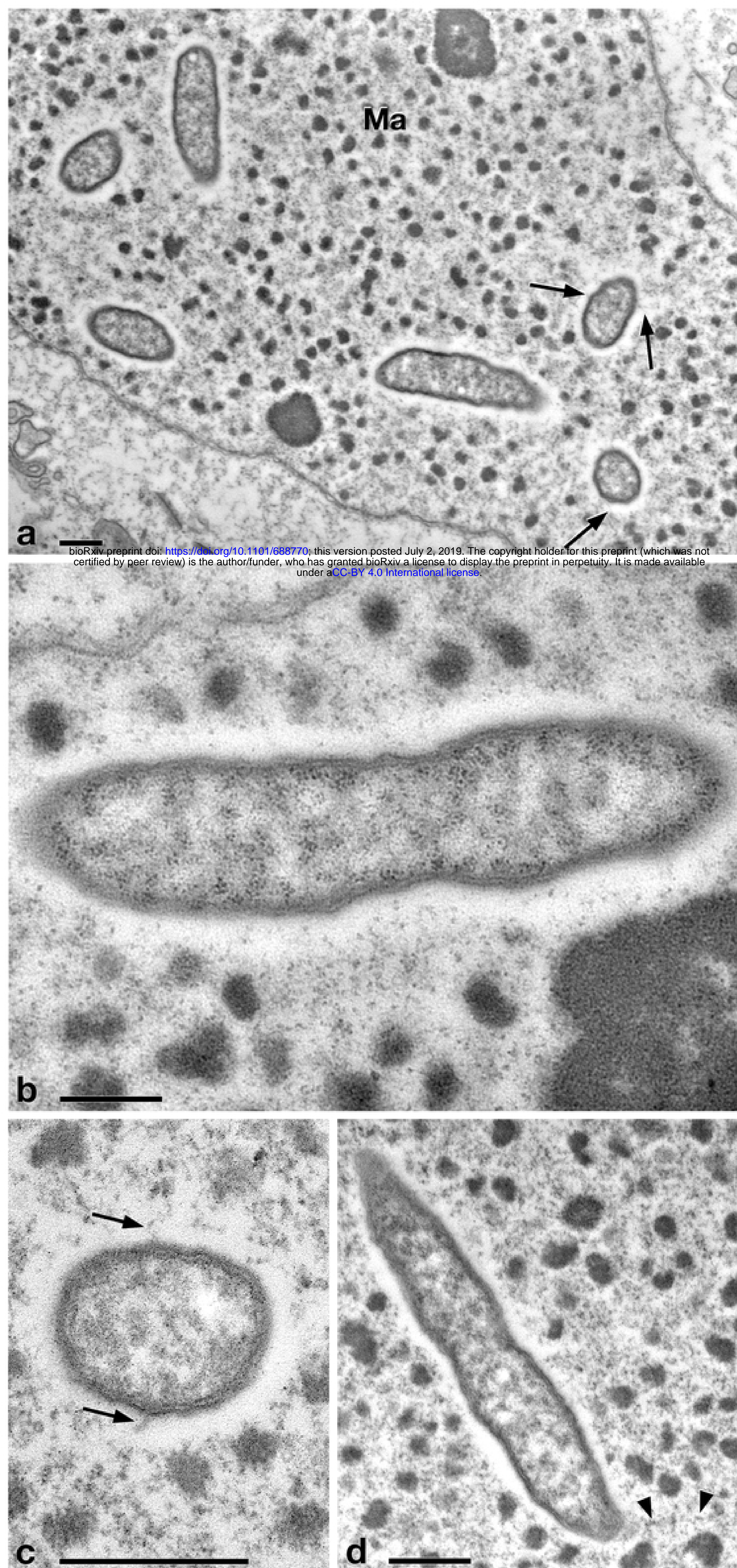


Figure 1



bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Figure 2

bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

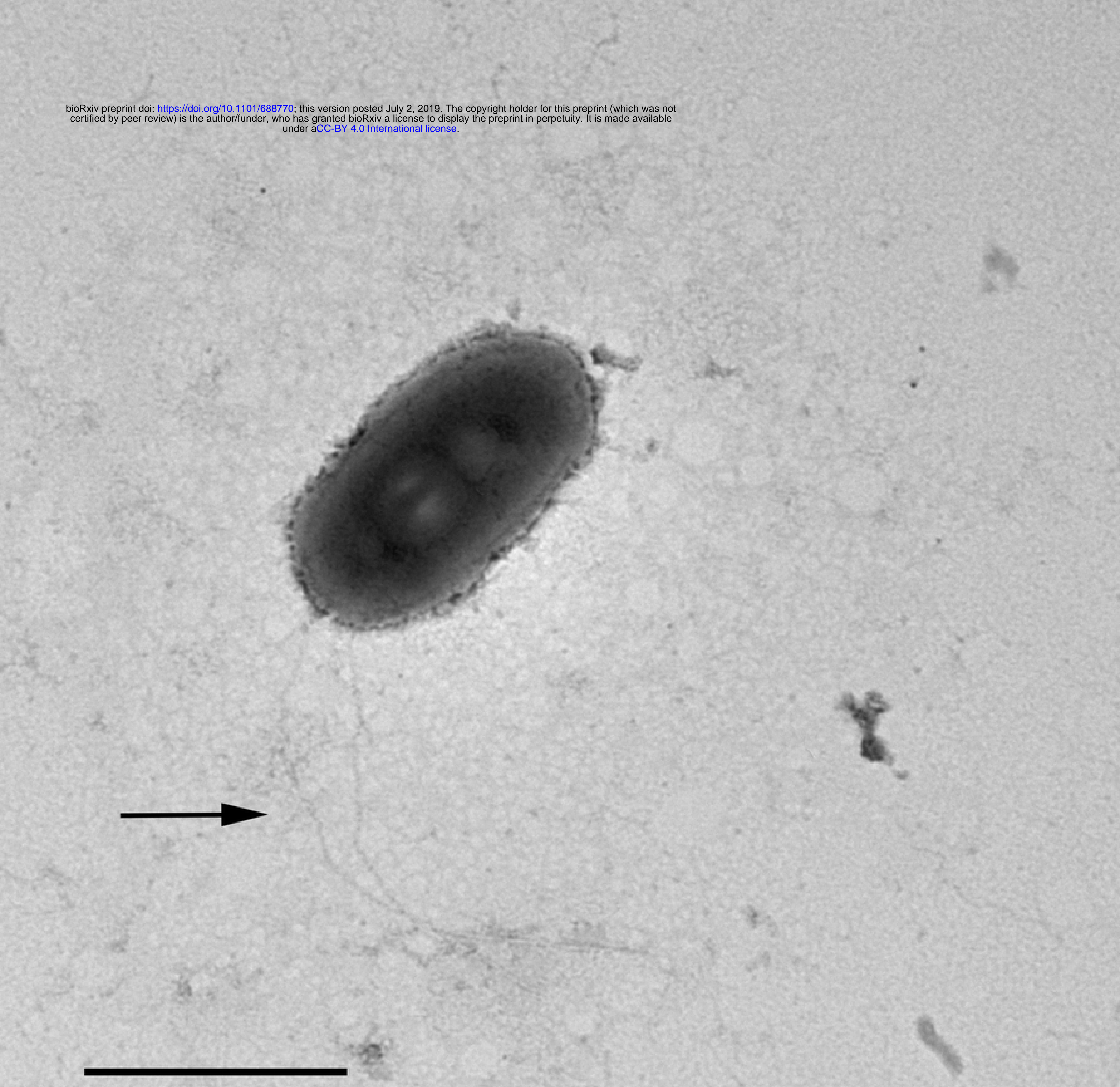
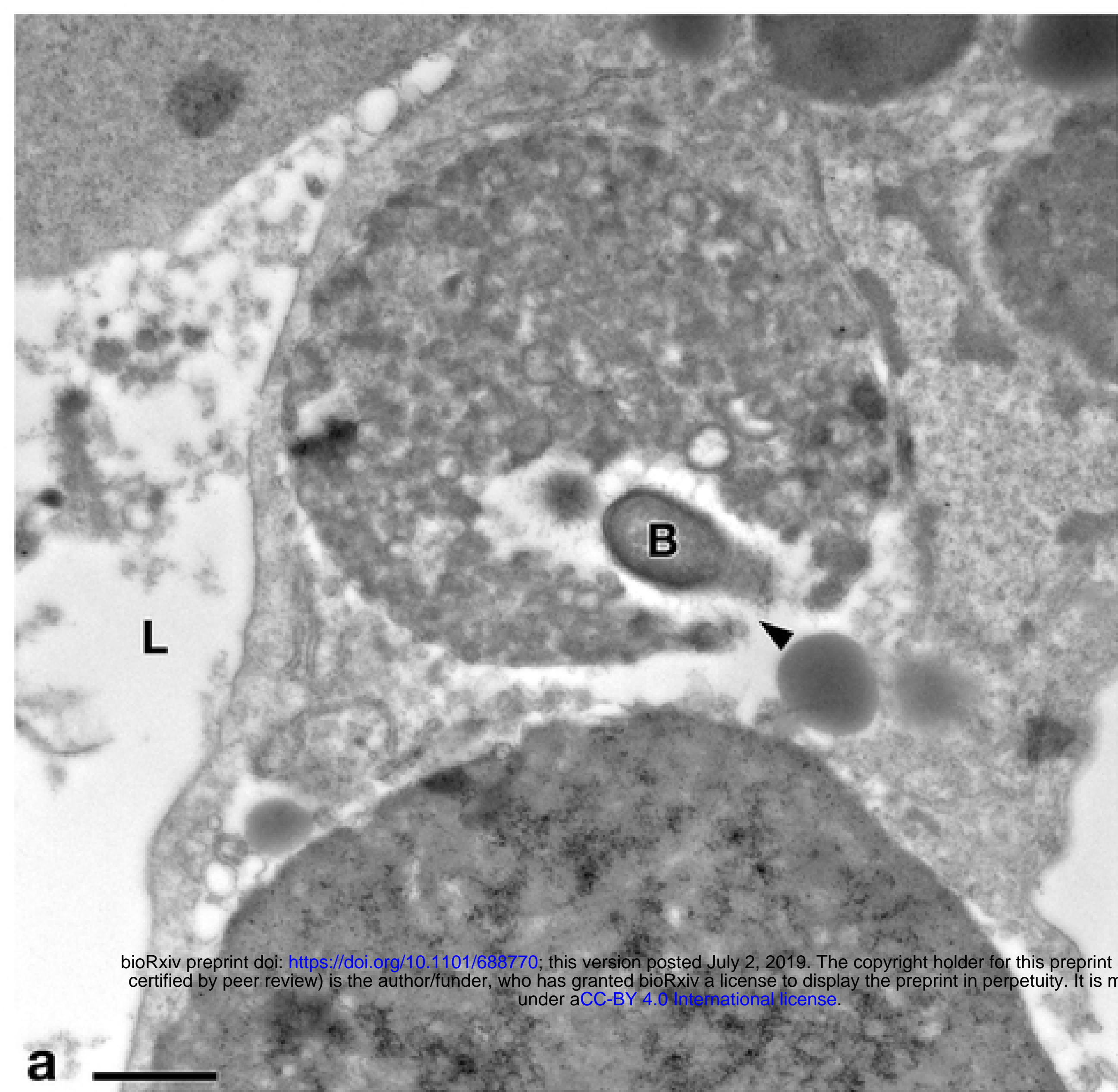
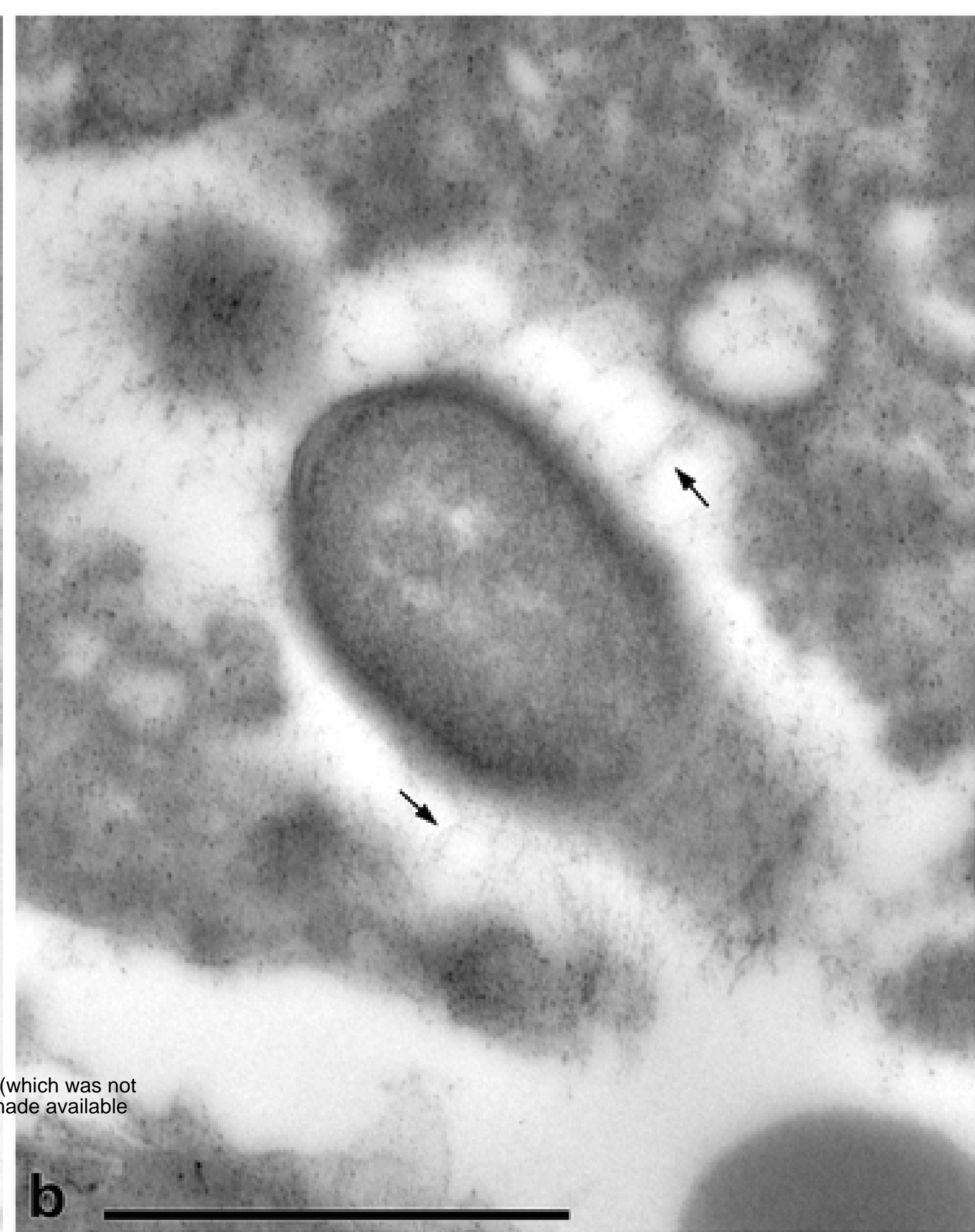
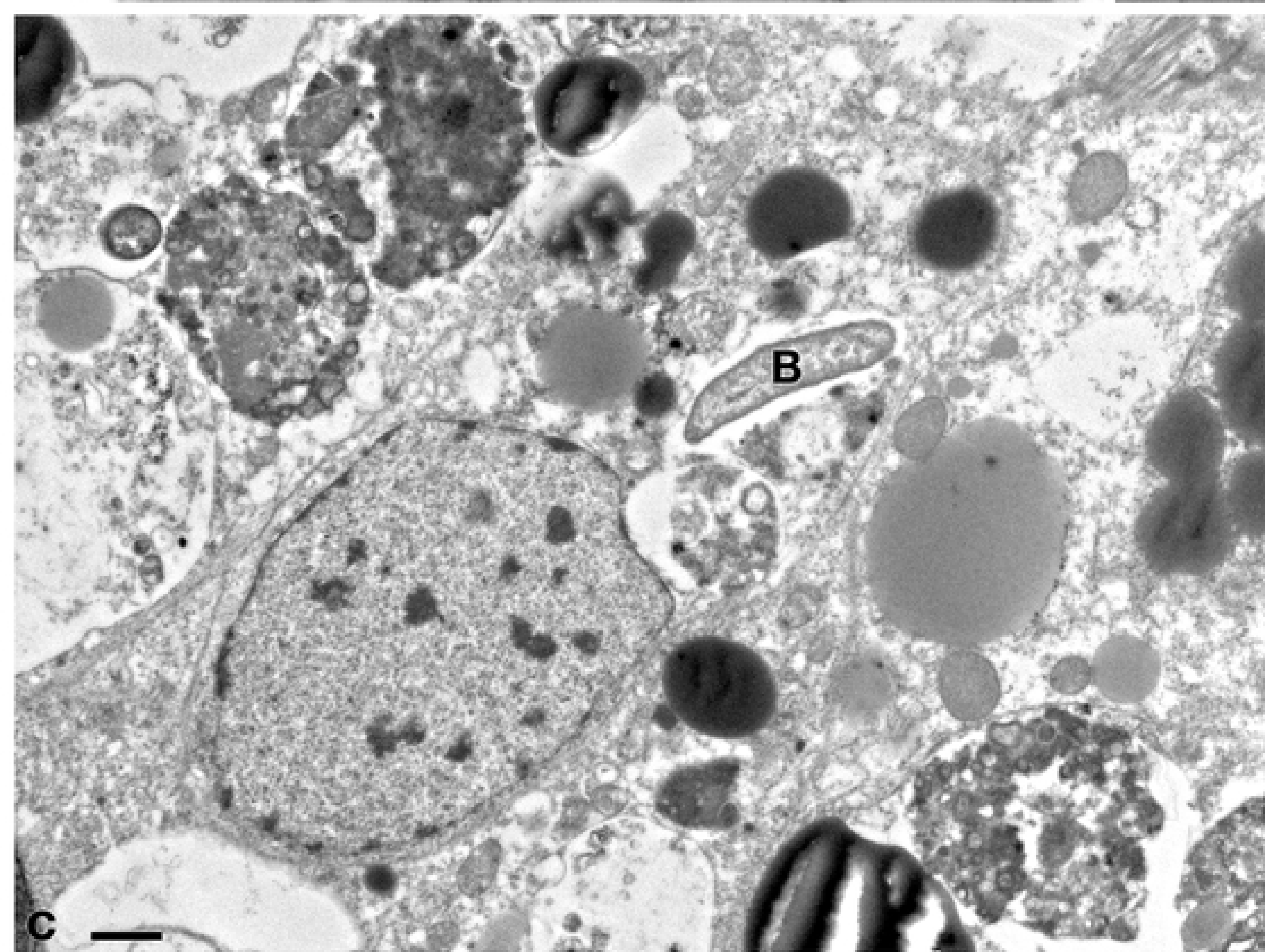


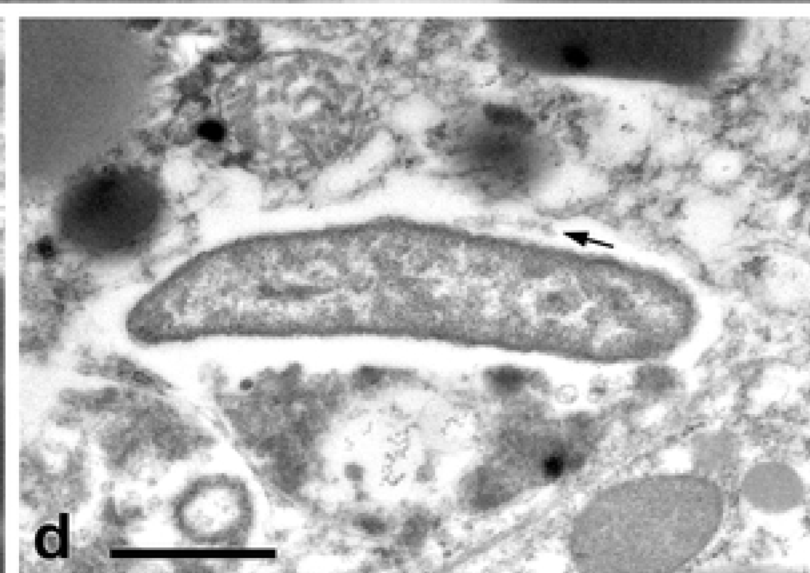
Figure 3

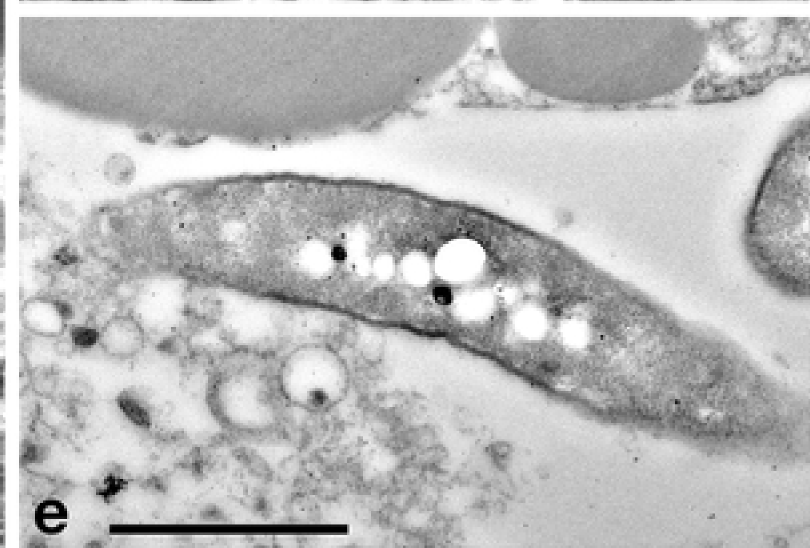


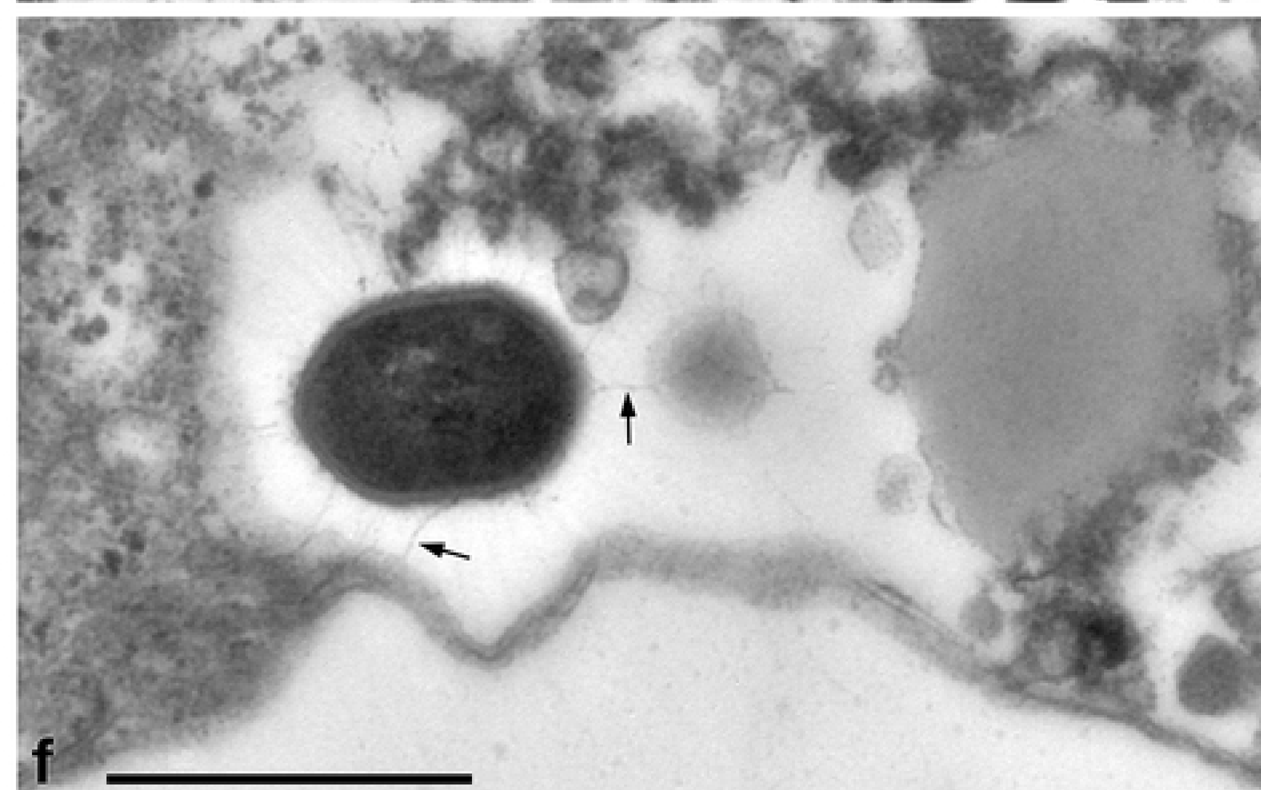
bioRxiv preprint doi: https://doi.org/10.1101/688770; this version posted July 2, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.











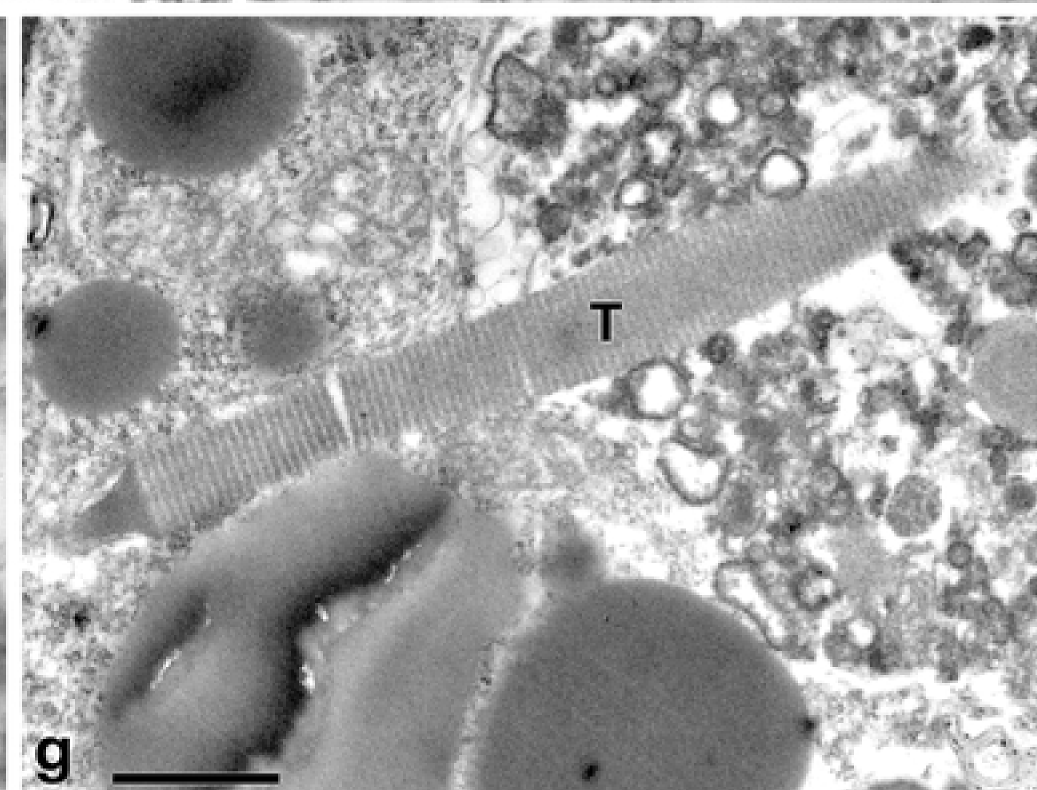
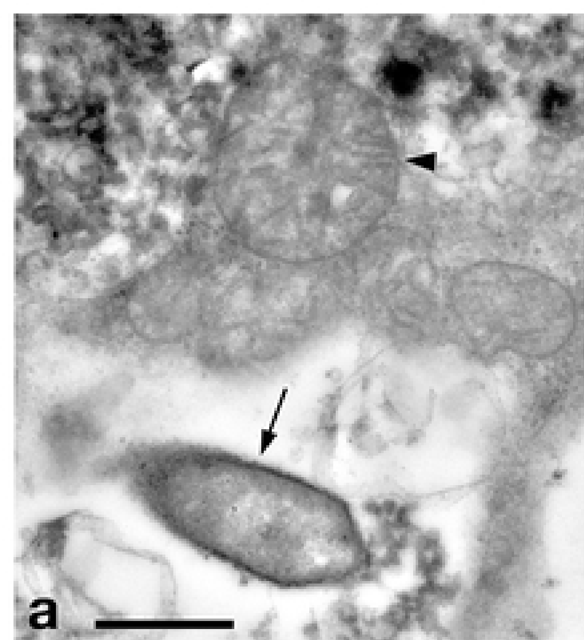


Figure 4



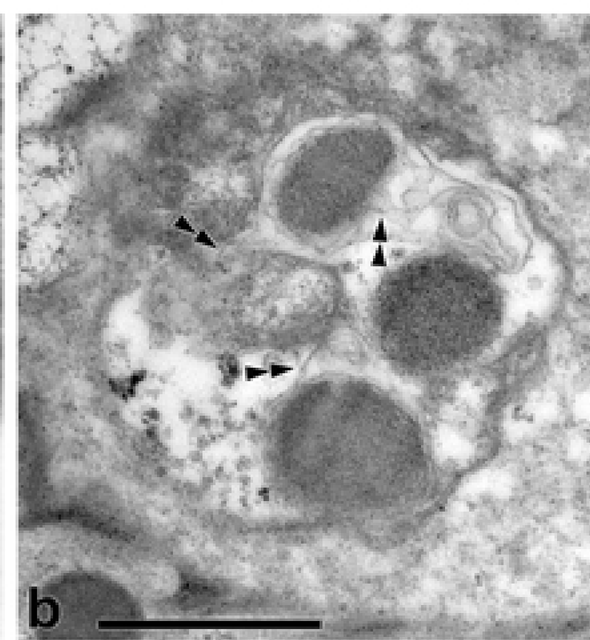


Figure 5