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Allovahlkampfia minuta nov. sp., (Acrasidae, Heterolobosea, Excavata) a New Soil Amoeba at the Boundary of the Acrasid Cellular Slime Moulds

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Abstract. We report the isolation of a new species of *Allovahlkampfia*, a small cyst-forming heterolobosean soil amoeba. Phylogenetic analysis of the 18S rDNA and the internal transcribed spacers indicates that *Allovahlkampfia* is more closely related to the acrasids than to other heterolobosean groups and indicates that the new strain (GF1) groups with *Allovahlkampfia tibetiensis* and *A. nederlandiensis* despite being significantly smaller than these and any other described *Allovahlkampfia* species. GF1 forms aggregated cyst masses similar to the early stages of *Acrasis* sorocarp development, in agreement with the view that it shares ancestry with the acrasids. Time-lapse video microscopy reveals that trophozoites are attracted to individuals that have already begun to encyst or that have formed cysts. Although some members of the genus are known to be pathogenic the strain GF1 does not grow above 28°C nor at elevated osmotic conditions, indicating that it is unlikely to be a pathogen.

INTRODUCTION

The class heterolobosea was first created on morphological grounds to unite the schizopyrenid amoebae/amoeboflagellates with the acrasid slime moulds (Page and Blanton 1985), and subsequent molecular genetic data supported this union (Roger *et al*. 1996; Keeling and Doolittle 1996). The locomotory habit of these amoebae is to tend to produce an eruptive pseudopod, often alternatively from one side and then the other, of the advancing front. The heterolobosea are not directly related to other amoebae such as those within the Amoebozoa, despite being similar in appearance and habit. The heterolobosean acrasid slime moulds are very similar to the amoebozoan slime moulds too in life cycle, but these remarkable similarities in appearance and function are most probably due to parallel evolution.

It is not usually possible to classify the trophozoites of this group morphologically since they share very similar habits and appearances but many heterolobosean amoebae are also capable of transforming into flagellates, these are so called amoeboflagellates. In addition, some heterolobosean groups are comprised of flagellates only (*e.g*. *Percolomonas* and *Stephanopogon*) having apparently lost the ability to adopt the ancestral amoeboid habit. Heterolobosean amoebae which do not form flagellates were traditionally placed in the genus *Vahlkampfia* (Page 1976) but it has since been shown that this group is not monophyletic and other genera such as *Paravahlkampfia* and *Neovahlkampfia* were

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formed (Brown and De Jonckheere 1999), to join other non-flagellate genera raised on morphological grounds. Further analysis of 18S RNA genes has allowed many other organisms to be included with the Heterolobosea and it is increasingly clear that it is an order with huge diversity (Pánek and Čepička 2016; Pánek *et al*. 2017). In 2009 it was necessary to raise a further genus, named *Allovahlkampfia* to place a new heterolobosean that did not fit elsewhere (Walochnik and Mulec 2009). A number of other isolates have now been added and as the genus *Allovahlkampfia* is presently configured, does not contain flagellates or amoeboflagellates but all members so far characterised, produce cysts without opercula. Members of *Allovahlkampfia* vary significantly in SSU rRNA sequence, with inserts present in some (Geisen *et al*. 2015). They are variable in size but the strain described in the present study which we name *Allovahlkampfia minuta*, is the smallest.

One heterolobosean amoeboflagellate, *Naegleria fowleri* is dangerously pathogenic for humans (Carter 1970; Siddiqui *et al*. 2016). Additionally, *Allovahlkampfia spelaea* is reported to infect the human eye (Huseein *et al*. 2016; Tolba *et al*. 2016) and to host bacteria known to be human pathogens (Mohamed *et al*. 2016) making this genus particularly worthy of study.

METHODS AND MATERIALS

Soil samples were collected from the Glenfinnan viaduct at the north end of Loch Shiel, Scotland, UK. Small quantities were settled on non-nutrient agar overlain with Neff's saline overnight. Strips of this were then inverted onto non-nutrient agar plates spread with *E. coli* (BL21) and monitored over a week for amoebal out growth. Small blocks were cut from the leading outgrowth onto fresh agar plates spread with *E. coli* until clones of strain GF1 were established. Large quantities of cloned GF1 amoebae were cultured on 2% agar YME plates containing 0.01% yeast extract and 0.025% malt extract overlain with Neff's saline at room temperature with *E. coli* as food organisms. For long term storage, the amoebae and cysts used in this study were taken up in Neff's saline with 10% DMSO and placed overnight in a freezer at –20°C, then transferred to –80°C freezer. Amoebae were also stored long term in clay pellets as previously described (Lorenzo-Morales and Maciver 2006).

To test tolerance to osmotic stress, amoebae were seeded on *E. coli* spread non-nutrient agar plates supplemented with 0.5 M, 1 M and 1.5 M mannitol as previously described (Khan *et al*. 2001). Temperature tolerance was also tested by seeding amoebae on *E. coli* spread non-nutrient agar plates incubated at various temperature and monitored for growth over a ten day period.

We investigated the ability of *A. minuta* to develop a flagellate stage with a variety of stimuli included the addition of distilled water, rapid changes in pH, CO_2 and O_2 levels in addition to physical agitation (Perkins and Jahn 1970).

DNA was isolated from GF1 as previously described (Lorenzo-Morales *et al*. 2005). Amoebae were lysed in buffer and treated with proteinase K at 60°C for 2 hours followed by phenol-chloroform extraction. DNA was then concentrated by precipitation with isopropanol. The resulting DNA was quantified using a "nanodrop" spectrophotometer (ThermoFisher). An aliquot of 100 ng of genomic DNA was used per PCR reaction, using GoTaq Green Master Mix polymerase (Promega).

Sequences were obtained from GenBank and other sources and compiled together with the new sequences from this study using "Seaview", version 4 (Gouy *et al*. 2010). "BioEdit" (Hall 1999) was used to trim sequences and to determine levels of homology between sequences. Sequences were aligned using seaview 4 which was also used to implement the PhyML algorithm to produce Maximum likelihood phylogenetic trees (Guindon and Gascuel 2003) using the GTR model. The non-parametric analysis was performed with 100 bootstrap pseudo-replicates, using representative sequences from other heterolobozoans (*Naegleria*, *Tetramitus* and *Vahlkampfia*) as the outgroup. Binomial names of the strains described previously (Geisen *et al*. 2015) were obtained from the CCAP website (https://www.ccap.ac.uk).

RESULTS

The locomotive form is noticeably smaller than that of previously described members of this genus (Table 1). It is often wider than it is long although this is usually temporary. Pseudopods are produced eruptively from the anterior in a manner typical of the heterolobosean amoebae (Fig. 1). The trophozoites can also adopt a flabellate-like morphology. In culture GF1 trophozoites and cysts were observed to collect at the water air boundary and migrated on the meniscus. Strain GF1 was found to produce aggregates of cysts on agar plates (Fig. 2). Time-lapse video microscopy (see supplementary data and Fig. 3) reveals that the aggregated nature of the cyst distribution results from trophozoites actively migrating toward preformed cysts and not as a result of cyst aggregating by a passive mechanism after formation. GF1 was found to grow at 4°C and slowly at 28°C but at 30°C it encysts. The optimal temperature for growth was 24°C. GF1 could not grow on plate supplemented with 0.5 M mannitol or above.

Phylogenetic analysis tentatively suggests that genus *Allovahlkampfia* is comprised of eight or nine groups, probably representing as many natural species. More data from more genes and isolates are required to clarify this of course. No flagellated cells have been found in culture despite the application of a range of previously reported flagellate inducing stimuli. Strain GF1 is closely related to two previously reported species (*A. nederlandiensis* and *A. tibetiensis*) (Fig. 4A)

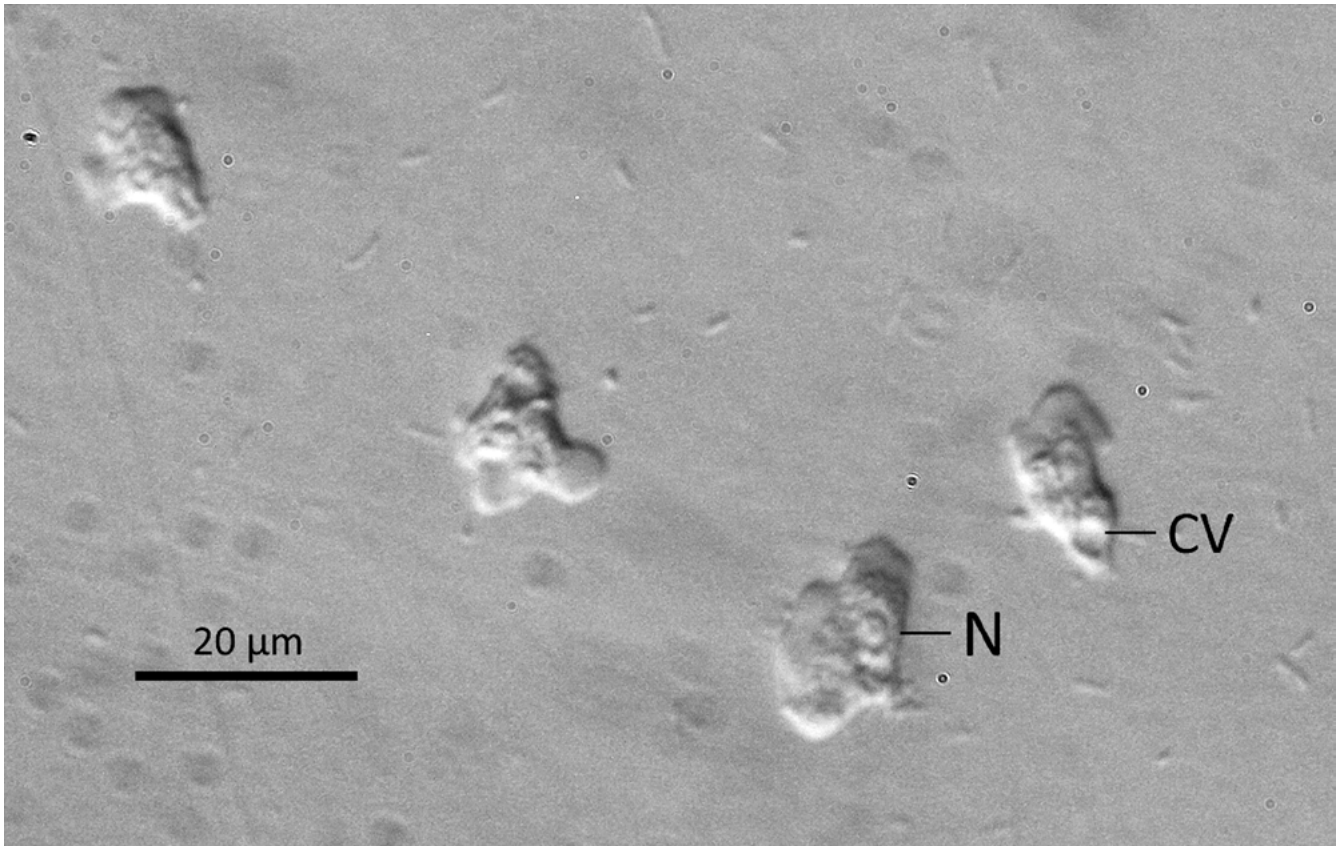

Fig. 1. Trophozoites of *Allovahlkampfia minuta* showing typical morphology with eruptive pseudopods. Nucleus (N) has a central nucleolus and the contractile vacuole (CV) is typically positioned at the rear of the amoeba. The scale bar is 20 µm. Food organisms, *E. coli* are visible in the background.

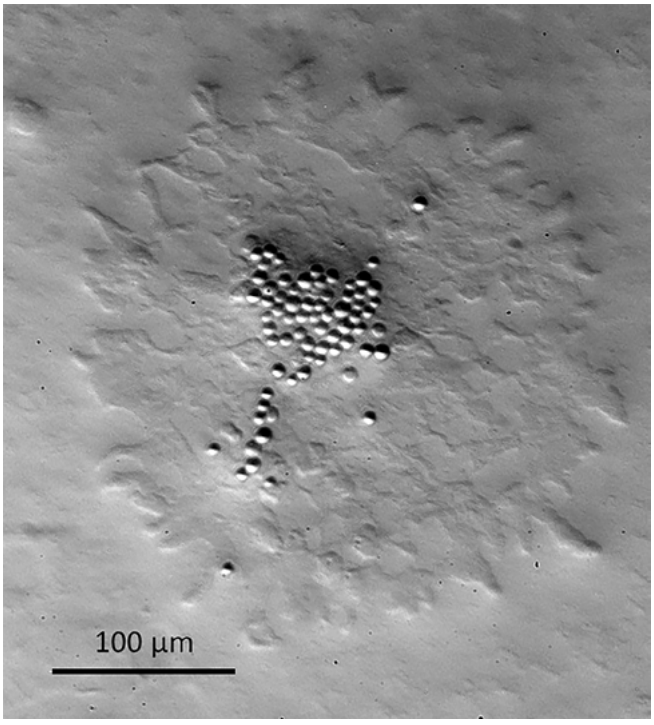

Fig. 2. Trophozoites gather and encyst on non-nutrient agar plate surface as the *E. coli* food organisms become depleted and as the conditions become less moist.

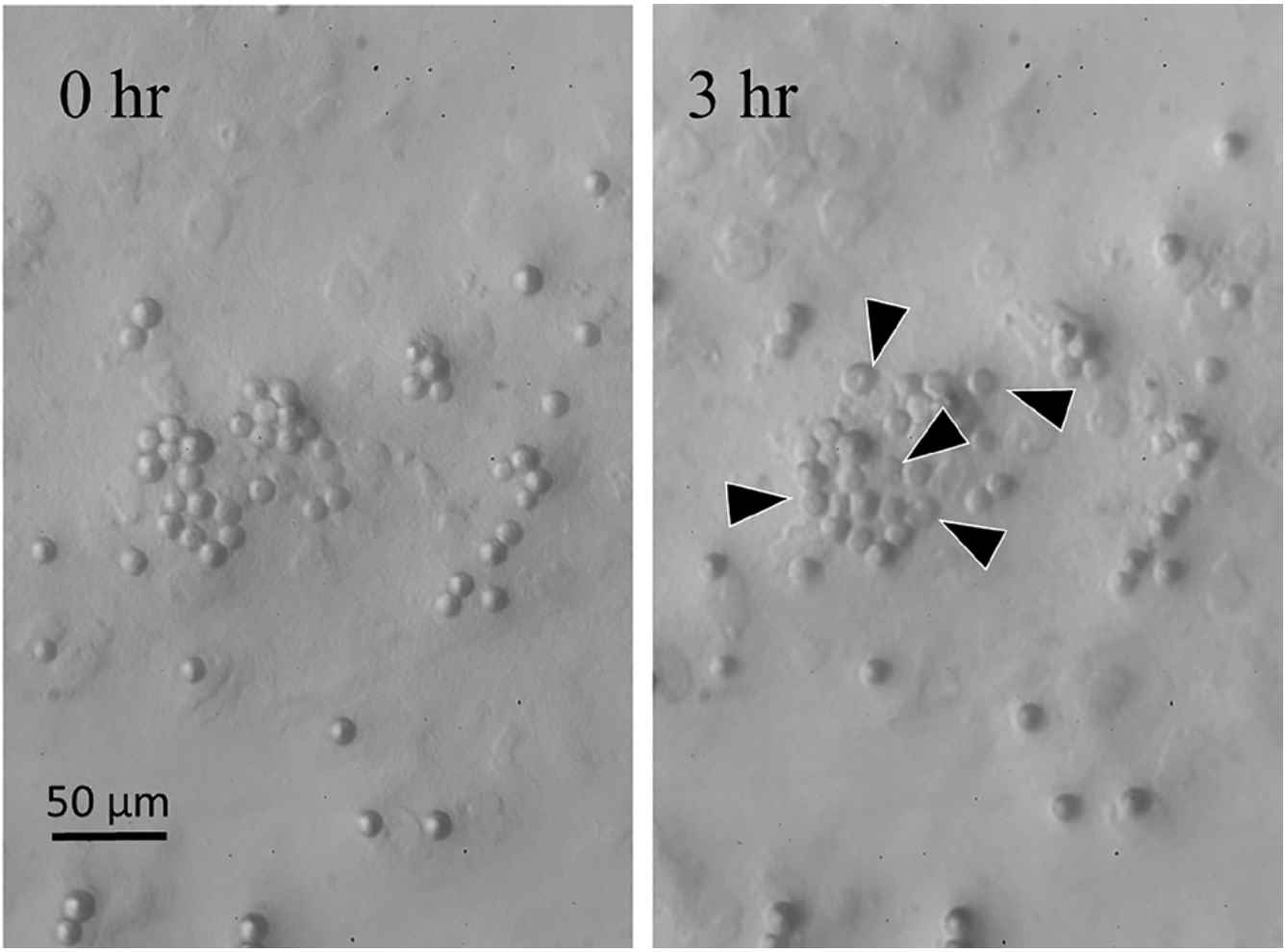

Fig. 3. Cooperative cyst formation. Trophozoites were placed on an agar plate with a monolayer of *E. coli*. Encystment took place when the plate became dry and the food source locally exhausted. Trophozoites aggregated around the first to encyst, forming a slightly raised mound of cysts. Left panel – This image is the first frame of a video (see supplementary data) taken to follow cyst aggregate formation. Right panel – This is the last image of the same video where black arrowheads point to cysts that have been produced during the time that the video was shot.

and these are very similar in appearance also, especially regarding the tendency to adopt a temporary flabellate locomotory habit. However, these other members of this group are significantly larger (Geisen *et al.* 2015) (Table 1).

DISCUSSION

The most notable feature of *A. minuta* is its small size but similarly sized heterolobosean amoebae have been described. For example, *Neovahlkampfia nana* is 12 µm in length (Tyml *et al.* 2017). Compared to other heteroloboseans including the genus *Allovahkmapfia*, the locomotion of *A. minuta* seems chaotic and inefficient with frequent direction changes and the production of seemingly immediately redundant pseudopods. *A. minuta* was observed to adopt a temporal flabellate morphology as is the case for other heteroloboseans for example *Heteramoeba clara* (Droop 1962) and *Vahlkampfia signyensis* (Garstecki *et al.* 2005). The habit of the cysts and trophozoites of *A. minuta* to collect at the meniscus has been reported to be the case with other free-living amoebae (Preston 2003).

The genus *Allovahlkampfia* is genetically diverse but morphologically uniform and represented so far only by freshwater and soil species. It is not possible to distinguish *Allovahlkampfia* from several other het-

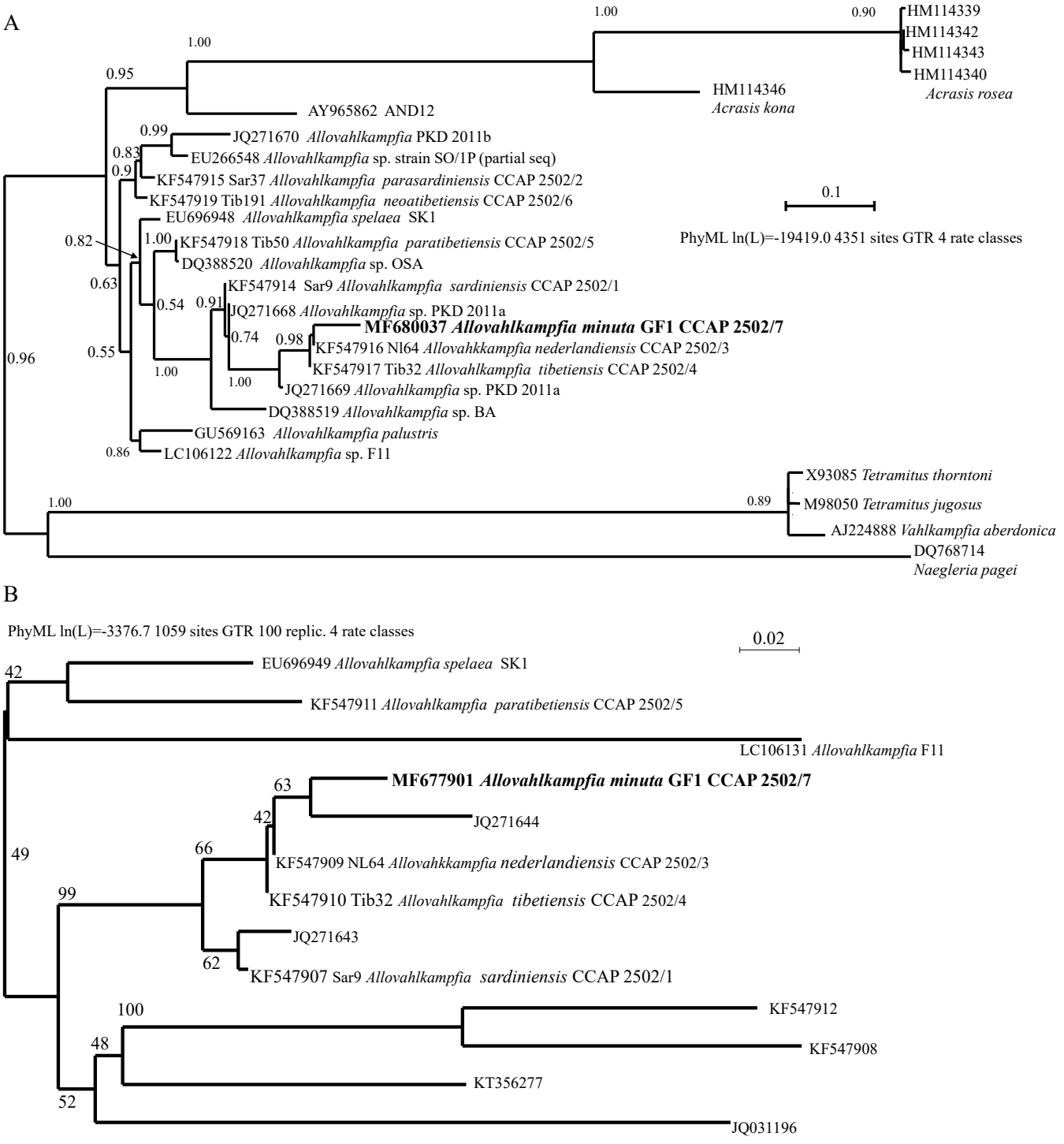

Fig. 4. A. A PhyML phylogenetic tree (GTR model) of members of some heterolobosean of the genus *Allovahlkampfia* and *Acrasis* based on 18S rDNA gene. Branch support values at each node indicated as percentages. The GenBank accession code of each sequence is followed by the species names, then strain name (where available). The tree has been rooted using a number of other heterolobosean genera (*Tetramitus*, *Naegleria* and *Vahlkampfia*) as the outgroup. Scale bar represents evolutionary distance. Binomials names for some strains (CCAP 2502/1 to 2502/6) were from the culture collection website (https://www.ccap.ac.uk) as they were not given in the original paper (Geisen *et al*. 2015). The subject of the present study is highlighted in bold. **B**. A PhyML phylogenetic tree (GTR model) of members of some heterolobosean of the genus *Allovahlkampfia* and *Acrasis* based on the fragment coding the internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2.

Table 1

Species/strain	Length µm Average &/or range	Breadth µm Average & range	Cyst diameter µm Average &/or range	Reference
A. palustris	31 (14–43)	6.3 (4–12)	8–10	Anderson *et al*. 2011
A. spelaea	20–40	n/a	16–25	Walochnik and Mulec, 2009
AND12	20–30	n/a	n/a	Lara *et al.* 2007
A. tibetiensis	25.2 (21.2–31.2)	6.6 (5.6–9.2)	6.6 (5.0–8.2)	Geisen *et al.* 2015
A. nederlandiensis	33.2 (21.8–50.8)	5.8 (3.6–8.6)	6.2 (5.0–7.8)	Geisen *et al.* 2015
F11	35.8	11.4	11.5	Tyml *et al.* 2016
A. minuta	13.4 (9.5–16.7)	8.1 (4.6–15.6)	6.5 (4.7–8.9)	This study

erolobosean genera based on appearance alone (Geisen *et al*. 2015), even at the level of electron microscopy (González-Robles *et al*. 2012). The cysts of all presently named species of the genus *Allovahlkampfia*, including *A. minuta* described here, lack opercula (Anderson *et al*. 2011; Walochnik and Mulec 2009; Geisen *et al*. 2015). This is a feature shared by *Acrasis rosea* (Hohl and Hamamoto 1969). It is the small size of *A. minuta* that distinguishes it from others in the genus. None of the previously characterised members of the genus is close in size to the trophozoites or the cysts.

Since reports of morphological similarities between vahlkampfids and acrasids were published (Page 1978; Page and Blanton 1985), several authors have reported a close genetic affinity also (Keeling and Doolittle 1996; Roger et al. 1996). Comparing ribosomal genes allowed *Allovahlkampfia* and *Acrasis* to be placed in clade "Acrasidia" within the Tetramitia (De Jonckheere *et al*. 2011; Harding *et al*. 2013; Geisen *et al*. 2015; Pánek *et al*. 2017). In this connection, the Allovahlkampfid strain AND12 (Lara *et al*. 2007) is especially interesting as it is currently the most closely known relative of the genus *Acrasis*. However, it was only partially characterized and because it was observed in liquid culture, its ability to develop fruiting bodies is untested (Lara *et al*. 2007). Unfortunately, this strain is no longer available in culture (Ekelund, Pers. Comm), but amoebae at the boundary between *Allovahlkampfia* and *Acrasis* may reveal details on the development of sporogenesis.

The aggregation of *A. minuta* trophozoites during encystment, produces images very similar to those seen in the acrasids especially the morphologically simple *Acrasis helenhemmesae* (see figures 14–16 in Brown *et al*. 2010). This tendency to aggregate may be shared with others of the genus as *A. palustris* and *A. spelaea* cysts are also reported to occur in clumps, although no further information about this was given (Anderson *et al*. 2011; Tolba *et al.* 2016). The significance of this aggregation is not yet clear but we have found time lapse video evidence (see supplementary data) for chemotaxis towards the encysting centre. There may be increased survival of aggregated cysts, or the aggregation may, like is this case in the acrasids be a prelude to the production of Allovahlkampfid fruiting bodies that may form under as-yet unknown circumstances. In this regard, it is interesting to note that a simple linear fruiting body has been observed in *Allovahlkampfia* strain BA (Brown *et al*. 2012), however this was observed on only one occasion. Other hints that *Allovahlkampfia* can produce multicellular aggregates such as fruiting exist. The isolate, *Allovahlkampfia* strain OSA, has been described as "an orange stalk-like fungal growth habit" (Shutt 2006) and while this description is a little vague, it is compatible with it having a multi-cellular or at least aggregative stage. Confusingly however, neither BA nor OSA formed fruiting bodies according to the worker who isolated them (Schutt 2006). Strains BA and OSA were assigned to *A. spelaea* (Brown *et al*. 2012) but this suggestion has not been adopted by later workers (Geisen *et al.* 2015) and is not supported by our 18S rDNA analysis. The aggregation that we observed with encysting *A. minuta* amoeba is superficially similar to the aggregation during the encystment of the amoebozoan, *Vannella pentlandii* (Maciver *et al*. 2017). Also, the closely related *Vannella fimicola* produces an actual fruiting body (Olive, 1962). The ability to fruit has evolved apparently independently, in other amoebozoan groups, including *Luapeleamoeba hula* within family Acanthamoebidae (Shadwick *et al*. 2016), and surprisingly, including the genus *Acanthamoeba* itself (Tice *et al*. 2016). Amoebae other than the Amoebozoa

are also known to produce fruiting bodies (Kang *et al*. 2017). There is little evidence of sexual recombination in the acrasids (Brown *et al*. 2012), but it is likely that the purpose of these fruiting bodies in the acrasids may be a dispersive strategy carrying the spores off the substrate to be carried by air currents, or passing birds and insects.

Many genera of free living amoeba contain species pathogenic to humans and while most of these including *Acanthamoeba*, *Balamuthia* and *Sappinia* are members of the amoebozoa some are heteroloboseans. *Acanthamoeba* is notorious for causing *Acanthamoeba* keratitis (Lorenzo-Morales *et al.* 2015), a sight threatening and extremely painful infection of the eye but there are reported cases of a similar keratitis associated with the presence of amoebae tentatively identified as being *Vahlkampfia* but in the presence of other organisms (Aitken *et al.*1996; Alexandrakis *et al*. 1998; Niyyati *et al*. 2010; González-Robles *et al*. 2012). These reports, while not concluding a directly causal link, do reveal that that heteroloboseans can survive on the human eye. Stronger evidence suggests that an amoeba SO/1P now known to belong in genus *Allovahlkampfia* (Fig. 2), cause keratitis in humans with herpes virus (Ozkoc *et al*. 2008) and that this same strain produced a similar infection in rat models (Huseein *et al*. 2016) (presumably in the absence of herpes virus since it was not mentioned). A second case where an amoeba closely related to *Allovahlkampfia spelaea* was reported to cause keratitis has also been reported (Tolba *et al*. 2016) and this strain also caused keratitis in a rabbit model. Whereas 93% of *Acanthamoeba* keratitis cases are associated with contact lens use (Radford *et al*. 1998), the few cases reported for heterolobosean amoebae have been dominated by trauma to the eye surface (Alexandrakis *et al*. 1998; Ozkoc *et al*. 2008; Tolba *et al.* 2016). It is firmly established that another heterolobosean, *Naegleria fowleri* causes the usually fatal brain infection primary amoebic meningoencephalitis in humans (Siddiqui *et al*. 2016). The finding that *A. minuta* does not grow above 28°C, and its failure to cope with elevated osmolarity, would seem to rule it out as a potential human eye pathogen where the temperature varies between 32 and 36°C (Purslow *et al.* 2005).

Allovahlkampfia minuta n. sp.

Description. The locomotive morphology of this species is typical for small heterolobosean amoebae. Amoeba length 13.4 µm (range 9.5–16.7) and width 8.1 µm (range 4.6–15.6). Length:breadth ratio is 1.65. A well-developed uroid is not often visible but trailing uroidal filaments are occasionally observed on glass and tissue culture plastic. The spherical nucleus is variable in diameter but it ranges between 1.5–2.5 µm. There is a central nucleolus. A contractile vacuole is usually visible in trophozoites in Neff's saline or distilled water. This is usually located at the rear of the amoeba, often within the bulbous uroid, if it is present. Growth on *E. coli* is observed between 4 and 28°C, with an optimum at 24°C. The pelleted trophozoites have a distinct pink/orange colour.

Cysts. Spheroidal diameter 6.5 µm (range 4.7–8.9) with a single cell wall and lacking pores. The mononuclear cysts are usually formed in aggregates.

Etymology. This isolate is significantly smaller than any other described *Allovahlkampfia* species, hence the name ''*minuta*''.

Type locality. Bank of Loch Shiel (freshwater) near the Glennfinnan monument (latitude 56.8687; longitude – 5.4378), Scotland, UK. The elevation is 4 m.

Habitat. Alluvial soil.

Type material. A culture of *A. minuta* has been deposited with the Culture Collection of Algae and Protists, accession number CCAP 2502/7, and gene sequences deposited with GenBank, MF680037 and MF677901 for the 18S rRNA gene and the ITS region respectively.

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Supplementary data available on journal website

Video 1. Locomotion of *Allovahlkampfia minuta* trophozoites. Real time video micrography shows typical eruptive locomotion but also show the chaotic nature of the progression of this species.

Video 2. Cyst chemotaxis. Time lapse video with frames every 15 seconds, total time 3 hours. While some trophozoites continue to move feed and divide, others are drawn toward existing cysts and they too then encyst. Contractile vacuole activity gradually lessens as cyst development takes place.