7-2014

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

Daniel P. Molloy

Sally L. Glockling

Clifford A. Siegfried

Gordon W. Beakes

Timothy Y. James

See next page for additional authors

Recommended Citation

Molloy DP, Glockling SL, Siegfried CA, Beakes GW, James TY, Mastitsky SE, Wurdak ES, Giamberini L, Gaylo MJ, Nemeth MJ. 2014. *Aquastella gen. nov.*: A new genus of saprolegniaceous oomycete rotifer parasites related to *Aphanomyces*, with unique sporangial outgrowths. *Fungal Biology* 118(7): 544-558.

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

Sally L. GLOCKLING [a], *Daniel P. MOLLOY* [b,c*], *Gordon W. BEAKES* [d], *Timothy Y. JAMES* [e], *Clifford A. SIEGFRIED* [b], *Sergey E. MASTITSKY* [f], *Elizabeth WURDAK* [g], *Laure GIAMBERINI* [h]

[a] 135 Brodrick Road, Eastbourne BN22 9RA, East Sussex, UK

[b] Division of Research & Collections, New York State Museum, New York State Education Department, Albany, NY 12230, USA

[c] Department of Biological Sciences, State University of New York, 1400 Washington Avenue, Albany, NY 12222, USA

[d] School of Biology, Newcastle University, Newcastle upon Tyne NE1 7RU, UK

[e] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109, USA

[f] RNT Consulting, Ontario, Canada

[g] Department of Biology, Saint John's University, Collegeville, MN 56321, USA

[h] Université de Lorraine, CNRS UMR 7146, Campus Bridoux, Rue du Général Delestraint, F-57070 Metz, France

* Corresponding author. Department of Biological Sciences, State University of New York, 1400 Washington Avenue, Albany, NY 12222, USA. Telephone: +1 518 435 5126; fax: 1 518 677 5236.

E-mail address: dmolloy@albany.edu (D. P. Molloy)

ABSTRACT

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

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

Running title: New genus of saprolegniaceous oomycete rotifer parasites

Introduction

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

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

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

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

Materials and methods

Sample collection and processing

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

Molecular techniques

Infected field-collected rotifers and cultivated material (see *Oomycete cultivation* section) were preserved in 2X CTAB (James *et al.* 2008) until DNA analysis could be performed. Cells were homogenized using glass beads (a mixture of 3.0 and 0.3 mm) in a Retsch MM301 ball mill. Homogenates were extracted once with (24:1) chloroform-isoamyl alcohol, and DNA was precipitated with an equal volume of isopropanol overnight at -20 ºC. DNA extracts were re-suspended in 25 µl of H_2O.

PCR of the 18S ribosomal RNA gene was performed using ExTaq DNA Polymerase (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 ºC for 3 min; 35 cycles of 94 ºC for 30 s, 54 ºC
118 for 30 s, 72 ºC for 1.5 min; and a final extension of 72 ºC 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*

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

Results

Phylogenetic analysis

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

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

Aquastella life cycles and morphology

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

Development of Aquastella attenuata

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

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

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

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

Development of Aquastella acicularis

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

Infection and very early stages of thallus formation were not observed in *A. acicularis*, although we suggest that, as in the previous species, infection is initiated by the encystment on the host lorica of infective secondary zoospores (Fig 2b - 6 and 1). In this species, the thallus in infected rotifers quickly becomes broad, saccate, and multi-lobed (Figs 19, 29). The thalli ultimately develop into a complex array of broad, saccate thalli that fill the host body cavity, often completely obscuring thallus detail (Figs 20, 25). Serial TEM sectioning confirmed that the thallus inside the rotifer body was a single, aseptate, coenocytic unit, with broad lobes which were constricted at the point of branching (Fig 29). Spherical lobes in *P. vulgaris* were seen by TEM to often be located just inside the rotifer cuticle (Figs 28, 29). TEM of young stages in thallus development

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

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

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

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

In vitro cultivation of rotifer parasites

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

Taxonomy

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

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

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

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

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

Etymology: '*attenuata'* (Latin), meaning attenuating, referring to the external sporangial outgrowths.

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

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

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

Etymology: '*acicularis'* (Latin) needle-like, referring to the external sporangial outgrowths.

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

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

Discussion

Taxonomic and phylogenetic placement of Aquastella

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

Because of the morphological differences between these two new species and other known oomycete rotifer parasites, and also due to their phylogenetic placement within the Saprolegniales, we erected the new genus, *Aquastella*, to accommodate these novel parasites with external thallus outgrowths. The SSU rRNA tree shows that these two *Aquastella* species share a common ancestor with two other animal parasitic *Aphanomyces* spp., and together form a discrete early diverging clade within the Saprolegniales (Fig 1). The ≈2% divergence in 18S rRNA sequence strongly suggests that the host specific *Aquastella* parasites are different species, this being considerably greater than the ≈0.5% divergence between *Saprolegnia ferax* and *S. parasitica* and similar to the divergence between *Phytophthora infestans* and *Plasmopara viticola* (≈2%). The most obvious relative to the *Aquastella* parasites would be *Sommerstorffia 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

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

Growth and development

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

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

Spore production and Infection

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

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

Outgrowths

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

460 **Acknowledgements**

461

462 We thank Michael Gaylo, Patricia Sprague, and Pamela Bolton for laboratory technical
463 assistance, and Bob Cheney, Denise Mayer, Mary Beth Kolozsvary, Jeremy Farrell, Gary
464 Lee, Bill Kitchen, Ron Andersson, John Hart, and Bob Daniels for field work assistance.
465 Special thanks to Bob Wallace for his guidance and encouragement at the initiation of
466 this project. We gratefully acknowledge the collaboration of Scott Quinn and Jay
467 Bloomfield for arranging helicopter transport to Brooktrout Lake. SLG thanks Julian
468 Thorpe, Department of Biology and the Medical School, University of Sussex, UK, for
469 microscope use.
470
471
472 ---

473 **References**

474

475 Akamatsu Y, Saikawa M, 2005. Giant mycelium by *Sommerstorffia spinosa*. *Proceedings*
476 *of the Annual Meeting of the Mycological Society of Japan* **49**: 79.

Apstein C, 1910. *Synchaetophagus balticus*, ein in *Synchaeta lebender* Pilz. *Wissenschaftliche Meeresuntersuchungen der Kommision zur Wissenschaftlichen Untersuchungen der Deutchen Meere, Abteilung Kiel* **12**: 163-166.

Arnaudow N, 1923a. Untersuchungen über *Sommerstorffia spinosa* nov. gen., nov. sp. *Jahrbuch der Sofianer Universität* **19, Heft 2, Abt. 1a**: 161-196.

Arnaudow N, 1923b. Ein neuer Rädertiere (Rotaria) fangender Pilz. (*Sommerstorffia spinosa*, nov. gen., nov. sp.). *Flora* **116**: 109-113.

Barron GL, 1976. Nematophagous fungi: Three new species of *Myzocytium*. *Canadian Journal of Microbiology* **22**: 752-762.

Barron GL, 1990. A new and unusual species of *Haptoglossa*. *Canadian Journal of Botany* **68**: 435-438.

Bartsch AF, Wolf FT, 1938. Two new saprolegniaceous fungi. *American Journal of Botany* **25**: 392-395.

Beakes, GW, 1994. Sporulation of lower fungi. In: Gow NAR, Gadd GM (eds), *The Growing Fungus*, Chapman and Hall, London, pp. 337-366.

Beakes GW, Glockling SL, 1998. Injection tube differentiation in gun cells of a *Haptoglossa* species which infects nematodes. *Fungal Genetics and Biology* **24**: 45-68.

Beakes GW, Honda D, Thines M, 2013. Systematics of the Straminipila: Labyrinthulomycota, Hyphochytriomycota and Oomycota. In: McLaughlin DJ, Spatafora J (eds), *The Mycota Vol. VIIA. Fungal Taxonomy and Systematics*, Springer, New York. In press, expected publication Autumn 2013.

499 Czeczuga B, Próba D, 1980. The characteristics of the environment of *Sommerstorffia*
500 *spinosa* (Oomycetes: Saprolegniales), a parasite of certain rotifers. *Mycologia* **72**:
501 702-707.
502 D'Eliscu PN, 1977. *Endosphaerium funiculatum*, gen. nov., sp. nov., a new predaceous
503 fungus mutualistic in the gills of freshwater pelecypods. *Journal of Invertebrate*
504 *Pathology* **30**: 418-421.
505 Dick MW, 2001. *Straminipilous Fungi. Systematics of the Peronosporomycetes Including*
506 *Accounts of the Marine Straminipilous Protists, and the Plasmodiophorids and*
507 *Similar Organisms*. Kluwer Academic Publishers, Dordrecht.
508 Diéguez-Uribeondo J, Garcia MA, Cerenius L, Kozubikova E, Ballesteros I, Windels C,
509 Weiland J, Kator H, Soderhall K, Martin MP, 2009. Phylogenetic relationships
510 among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes).
511 *Fungal Genetics and Biology* **46**: 365-376.
512 Drechsler C, 1946. A nematode-destroying phycomycete forming immotile spores in
513 aerial evacuation tubes. *Bulletin of the Torrey Botanical Club* **73**: 1-17.
514 Glockling SL, Beakes GW, 2000. A review of the taxonomy, biology and infection
515 strategies of “biflagellate holocarpic” parasites of nematodes. *Fungal Diversity* **4**:
516 1-20.
517 Glockling SL, Beakes GW, 2006. An ultrastructural study of development and
518 reproduction in the nematode parasite *Myzocytiopsis vermicola*. *Mycologia* 98:1-15.
519 Glockling SL, Dick MW, 1997. New species of *Chlamydomyzium* from Japan and pure
520 culture of *Myzocytiopsis* species. *Mycological Research* **101**: 883-896.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696-704.

James TY, Stenlid J, Olson A, Johannesson H, 2008. Evolutionary significance of imbalanced nuclear ratios within heterokaryons of the basidiomycete fungus *Heterobasidion parviporum*. *Evolution* **62**: 2279-2296.

Johnson TW, Seymour RL, Padgett DE, 2004. Biology and systematics of the Saprolegniaceae. http://dl.uncw.edu/digilib/biology/fungi/taxonomy%20and%20systematics/padgett%20book/

Karling JS, 1936. A new predacious fungus. *Mycologia* **28**: 307-320.

Karling JS, 1944. New lagenidiaceous parasites of rotifers from Brazil. *Lloydia* **7**: 328-342.

Karling JS, 1952. *Sommerstorffia spinosa* Arnaudow. *Mycologia* **44**: 387-412.

Maddison D, Maddison W, 2000. *MacClade Version 4: Analysis of Phylogeny and Character Evolution. Version 4.08.* Sinauer Associates, Sunderland, Massachusetts.

Molloy DP, Siegfried CA, Wurdak E, Glockling SL, Mastitsky SE, Gaylo MJ, Giamberini L, Nemeth MJ, 2013 – in press. An investigation of oomycetes infecting rotifers in Brooktrout Lake, New York – Lethal parasites that produce outgrowths with distinctive morphologies but of unknown function. *Fungal Biology*

Parrent JL, Vilgalys R, 2009. Expression of genes involved in symbiotic carbon and nitrogen transport in *Pinus taeda* mycorrhizal roots exposed to CO_2 enrichment and nitrogen fertilization. *Mycorrhiza* **19**: 469-479.

543 Posada D, 2008. jModelTest: Phylogenetic model averaging. *Molecular Biology and*
544 *Evolution* **25**: 1253-1256.
545 Prowse GA, 1954. *Sommerstorffia spinosa* and *Zoophagus insidians* predacious on
546 rotifers, and *Rozellopsis inflata* the endoparasite of *Zoophagus*. *Transactions of the*
547 *British Mycological Society* **36**: 134-150.
548 Saikawa M, Hoshino J, 1986. Electron microscopy on *Sommerstorffia spinosa*, a
549 watermold parasitic on rotifers. *Mycologia* **78**: 554-561.
550 Scott WW, 1961. A monograph of the genus *Aphanomyces*. *Virginia Agricultural*
551 *Experimental Station, Technical Bulletin* **151**: 1-95.
552 Skvortzow BW, 1925. Zur Kenntnis der Phycomyceten aus der Nordmandshurei, China.
553 *Archiv für Protistenkunde* **51**: 428-433.
554 Sparrow Jr FK, 1929. A note on the occurrence of two rotifer-capturing Phycomycetes.
555 *Mycologia* **21**: 90-96.
556 Sparrow FK, 1960. *Aquatic Phycomycetes.* 2nd edn. Michigan University Press, Ann
557 Arbor, Michigan.
558 Thomas SH, Housley JM, Reynolds AN, Penczykowski RM, Kenline KH, Hardegree N,
559 Schmidt S, Duffy MA, 2011. The ecology and phylogeny of oomycete infections in
560 *Asplanchna* rotifers. *Freshwater Biology* **56**: 384-394.
561 Valkanov A, 1931. Über Morphologie und Systematik der rotatorienbefallenden Pilze.
562 *Archiv für Protistenkunde* **74**: 5-17.
563 Valkanov A, 1932. Nachtrag zu meiner Arbeit über rotatorienbefallenden Pilze. *Archiv*
564 *für Protistenkunde* **78**: 485-496.

565 Vilgalys R, Hester M, 1990. Rapid genetic identification and mapping of enzymatically
566 amplified ribosomal DNA from several *Cryptococcus* species. *Journal of*
567 *Bacteriology* **172**: 4238-4246.
568 Voigt M, 1957. *Rotaria. Die Rädertiere Mitteleuropas.* Vol. I & II. Gerbrüder
569 Borntraeger, Berlin-Nikolassee.
570

Legend for Figures

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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