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Aquastella gen. nov.: A new genus of saprolegniaceous oomycete rotifer parasites related to *Aphanomyces*, with unique sporangial outgrowths

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1 ***Aquastella* gen. nov.: a new genus of saprolegniaceous oomycete rotifer parasites**
2 **related to *Aphanomyces*, with unique sporangial outgrowths**

3

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24

25 **ABSTRACT**

26 The new oomycete genus *Aquastella* is described to accommodate two new species of
27 parasites of aquatic rotifers observed in Brooktrout Lake, New York State, USA.
28 Sequencing of 18S rRNA and phylogenetic analysis of both species placed them within
29 the oomycetes in the order Saprolegniales, in a clade closely related to *Aphanomyces*.
30 The parasites formed a lobed, coenocytic thallus within the rotifer body. The two
31 *Aquastella* species were morphologically distinct from other rotifer parasites as the
32 developing sporangia penetrated out through the host body to produce tapered
33 outgrowths. These outgrowths did not function as discharge tubes for spore release and
34 were not involved in the capture of prey. *Aquastella attenuata* produced long, narrow,
35 tapering, finger-like outgrowths, whilst *Aquastella acicularis* produced shorter, spike-like
36 outgrowths that tapered to a sharp point. Spore cleavage was intrasporangial with spore
37 release through exit tubes. *Aquastella attenuata* produced primary zoospores, whereas *A.*
38 *acicularis* released spherical primary aplanospores (cysts), more typical of other genera
39 in the *Aphanomyces* clade.

40

41 Keywords: *Aquastella*, Saprolegniales, Oomycetes, rotifer parasite, outgrowths

42 Running title: New genus of saprolegniaceous oomycete rotifer parasites

43

44 **Introduction**

45

46 Records of oomycete parasites of rotifers are uncommon with only about 15 recognised
47 species, from both peronosporalean and saprolegnialean lineages as well as some early
48 diverging clades. The majority of these infect bdelloid rotifers in wet terrestrial habitats,
49 with only a few species infecting rotifers in aquatic habitats, such as ponds and lakes.
50 For instance, some species of *Haptoglossa* infect bdelloid *Adenita* rotifers (Barron 1990),
51 and parasitism of loricate *Distyla* rotifers and their eggs (now genus *Lecane*) by
52 *Myzocytiopsis* (Karling 1944) and *Chlamydomyzium* (Glockling & Dick 1997) species
53 has been reported. Parasitism and predation of aquatic rotifers by species in the
54 Saprolegniales appear rare, with the best known example being *Sommerstorffia spinosa*
55 Arnaudow (Arnaudow 1923a,b; Sparrow 1929; Karling 1952; Prowse 1954; Czczuga &
56 Próba 1980; Saikawa & Hoshino 1986). This species was reported to capture *Distyla*
57 rotifers on the apices of short predacious hyphal branches (Arnaudow 1923a,b). Karling
58 (1952) found that *Sommerstorffia* could capture *Monostyla* (*Lecane*) and *Colurella*
59 (*Colorus*) rotifers both by means of the narrow (rostrate) tips of predacious branches as
60 described by Arnaudow (1923a,b) and by specialized adhesive flask-shaped infective
61 spores which trapped rotifers which tried to ingest them. Arnaudow (1923a,b) regarded
62 *Sommerstorffia* as being closely related to *Aphanomyces*, and this placement was
63 accepted by Johnson *et al.* (2004). Sparrow (1929) and Karling (1952) agreed with the
64 classification of *Sommerstorffia* in the Saprolegniales because of its achlyoid mode of
65 spore release and subsequent zoospore development. However, no DNA sequence data
66 are available yet to confirm the placement of *Sommerstorffia* in the *Aphanomyces* clade.

67 A small number of other rotifer-infecting species have been described in saprolegnialean
68 genera including *Aphanomyces* and *Hydatinophagus*, and the monotypic genera
69 *Synchaetophagus* and *Endosphaerium*. However, all of these descriptions are based on
70 single published observations. The genus *Aphanomyces* contains a species that is
71 reported to infect rotifers, *A. gordajeverae* (Skvortzow 1925). The rotifer infecting genus
72 *Hydatinophagus* contains two described species, *H. apsteinii* (Valkanov 1931, 1932) and
73 *H. americanus* (Bartsch & Wolf 1938), although Scott (1961) later transferred this genus
74 to *Aphanomyces* (Scott 1961). Of the monotypic genera *Synchaetophagus balticus* was
75 described as a parasite of marine rotifers in the Baltic Sea (Apstein 1910), but no
76 information about reproduction was given. *Endosphaerium funiculatum* (D'Eliscu 1977)
77 was described as a parasite of rotifers and nematodes living in the mantle cavity of
78 bivalve molluscs. Many of these early descriptions lacked detailed morphological criteria
79 to include them as bona fide saprolegnialian genera and most are considered by Dick
80 (2001) to be species *incertae sedis*. Recently, an oomycete rotifer parasite was isolated
81 from *Asplanchna* rotifers and confirmed by its 18S sequence analysis to be associated
82 with a *Pythium* clade (Thomas *et al.* 2011).

83 Plankton samples from Brooktrout Lake in the Adirondack Mountains in New York
84 State, collected between 2005 and 2011, revealed rotifers which were infected with two
85 oomycete species with distinctive morphologies (Molloy *et al.* 2013). One species was
86 specific to *Keratella taurocephala* and the other was specific to both *Polyarthra vulgaris*
87 and *Ploesoma truncatum* (Molloy *et al.* 2013). Infection produced a characteristically
88 saccate and lobed holocarpic thallus inside the rotifer bodies. Uniquely, in addition to
89 exit tubes typical of oomycete parasites, tubular thallus outgrowths were also produced

90 within 24 h of host death. Prior to the report of Molloy *et al.* (2013), such external
91 outgrowths had not been recorded for any rotifer parasite. Here we propose the new
92 genus *Aquastella* for these new rotifer parasites and describe the taxonomy, phylogeny,
93 and structure of two new species, *Aquastella attenuata* and *Aquastella acicularis*.

94

95

96

97 **Materials and methods**

98

99 *Sample collection and processing*

100 Plankton samples were collected and prepared for light microscopy and SEM in
101 accordance with Molloy *et al.* (2013). In addition, specimens of *P. vulgaris* infected with
102 *A. acicularis* were prepared for TEM following Beakes & Glockling (1998).

103

104 *Molecular techniques*

105 Infected field-collected rotifers and cultivated material (see *Oomycete cultivation* section)
106 were preserved in 2X CTAB (James *et al.* 2008) until DNA analysis could be performed.

107 Cells were homogenized using glass beads (a mixture of 3.0 and 0.3 mm) in a Retsch

108 MM301 ball mill. Homogenates were extracted once with (24:1) chloroform-isoamyl

109 alcohol, and DNA was precipitated with an equal volume of isopropanol overnight at -20

110 °C. DNA extracts were re-suspended in 25 µl of H₂O.

111 PCR of the 18S ribosomal RNA gene was performed using ExTaq DNA Polymerase

112 (TaKaRa) using 5 µl of DNA extract in a reaction volume of 12.5 µl. Amplification was

113 performed on the *P. vulgaris* parasite using oomycete specific primers SRSt-1F (5'-
114 AAACTGCGAATGGCTCATTAT-3') and SRSt-1R (5'-
115 AGTTTATGGTTAAGACTACGATG-3'). Amplification of the *K. taurocephala*
116 parasite utilized primers SR1R (Vilgalys & Hester 1990) and SR6.1 (Parrent & Vilgalys
117 2009). The amplification profile was: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 54 °C
118 for 30 s, 72 °C for 1.5 min; and a final extension of 72 °C for 7 min. PCR amplicons
119 were purified with ExoSAP-IT (USB), and sequenced on an ABI 3730 at the University
120 of Michigan Sequencing Core (Ann Arbor, Michigan). The 18S rRNA sequences for *A.*
121 *acicularis* and *A. attenuata* have been deposited in GenBank under accession numbers
122 KF294791 and KF294792, respectively.

123

124 *Molecular data analysis*

125 Sequence chromatograms were edited and assembled using Sequencher (Gene Codes).
126 Oomycete 18S rRNA sequences were retrieved from GenBank, and manually aligned to
127 the rotifer parasite sequences in MacClade (Maddison & Maddison 2000). After
128 removing ambiguous regions of the alignment, 1700 characters remained. The best-
129 fitting model of evolution under maximum likelihood was selected using the program
130 jModelTest 0.1.1 (Posada 2008). The best-fitting model using the Akaike Information
131 Criterion was TIM3+I+ Γ . This model was used to start a maximum likelihood search
132 using the program PhyML 3.0 (Guindon & Gascuel 2003) with support estimated using
133 100 bootstrap pseudo-replicates.

134

135 *Oomycete cultivation*

136 Specimens of infected rotifers were isolated at a pre-cleavage stage in development when
137 outgrowths were just beginning to protrude through the rotifer body. Specimens were
138 placed into separate slide cultures using the hanging-drop technique. The infected rotifer
139 was picked up with a glass needle and put on a sterile glass cover slip in a drop (10 μ l) of
140 insect cell culture media (SF-900 II SFM) diluted to 50% and containing 1 μ l of the
141 antibiotics penicillin and streptomycin. The cover slip was inverted onto a sterile cavity
142 slide and sealed with sterile water. The slide culture was placed in a Petri dish with some
143 filter papers moistened with sterile water, sealed with Parafilm and held at 4 °C or
144 ambient room temperature. Slides were checked daily for any contamination and any
145 signs of growth.

146

147

148

149 **Results**

150

151 *Phylogenetic analysis*

152 Two related but non identical (98.2% identity) 18S rRNA sequences were obtained for
153 the two parasites. One sequence was obtained from infected *K. taurocephala*. The other
154 was obtained from infected *P. vulgaris* and also from cultivated material grown from an
155 infected *P. vulgaris*. The latter two sequences showed 100% identity. The 18S rRNA
156 phylogeny showed the two sequences grouped within the Saprolegniales together with
157 *Aphanomyces* isolates from fish (*Aphanomyces invadans*) and from *Daphnia*
158 (*Aphanomyces* sp. APH1) (Fig 1). The sequences were not related to the sequence from a

159 parasite of *Asplanchna* rotifers (GU270938.1) (Thomas *et al.* 2011), which grouped with
160 the Pythiales (Fig 1). In addition to showing the placement of *Aquastella*, the tree also
161 shows how widespread pathogens of invertebrates and vertebrates are within the
162 oomycetes.

163

164 *Aquastella* life cycles and morphology

165 The life cycles of the two parasites are incompletely known, but their probable life cycles
166 are presented in Fig 2. *Aquastella attenuata* appears to release zoospores, although our
167 evidence to support this is scant (Fig 2a, stage 5). *Aquastella acicularis* produces
168 primary aplanospores (Fig 2b, stage 5). We presume that a secondary type zoospore
169 emerges in both species (Fig 2). The morphology of the two parasites in their different
170 rotifer hosts differed subtly, but both species produced external sporangial outgrowths,
171 which projected out from the host, giving them a generally star-like appearance (Fig 2).
172 The thallus in both species appeared to be irregularly lobed, aseptate, and coenocytic.

173

174 *Development of Aquastella attenuata*

175 *Keratella taurocephala* is a loricate species, and dead infected individuals had long, rigid,
176 tapering outgrowths of *A. attenuata* penetrating out from the soft tissue in areas not
177 covered by the lorica at the anterior and posterior ends (Figs 2a, 3, 11). Infection of *K.*
178 *taurocephala* appeared to be via encysted zoospores as spherical cysts, about 5.0 μm
179 diam, were observed penetrating a rotifer egg with narrow germ tubes (Fig 10). Similar
180 cysts were also recorded attached to the lorica of a *K. taurocephala* observed under the
181 SEM (Fig 14). Early stages in thallus growth were only seen in histological specimens of

182 infected *K. taurocephala* (Fig 8). The young thalli were already branching and spreading
183 throughout the rotifer tissues, and at this stage appeared narrow with vacuolate regions
184 (Fig 8). A slightly more advanced stage in thallus development, seen in serial sections
185 several microns apart, indicated a broader thallus which appeared continuous and
186 coenocytic (Fig 16a-b). The thallus profiles were dispersed throughout the host body
187 tissues and organs. Thalli grew irregularly inside the host body and were often saccate
188 and lobed (Fig 10). Serial sections through mature infections showed an extensive and
189 convoluted hyphal-like thallus, which contained large vacuoles and peripheral cytoplasm
190 (Fig 17a-c). At this stage of infection there was little remaining rotifer tissue (Fig 17a-c)
191 and tapered sporangial outgrowths penetrated out from the host at the anterior and
192 posterior ends (Figs 3, 11, 17a-c). There was no penetration of outgrowths from the
193 dorsal or mid ventral sides of the rotifer body (Figs 11, 17). Infected rotifers had as few
194 as 1 and as many as 7 outgrowths, but typically between 2 and 5 were produced (Figs 3,
195 4, 11). Fully extended outgrowths were long and slender and tapered very gently from
196 the base to the rounded apex (Figs 3, 11). The outgrowths grew to up to 150 μm in
197 length, but were more commonly about 120 μm long. Exit tubes were produced prior to
198 spore release and, although similar to the sporangial outgrowths, could be distinguished
199 because they were considerably shorter (about 30 μm) and of relatively constant diameter
200 (Figs 4, 7, 15). It was not unusual for more than one exit tube to form and up to 3 or 4
201 exit tubes were sometimes observed (Fig 7). Unlike the outgrowths projecting from the
202 anterior and posterior of the host, the exit tubes penetrated from anywhere on the body
203 including the mid ventral surface (Figs 4, 18a-c). Exit tubes developed only when the

204 infection was in an advanced stage of development and the rotifer tissue had been
205 assimilated by the parasite (Fig 18a-c).

206 Histological studies showed that thallus development varied between different parts of
207 the same thallus so that spore formation was not synchronous. A mature specimen had
208 thallus profiles in various stages of development between early cleavage and complete
209 separation of what appear to be zoospore profiles (Fig 12). We did not see the expulsion
210 of zoospores from the sporangia, but biflagellate zoospores were evident on a lactophenol
211 blue-stained slide of an infected *K. taurocephala* prepared at a very mature stage in
212 development (Fig 13). Using video microscopy, zoospore movement was also recorded
213 in a *K. taurocephala* that had been isolated into a clean glass dish (Supplementary
214 Video). This infection had recently discharged most of its spores (Fig. 7), but there were
215 still a few motile zoospores inside the rotifer body, which is never observed in
216 *Aphanomyces* spp. Outside of the thallus many of the recently discharged spores were
217 spherical and immotile and had presumably encysted. This suggests the primary
218 zoospore stage is extremely short-lived. This latter infected *K. taurocephala* had released
219 spores in several bursts, and three open exit tubes were present whilst another exit tube
220 remained intact (Fig 7). A large number of spherical cysts were recorded around this
221 specimen (Fig 7), but they did not form the tight balls of encysted spores at the mouth of
222 the exit tubes that characterises typical aphanomycoïd spore discharge. The cysts
223 adhered to the bottom of the glass Petri dish and could not be dislodged by water currents
224 created with a pipette (Fig 6). A stained histological median section of a cyst showed it
225 to have a smooth outer profile and a central nucleus surrounded by several organelles
226 which could be mitochondrial profiles (Fig 9). It is likely that a secondary type zoospore

227 is produced as some empty cysts were observed outside a specimen that had discharged
228 its spores. In addition, a cyst with a discharge pore is shown on a *K. taurocephala* lorica
229 in Fig 15. It is presumably these secondary type zoospores (Fig 2a – 7) which settle and
230 encyst to initiate infection (Figs 2a-1, 10).

231

232 *Development of Aquastella acicularis*

233 There were some general morphological differences in *A. acicularis* between the two
234 infected rotifer host species. *Polyarthra vulgaris*, which is an illoricate species, appeared
235 star-like and echinulate in mature stages of infection as the spike-like outgrowths of *A.*
236 *acicularis* projected through the body wall at various places (Figs 2b, 20, 21). When
237 *Ploesoma truncatum*, a loricate species, was infected by *A. acicularis*, individuals did not
238 typically appear as star-like as they had fewer outgrowths, which frequently only
239 penetrated out from the ventral side of the host (Fig 26).

240 Infection and very early stages of thallus formation were not observed in *A. acicularis*,
241 although we suggest that, as in the previous species, infection is initiated by the
242 encystment on the host lorica of infective secondary zoospores (Fig 2b - 6 and 1). In this
243 species, the thallus in infected rotifers quickly becomes broad, saccate, and multi-lobed
244 (Figs 19, 29). The thalli ultimately develop into a complex array of broad, saccate thalli
245 that fill the host body cavity, often completely obscuring thallus detail (Figs 20, 25).

246 Serial TEM sectioning confirmed that the thallus inside the rotifer body was a single,
247 aseptate, coenocytic unit, with broad lobes which were constricted at the point of
248 branching (Fig 29). Spherical lobes in *P. vulgaris* were seen by TEM to often be located
249 just inside the rotifer cuticle (Figs 28, 29). TEM of young stages in thallus development

250 showed lobed thalli, with fairly dense content, few nuclear profiles, small vacuoles, and
251 many scattered dense body vesicles (DBVs) (Fig 28). The developing pre-cleavage
252 thallus was highly vacuolate (Figs 29, 30, 31), with peripherally distributed cytoplasm in
253 which the nuclei were evenly distributed (Fig 31). Developing thalli contained
254 cylindrical or ovoid mitochondrial profiles with tubular cristae (Fig 32). As the thalli
255 matured, the nuclei became pyriform in profile, with the pointed apex, that was often
256 associated with apical centrioles/kinetosomes (Fig 33) oriented towards the thallus wall
257 (Fig 34). The nuclei were frequently surrounded by two or three cisternae of rough
258 endoplasmic reticulum and often one or two Golgi dictyosomes, which at this stage were
259 generating small electron-transparent vesicles (Fig 33). In these pre-spore formation
260 thalli, there was no evidence of K-bodies or encystment vesicles normally associated with
261 sporangia in the Leptomitales and Saprolegniales (Beakes 1994).

262 As thallus development proceeded, the peripheral cytoplasm appeared to be forming
263 spore initials, in which the nuclei were surrounded by DBVs (Fig 34).

264 Outgrowths later protruded through the rotifer cuticle and could be seen to be external
265 extensions of the thalli (Figs 20, 21, 35). The outgrowths were broad at the base,
266 appearing rigid and spike-like as they tapered sharply to a point at the apex (Figs 20, 21,
267 22, 37). The outgrowths extended outwards from *P. vulgaris* from any part of its body
268 and as many as 15 outgrowths could be seen in a single specimen, although more usually
269 there were 4-8 (Fig 20). The developing outgrowths were packed with cytoplasm and
270 small vacuoles (Fig 36), which extended into the narrow tip (Fig 37). The tip of the
271 outgrowth did not contain any of the type of vesicles associated with adhesive or trapping
272 structures (Fig 37). TEM showed that the wall of the thallus thickened and appeared

273 more electron dense than the wall of the internal thallus (Figs 35, 36). Exit tubes formed
274 at a late stage in development. In infected *P. vulgaris* the exit tube was usually of a
275 constant diameter of around 8-10 μm and was shorter than the outgrowths, being about
276 30-50 μm long (Figs 21, 22, 23). In infected *P. truncatum*, however, exit tubes were
277 often longer and sometimes up to 100 μm long (Fig 25). Some rather poorly fixed TEM
278 of a *P. vulgaris* infected with *A. acicularis* revealed that the sporangium was packed with
279 fully differentiated walled primary cysts (Fig 38). A squashed and stained *P. vulgaris*
280 with a mature infection also revealed cysts (Fig 24). This suggests that primary cysts are
281 usually discharged from the mature sporangial thallus in this species. The cysts were uni-
282 nucleate and contained a large vacuole and mitochondria. Little detail could be gleaned
283 from this poorly fixed specimen, but one cyst had packets of tubular tripartite hairs
284 (TTH) running along the side of the nuclear envelope (Fig 39), which is typical of cysts
285 prior to formation of secondary zoospores.

286

287 *In vitro* cultivation of rotifer parasites

288 Growth of the oomycete species from infected *P. vulgaris* and *P. truncatum* rotifers was
289 very slow in liquid media (and had to mainly be grown at 4 °C to inhibit bacterial growth)
290 and initially produced many short finger-like processes from the thalli, which later
291 swelled, giving rise to spherical, cylindrical, lobed and irregular thalli (Fig 27). Growth
292 from both infected *P. vulgaris* and infected *P. truncatum* appeared identical and was
293 morphologically similar to its growth inside the rotifer host. Attempts to cultivate the
294 oomycete species isolated from *K. taurocephala* were unsuccessful, with no growth
295 noted.

296

297

298

299 **Taxonomy**

300

301 *Aquastella* D. P. Molloy & S. L. Glockling, **gen. nov.**

302 **Etymology:** ‘*Aquastella*’ (Latin), meaning ‘water star’, relating to the general star-like
303 appearance of the mature infection in the aquatic rotifer hosts.

304 **Description:** Thalli endobiotic, coenocytic, aseptate, and convoluted, sometimes initially
305 narrow, 5-15 µm diam, becoming broader with several irregular, saccate or spherical
306 lobes, 15-30 µm diam, producing elongate, spiked or tapered tubular outgrowths towards
307 maturity, 60-150 µm long X 2-10 µm wide. Sporangium producing one or more exit
308 tubes at maturity, up to 100 (usually 30) µm long x 3-7 µm diam. Sporogenesis
309 intrasporangial; zoospores or cysts released via the exit tube. Zoospores biflagellate,
310 encysting after discharge to form spherical cysts 4.0-6.0 µm diam. Sexual reproduction
311 not observed. Parasitic in the rotifers *Keratella taurocephala*, *Ploesoma truncatum*, and
312 *Polyarthra vulgaris*.

313 **Type:** *Aquastella attenuata* D. P. Molloy & S. L. Glockling

314

315 *Aquastella attenuata* D. P. Molloy & S. L. Glockling, **sp. nov.** (Figs 3-18)

316 **Etymology:** ‘*attenuata*’ (Latin), meaning attenuating, referring to the external sporangial
317 outgrowths.

318 **Description:** Thallus initially narrow and cylindrical, 5-12 μm diam., coenocytic,
319 aseptate, extensive, and convoluted, becoming broader, lobed, and irregular, 6-20 μm
320 diam., giving rise towards maturity to up to 7 long, gently tapering, rigid, finger-like
321 outgrowths extending outside the host from the ventral anterior and/or posterior ends.
322 Sporangial outgrowths up to 125 μm long (usually 80-100 μm long) x 4.5-5.5 μm
323 diameter at the base, tapering gradually to 2 μm diameter at the apex. Exit tube(s) up to
324 30 μm long (usually 15-25 μm long) x 3-6 μm diameter produced vertically from ventral
325 surface or from ventral anterior or posterior end of host. Primary zoospores encysting
326 shortly after release. Cysts spherical, 4.0 - 5.0 μm diam. Infecting *Keratella*
327 *taurocephala* rotifers.

328 **Holotype:** Fig 4; collected by D. P. Molloy on July 20, 2010 at Brooktrout Lake (43° 36'
329 00" N, 74° 39' 45" W), New York State, USA; in *Keratella taurocephala*.

330

331 *Aquastella acicularis* D. P. Molloy & S. L. Glockling, **sp. nov.** (Figs 19-39)

332 **Etymology:** ‘*acicularis*’ (Latin) needle-like, referring to the external sporangial
333 outgrowths.

334 **Description:** Thallus irregular, coenocytic, aseptate, and convoluted, with broad saccate,
335 subspherical or spherical lobes, up to 30 μm diam.; giving rise to up to 15 (usually 2-8)
336 rigid, spiked outgrowths projecting out from the host, up to 90 μm long (usually 60-70
337 μm long) x 7-10 μm wide at the base, tapering to a sharp point at the apex. Exit tube(s)
338 up to 100 μm long (usually 30-50 μm in *P. vulgaris*, 60-80 μm in *Ploesoma truncatum*) x
339 8-10 μm diam. Spore cleavage intrasporangial, forming walled cysts. Cysts 3.5-5.0 μm
340 diam. Infecting *Ploesoma truncatum* and *Polyarthra vulgaris* rotifers.

341 **Holotype:** Fig 23; collected by C. A. Siegfried on September 16, 2006 at Brooktrout
342 Lake (43° 36' 00" N, 74° 39' 45" W) New York State, USA; in *Polyarthra vulgaris*.

343

344

345 **Discussion**

346

347 *Taxonomic and phylogenetic placement of Aquastella*

348 Cultivation of *A. acicularis* in liquid insect cell culture media facilitated the sequencing
349 of this species using specific oomycete primers, although its growth in artificial culture
350 was extremely slow. Attempts to cultivate *A. attenuata* from infected *K. taurocephala*,
351 using the same methods as for *A. acicularis*, were unsuccessful.

352 Because of the morphological differences between these two new species and other
353 known oomycete rotifer parasites, and also due to their phylogenetic placement within
354 the Saprolegniales, we erected the new genus, *Aquastella*, to accommodate these novel
355 parasites with external thallus outgrowths. The SSU rRNA tree shows that these two
356 *Aquastella* species share a common ancestor with two other animal parasitic
357 *Aphanomyces* spp., and together form a discrete early diverging clade within the
358 Saprolegniales (Fig 1). The $\approx 2\%$ divergence in 18S rRNA sequence strongly suggests
359 that the host specific *Aquastella* parasites are different species, this being considerably
360 greater than the $\approx 0.5\%$ divergence between *Saprolegnia ferax* and *S. parasitica* and
361 similar to the divergence between *Phytophthora infestans* and *Plasmopara viticola*
362 ($\approx 2\%$). The most obvious relative to the *Aquastella* parasites would be *Sommerstorffia*
363 *spinosa*, which is mainly a predator of rotifers (Karling 1952) and is also considered to be

364 closely related to *Aphanomyces* (Johnson *et al.* 2004). Unfortunately, this species has not
365 yet been sequenced so we are unable to confirm a close phylogenetic relationship
366 between these two rotifer infecting genera. Recently Diéguez-Uribeondo *et al.* (2009), in
367 an ITS-based analysis of species in the genus *Aphanomyces*, have shown that the animal
368 parasites such as *A. astaci* and *A. invadans* fall into their own separate clade, as do the
369 plant pathogenic and saprophytic genera. In this study both *Aphanomyces* sequences
370 selected for comparison with *Aquastella* were from isolates infecting either *Daphnia*
371 (APH1) or fish (*A. invadans*). Unfortunately, there are no 18S sequences available for
372 these latter species to see whether these rotifer infecting species also fit into this overall
373 ecological pattern, or whether they may form yet another clade separate from
374 *Aphanomyces*. The only other parasite of aquatic rotifers for which sequence data are
375 available is a *Pythium* sp., which is not related to *Aquastella* as it is in the Pythiales
376 (Thomas *et al.* 2011). As the two *Aquastella* species grouped with sequences from
377 *Aphanomyces invadans* (fish parasite) and *Aphanomyces* sp. APH1 (*Daphnia* parasite), it
378 appears that a clade containing diverse parasites separate and sister to a clade containing
379 the more dominant water moulds in the Saprolegniaceae and Achlyaceae is emerging.
380 A recent revision of oomycete taxonomy has placed the genus *Aphanomyces*, together
381 with a number of soil born plant pathogens, such as *Pachymetra* and *Verrucalvus*, in an
382 amended Verrucalvaceae family (Beakes *et al.* 2013), and this appears to be the earliest
383 diverging clade with the Saprolegniales order. The key morphological characteristics of
384 this family is that they have relatively undifferentiated sporangia, even in eurcarpic
385 genera such as *Aphanomyces* and they form clusters of primary cysts that often form
386 spore balls around the orifice of the discharge tube or apical papillum. This feature, as

387 Karling (1952) noted for *Sommerstorffia*, is similar to holocarpic parasites of insect eggs
388 such as *Aphanomycoopsis*. In contrast, the mode of spore release in *Aquastella* differed
389 from that of *Sommerstorffia* and all other members of the group in that either zoospores
390 (in *A. attenuata*) or aplanospores (in *A. acicularis*) are released directly into the
391 environment and do not form the typical clusters of primary spores at the mouth of the
392 exit tube. The cysts of *Aquastella* are fairly uniform in size, being between 3.5 and 5.0
393 μm in diameter, whereas those of *Sommerstorffia* are reported to be larger, varying
394 between 6.8 and 10.2 μm (Karling 1952). The release of both aplanospore cysts and
395 zoospores does occur in some oomycete genera including those infecting nematodes and
396 rotifers such as *Chlamydomyrium* (Barron 1976; Glockling & Dick 1997; Glockling &
397 Beakes 2000), *Myzocytiopsis* (Barron 1976; Glockling & Beakes 2000; Dick 2001), and
398 *Haptoglossa* (Drechsler 1946; Glockling & Beakes 2000).

399

400 *Growth and development*

401 Young stage thalli of *A. attenuata* observed in histological section were already
402 branching and running through the rotifer tissues. Although these young, narrow, thalli
403 contained vacuolar regions, broader thalli, which we interpret as being more developed,
404 had a denser cytoplasm. We did not observe early infection in *A. acicularis* although the
405 specimen in Fig 19 contained a lobed thallus which had not yet produced any outgrowths.
406 Histology of *A. attenuata* and TEM of *A. acicularis* indicated that the young thallus has a
407 fairly dense cytoplasm, whereas the maturing, pre-cleavage thallus becomes highly
408 vacuolated, with a large central vacuole forcing all the cytoplasmic contents to the
409 periphery. The transient nature of the DBVs in *A. acicularis* and their ability to lessen

410 and lose their electron dense component during the maturation of the thallus, suggest that
411 they contribute to vacuole formation. Although we did not observe advanced stages in
412 sporogenesis, the DBVs and vacuoles appeared to organise the cytoplasmic content prior
413 to cleavage, spatially separating the nuclear units. Although seen at a much lower
414 resolution than TEM, the histology of mature thalli of *A. attenuata* also appeared to show
415 cleavage furrows (see Figs 12, 18). In the mature pre-cleavage thallus of *A. acicularis*,
416 nuclei lost their spherical profiles, becoming more pyriform and oriented towards the
417 thallus wall. These nuclei appeared active, with apical centrioles, and their associated
418 Golgi dictyosomes appeared to be generating vesicles.

419

420 *Spore production and Infection*

421 In *A. attenuata*, if zoospores are released as we have suggested, they encyst very quickly.
422 Profiles of what appear to be fully cleaved zoospores were recorded inside sporangia (Fig
423 12), and flagellate zoospores and encysting zoospores were observed near empty exit
424 tubes. Large areas of scattered cysts were seen shortly after spore release, and our
425 interpretation of this is that zoospores had been released and had encysted very shortly
426 afterwards. Empty cysts, seen in some lactophenol blue-stained specimens, and an open
427 empty cyst in an SEM specimen indicated the emergence of a secondary type zoospore in
428 *A. attenuata*. The walled cysts in mature sporangia of *A. acicularis* as seen by TEM are
429 evidently the primary spore produced by this species, but the presence of TTH packets in
430 the cysts suggest that a motile zoosporic phase will follow. It is likely that these primary
431 cysts give rise to a secondary type zoospore in order to actively locate a healthy
432 swimming rotifer host. However, we did not record zoospores or initiation of infection in

433 this species. Infection in *A. attenuata* was by means of encysted spores, which were
434 observed directly penetrating the egg of a *K. taurocephala* by means of narrow germ
435 tubes (Fig 10). Both the adult rotifer and the egg were already infected and contained
436 thalli.

437

438 *Outgrowths*

439 Several hypotheses were discussed by Molloy *et al.* (2013) as to the function of the
440 outgrowths in *Aquastella*. In comparison with infection in the apparently related genus
441 *Sommerstorffia*, it does not appear that the elongate appendages in this genus play any
442 direct role in attracting and ensnaring potential rotifer victims. *Sommerstorffia* forms an
443 external mycelium that has terminal tapering spines which serve to trap rotifers
444 (Arnaudow 1923a,b; Karling 1952; Akamatsu & Saikawa 2005). In addition, the
445 germinated secondary cysts develop into specialised infective sporelings, with a terminal
446 sticky knob which traps potential prey (Akamatsu & Saikawa 2005). However, the
447 spine-like appendages in *Aquastella*, although superficially reminiscent of the trapping
448 structures in *Sommerstorffia*, lack the apical accumulation of specialised vesicles, which
449 Akamatsu & Saikawa (2005) consider to contain adhesive material which helps trap the
450 rotifer. Rotifers were never observed attached to the tapering outgrowths of *Aquastella*
451 (Molloy *et al.* 2013). The outgrowths in *Aquastella* resemble rotifer body wall
452 projections, which are generally accepted to have evolved to deter rotifer predation and to
453 maintain their position in the water column by slowing their descent (reviewed in Molloy
454 *et al.* 2013). Molloy *et al.* (2013) hypothesized that the outgrowths formed by *Aquastella*

455 evolved to serve the same two functions, suggesting convergent evolution of host rotifer
456 and oomycete parasite morphological traits.

457

458

459

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461

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- 570

571 **Legend for Figures**

572 **Fig 1.** A maximum likelihood phylogeny estimated for the 18S rRNA sequences of
573 *Aquastella attenuata* and *Aquastella acicularis*. Values above nodes indicate bootstrap
574 support measures where above 60%. GenBank values are given for each sequence
575 following the taxonomic name.

576 **Fig 2.** Comparative life cycles of a) *Aquastella attenuata*. 1. Encysted zoospores on host;
577 2. Thallus inside host; 3. External outgrowths from maturing thallus; 4. Cleaving
578 sporangium with exit tube; 5. Zoospores released from sporangium via exit tube; 6.
579 Cysts; 7. Secondary zoospores. b) *Aquastella acicularis*. 1. Encysted zoospores; 2.
580 Thallus inside host; 3. External outgrowths from maturing thallus; 4. Cleaving thallus
581 with exit tube; 5. Cysts released from sporangium via exit tube; 6. Zoospores. (Not drawn
582 to scale).

583 **Fig 3.** *Keratella taurocephala* with several elongate outgrowths of *Aquastella attenuata*
584 (arrows). Scale = 50 μm

585 **Fig 4.** *Keratella taurocephala* with empty outgrowths (black arrows) and open exit tubes
586 (white arrows). Scale = 50 μm

587 **Fig 5.** Encysting zoospores and cysts. Scale = 10 μm

588 **Fig 6.** Cysts adhering to the bottom of a glass dish. Scale = 10 μm

589 **Fig 7.** *Keratella taurocephala* with several open exit tubes (white arrows) and one intact
590 exit tube (black arrow), having discharged many spores which have encysted. Scale = 50
591 μm

592 **Fig 8.** Stained histology section of host with profiles of young *Aquastella attenuata* thalli
593 (*) running through the rotifer tissues (t). Scale = 8 μm

594 **Fig 9.** Stained histology section through a cyst, revealing nucleus and probable
595 mitochondria. Scale = 5 μm

596 **Fig 10.** Lactophenol blue-stained *Keratella taurocephala* containing cylindrical and
597 saccate thalli (*), with an egg into which encysted spores are penetrating with narrow
598 germ tubes (arrows). Scale = 10 μm

599 **Fig 11.** SEM of *Keratella taurocephala* with long, narrow outgrowths (black arrows)
600 extending from under the lorica at the anterior and posterior ends of the host. An exit
601 tube (white arrow) is extending from a more central ventral region. Scale = 100 μm

602 **Fig 12.** Stained histology section of cleaved and cleaving sporangial profiles, showing
603 fully cleaved spores (z). Scale = 10 μm

604 **Fig 13.** Lactophenol blue-stained whole mount showing flagellate zoospores (white
605 arrows) near an open exit tube (e) and empty outgrowths (o). Scale = 8 μm

606 **Fig 14.** SEM of two cysts (c) on a *Keratella taurocephala*. Scale = 5 μm

607 **Fig 15.** SEM showing outgrowth (o) and exit tube (e) extending from the host. Note the
608 empty cyst (c) with what appears to be an apical opening. Scale = 8 μm

609 **Fig 16, a-b.** Serial histology section of young infection of *Aquastella attenuata* in
610 *Keratella taurocephala* showing thallus profiles (*) amongst the host tissues (t). Note the
611 thick covering of the lorica (white arrows). b) Some thallus profiles show nuclei with
612 nucleoli (black arrows). Scale = 8 μm

613 **Fig 17, a-c.** Serial histology sections of developing, vacuolated, thallus, showing
614 outgrowths at the anterior and posterior ends of the host (black arrows), penetrating the
615 body wall. The thick lorica covering the dorsal side is indicated with white arrows.
616 Scale = 10 μm

617 **Fig 18, a-c.** Serial histology sections through mid cross-section of host showing thick,
618 loricate dorsal covering (white arrows). Thallus is maturing into sporangium and has
619 cleavage furrows visible (*). An exit tube is penetrating out through the mid ventral body
620 wall (black arrows). Scale = 10 μm

621 **Fig 19.** Lobed thalli (*) of *Aquastella acicularis* inside *Polyarthra vulgaris*. Scale = 15
622 μm

623 **Fig 20.** Maturing infection with several outgrowths (arrows). Scale = 15 μm

624 **Fig 21.** *Polyarthra vulgaris* containing empty sporangia and outgrowths (black arrows).
625 Exit tube (white arrow) Scale = 10 μm

626 **Fig 22.** Empty saccate and lobed sporangium with spiked outgrowth (black arrow) and
627 open exit tube (white arrow). Scale = 15 μm

628 **Fig 23.** Lactophenol blue-stained whole mount of mature infection with cleaved content,
629 showing sporangial outgrowths (black arrows) and intact exit tube (white arrow). Scale =
630 15 μm

631 **Fig 24.** Lactophenol blue-stained fully cleaved cysts in sporangium in *Polyarthra*
632 *vulgaris*. Scale = 5 μm

633 **Fig 25.** Infection in *Ploesoma truncatum* showing dorsal, loricate side with many
634 spherical lobes underneath (*). Note the long exit tubes (white arrows). Scale = 30 μm

635 **Fig 26.** Lobed thalli inside *Ploesoma truncatum* with a spiked outgrowth and an exit tube.
636 Note the toes (*) under the lorica. Scale = 10 μm

637 **Fig 27.** Cultivated growth of *Aquastella acicularis*. Scale = 10 μm

638 **Fig 28.** TEM of young stage thallus with a thallus lobe just inside the host body wall
639 (arrow). Note the dense cytoplasm with few nuclear profiles (n) and many small dense
640 body vesicles (DBVs). Scale = 10 μm

641 **Fig 29.** Saccate thallus with large vacuole (v) and lobed thalli inside the *Polyarthra*
642 *vulgaris* host. Scale = 10 μm

643 **Fig 30.** Developing thalli with large vacuole (v) on one lobe. Scale = 10 μm

644 **Fig 31.** Developing thallus with several large central vacuoles (v). Note the peripheral
645 position of the nuclei (n) and dense body vesicles (d). Scale = 2 μm

646 **Fig 32.** Periphery of thallus showing thallus wall (arrows), mitochondria (m) and DBVs
647 (d). Scale = 1 μm

648 **Fig 33.** Nucleus (n) inside an outgrowth, with an associated Golgi dictyosome (g). Note
649 the paired centrioles/kinetosomes (*) at the nuclear apex. Scale = 1 μm

650 **Fig 34.** Mature pre-cleavage thallus containing vacuoles (v), mitochondria (m) and DBVs
651 (d). Note the pyriform nuclei (n) and apical centriole (*). Nuclei are oriented towards
652 the thallus wall (arrows). Scale = 2 μm

653 **Fig 35.** Section of outgrowth from a lobed thallus with a large vacuole (v). Note the wall
654 of the outgrowth (arrow). Scale = 10 μm

655 **Fig 36.** Basal section of outgrowth containing dense cytoplasm with nuclei (n) and small
656 vacuoles (v). Note the dense wall (w) of the outgrowth. Scale = 5 μm

657 **Fig 37.** Apical region of outgrowth showing the tip (arrow) and small vacuoles (v). Scale
658 = 2 μm

659 **Fig 38.** Fully cleaved sporangium containing cysts with single nucleus (n) and large
660 vacuole (v). Note the cysts wall (arrow). Scale = 2 μm

661 **Fig 39.** Cyst nucleus (n) with associated TTH packet (*). Scale = 250 nm