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A Hotspot of Amoebae Diversity: 8 New Naked Amoebae Associated with the Planktonic Bloom-forming Cyanobacterium *Microcystis*

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Abstract. The colonies of *Microcystis*, one of the most common bloom-forming cyanobacteria worldwide, harbor a diverse community of microorganisms. Among these, naked amoebae feeding on *Microcystis* cells can strongly influence natural *Microcystis* population dynamics. In this study, we investigated the species diversity of these amoebae based on 26 *Microcystis*-associated amoebae (MAA) strains from eutrophied water bodies in Belgium and elsewhere in western Europe. A detailed morphological characterization in combination with 18S rDNA (SSU) phylogenies revealed the presence of no less than 10 species. Nine of these belonged to the known genera *Vannella* (2 species), *Korotnevella* (2), *Copromyxa* (2), *Vexillifera* (1), *Cochliopodium* (1) and the recently described *Angulamoeba* (1). Only two were previously described, the others were new to science. One taxon could not be assigned to a known genus and is here described as *Schoutedamoeba* gen. n., representing a new variosean lineage. The discovery of so many new species from only one very specific habitat (*Microcystis* colonies) from a rather restricted geographical area indicates that the diversity of planktonic naked amoebae is much higher than previously appreciated and that only a tiny fraction of the total diversity of naked amoebae is currently known.

Key words: Naked amoebae, *Microcystis*, morphology, 18S rDNA, harmful algal blooms, grazing, Europe

INTRODUCTION

Although amoebae are often encountered suspended in the water column, they are generally ignored in studies of freshwater pelagic food webs (Arndt 1993, Sims *et al*. 2002). They are considered to be predominantly benthic organisms (Finlay and Esteban 1998) whose presence in the water column is supposed to be accidental (Velho *et al*. 2003) and importance in the pelagic food web generally assumed to be negligible (Rodríguez-Zaragoza 1994). Despite this, high amoebae densities are sporadically detected in the water column, often but not always in association with suspended particles (Anderson and Rogerson 1995, Rogerson and Gwaltney 2000, Rogerson *et al*. 2003, Anderson 2011), and sometimes a high local diversity of mainly herbivore

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and omnivore taxa is present (Arndt 1993, Garstecki and Arndt 2000). Moreover, it is becoming increasingly clear that these amoebae have the capacity to strongly influence natural phytoplankton population dynamics (e.g. Canter and Lund 1968, 1995; Bailey-Watts and Lund 1973; Cook and Ahearn 1976; Yamamoto and Suzuki 1984; Van Wichelen *et al*. 2006). Apart from mineral particles and detritus, suspended biota can also act as substrates for amoebae attachment (e.g. Anderson 1977, Rodríguez-Zaragoza 1994, Sheridan *et al*. 2002). *Microcystis,* one of the most widespread bloom-forming cyanobacteria (Visser *et al*. 2005), represents such a habitat in the pelagic zone of many standing freshwaters around the world. Its colonies, which generally consist of a few to several thousand cells embedded in a mucilage matrix, can harbor a rich microbial community (Li *et al*. 2011) including viruses (Honjo *et al*. 2006, Deng and Hayes 2008), bacteria (Maruyama *et al*. 2003, Shi *et al*. 2009), microalgae (Pankow 1986, Hindák and Hindáková 1995), chytrid fungi (Sen 1988, Van Wichelen *et al*. 2010) and amoeboid taxa (Whitton 1973, Yamamoto and Suzuki 1984, Nishibe *et al*. 2004). An intensive monitoring campaign of a *Microcystis* bloom in a small urban hypertrophic pond in Belgium showed the presence of *Microcystis*-grazing naked amoebae. In two consecutive years, a short-lived high cell-density peak of these amoebae led to a temporary collapse of the *Microcystis* population and a marked shift in the genetic structure of the bloom (Van Wichelen *et al*. 2010), caused by selective grazing of the amoebae (Van Wichelen *et al*. 2012). While a preliminary characterization based on overall morphology and 18S rDNA (SSU) sequences suggested that amoebae strains isolated from the bloom belonged to three unknown species from different genera, their species identity remained elusive (Van Wichelen *et al*. 2010).

Amoebae species taxonomy is mainly based on cultures because of methodological difficulties in preserving, observing and identifying naked amoebae in natural samples (Caron and Swanberg 1990). This is further complicated by their often complex life cycles (Rodríguez-Zaragoza 1994) and a general lack of distinctive morphological characters (Bovee 1953, Page 1988, Smirnov and Brown 2004, Nassonova *et al*. 2010). As a result, the assessment of amoebae diversity is a challenging research topic that is only poorly explored (Smirnov *et al*. 2011, Tekle 2014). The development of molecular tools in the last two decades and specifically sequencing of the 18S rDNA region significantly improved the assessment of phylogenetic relationships between amoebae. Currently, the classification of naked lobose amoebae and the discovery of new species is based on a combination of morphological characters and 18S rDNA phylogenies (Smirnov *et al*. 2005, 2009; Nassonova *et al*. 2010, Smirnov *et al*. 2011; Tekle 2014; Geisen *et al*. 2014). Recently, a strain (A1WVB) of one of the *Microcystis*-associated amoebae (MAA) investigated by Van Wichelen *et al*. (2010) was described as a member of the new genus *Angulamoeba* in the class Variosea (Berney *et al*. 2015). Observations of *Microcystis* bloom samples from other localities in Belgium and elsewhere during the past few years resulted in the cultivation of additional amoebae from *Microcystis* colonies. In this paper we present the morphological and molecular characterization, based on 18S rDNA sequences, of all available MAA strains. Based on this, we describe 8 species and 1 genus new to science.

MATERIALS AND METHODS

Microcystis bloom samples were prospected for the presence of MAA with the aid of a Leitz Diaplan light microscope using Nomarski interference illumination at a magnification of 400 x. Amoebae cultures were established by either picking out individual amoebae, or *Microcystis* colonies with one (or in rare cases a few) amoebae on them, with a sterile micropipette under a binocular microscope. They were put into 96-well culture plates (cellstar®, greiner bio-one), and grown on a monoclonal *Microcystis* culture (strain M31WVB, see Van Wichelen *et al*. 2010, available from NIES MCC with accession number NIES-3730) as food source. All amoebae strains were re-isolated 1 to 3 times to eliminate algal contaminants and ensure their monoclonal status. When present in sufficient densities, they were transferred to 24-well culture plates. Both the *Microcystis* source culture and the amoebae cultures were grown in WC medium (Guillard and Lorenzen 1972, without pH adjustment and vitamin addition), at 24°C, a photon flux density of ca. 120 µE $s^{-1}m^{-2}$ and a 12h/12h light/dark regime. For long-term maintenance, the culture plates were incubated at 18°C, a photon flux density of ca. 30 µE s^{-1} m^{-2} and a 12h/12h light/dark regime, and re-inoculated approximately every month. In addition to 7 previously established strains (Van Wichelen *et al*. 2010, 2012), 19 monoclonal amoebae cultures were obtained, of which the majority originated from different freshwater bodies in Belgium, two from a mesocosm experiment in Denmark and one from a Swedish lake (Table 1). Re-isolations were carried out for several strains belonging to the new genus *Angulamoeba* in order to ascertain that the large flagellated cells appearing in those cultures (especially after re-inoculation) represented a life cycle stage of the amoeba and not a contamination.

Light microscopy was done by placing drops of culture with living amoebae on glass slides under a cover slip. Pictures were taken with an Olympus DP50 or a Color view IIIu digital camera at 400–1000 X magnification. For each taxon, morphological mea-

surements of at least 100 individuals from different strains (when possible) and several cultures of varying age were made using ImageJ software. Material for transmission electron microscopy (TEM) was prepared according to Lambrecht *et al*. (2015). Formaldehyde-fixed (4%, borax buffered) amoebae were settled on Rat Tail Collagen (Type I, C7661 Sigma-Alderich, Sr Louis, US)-coated circular cover slips that were inserted at the bottom of a 24-well culture plate. The cover slips were rinsed with sodium cacodylate buffer 0,1 M (pH 7.4) and post-fixed with reduced osmium for 1 h at room temperature. After rinsing with double-distilled water, specimens were gradually dehydrated in an ethanol series and embedded in Spurr resin (EMS Hatfield UK, Spurr 1969). The resulting blocs were trimmed (Leica EM trim, Vienna, Austria) and ultra-thin sections (70–80 nm) were made with a Leica ultracut S ultramicrotome (Leica, Vienna, Austria) containing a diamond knife (Diatome Ltd., Biel, Switzerland) and mounted on Formvar-coated single slot copper grids (Agar Scientific, Stansed, UK). After staining with uranyl acetate and lead citrate (Leica EM stain), the sections were examined with a JEOL JEM 1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan). Pictures were digitized using the Ditabis system (Pforzheim, Germany).

For molecular analysis, amoebae were harvested by transferring 1–2 ml culture to sterile 2 ml Eppendorf vials, that were rotated at 3000 rpm in a Sigma 4K15 laboratory centrifuge for 15 min at 4°C. The supernatant was removed and the vials were stored at –80°C until further analysis. DNA was extracted according to Zwart *et al*. (1998) and purified on a Wizard column (Promega). The entire 18S rDNA was amplified using the primers described in van Hannen *et al*. (1998, external primers) and Huss *et al*. (1999, internal primers). The composition of the reaction mix was as in Janse *et al*. (2003). The program used was 5 minutes at 95°C followed by 40 cycles of 1 minute at 94°C, 1.5 minute at 55°C and 10 minutes at 72°C. Five µl of the PCR product was send to Macrogen for sequencing using the same primers. The 18S rDNA of all re-isolated *Angulamoeba* strains was sequenced as well, including the two strains for which a sequence was obtained earlier (Van Wichelen *et al*. 2010). The PCR product of 13 strains (see Table 1) had to be cloned (using the PGEM T vector system II kit from Promega) before good sequences could be obtained, mainly due to contamination with fungi and/or heterotrophic nanoflagellates. For each contaminated strain, the similarity of the cloned sequences was checked by automatically aligning them using the ClustalW algorithm as implemented in BioEdit version 7.0.3 (Hall 1999), and the longest sequence selected for phylogenetic analysis. For the vannellid strains A1SMB and A2SMB, for which the original sequence consisted of two parts, the cloned sequence was used to bridge the gap between both parts.

First, BLAST searches were used to identify the GenBank sequences most similar to ours. Also, the phylogenetic position of our MAA was determined using an Amoebozoan phylogeny. This was done by adding and aligning our 18S rDNA sequences manually to the alignment of Berney *et al*. (2015, Fig. S1), complemented with some additional taxa (Smirnov *et al*. 2011, Kudryavtsev and Pawlowski 2015). The final alignment contained 158 sequences from representatives of all major clades of Amoebozoa and various members of opisthokonts and apusomonads as outgroup. A total of 1414 unambiguously aligned positions were used for phylogeny reconstruction. The nucleotide substitution model was GTR+I+G, as determined in jModeltest 2.1.6 using the default settings and the BIC criterion. Bayesian Inference (BI, using MrBayes 3.2, Huelsenbeck and Ronquist 2001, Ronquist *et al*. 2012) and Maximum Likelihood (ML, using MEGA 6.06) were used for phylogeny reconstruction. The number of discrete gamma categories was four. For BI, no initial values were assigned to the model parameters. Two runs of four Markov Chains (one cold and three heated) were run for 10 million generations and sampled every 500 generations. This yielded a posterior probability (PP) distribution of 20,001 trees. After exclusion of 10,000 'burn-in' trees, PPs were calculated by constructing a 50% majority-rule consensus tree. For ML, tree inference was done excluding sites that were less than 50% complete, using the default settings and 1000 bootstrap replicates. After a first run, 3 sequences (*Vermistella antarctica* DQ229956, *Arcella hemisphaerica* EU273445 and *Parvamoeba rugata* JN202428) were removed from the dataset before re-analysis in order to reduce the amount of long-branch attraction artefacts which restored monophyly of Amoebozoa.

Finally, separate alignments were made for each genus to which our strains corresponded, including all available GenBank sequences of the genus. Representatives of related genera (*Korotnevella*, *Hartmannella/Copromyxa*, *Vannella*, *Cochliopodium*) or basal lineages within the genus (*Vexillifera*, *Angulamoeba*) were chosen as outgroup, based on Smirnov *et al*. (2007), Pawlowski and Burki (2009), Dyková *et al*. (2011), Corsaro *et al*. (2013), Geisen *et al*. (2014) and Berney *et al*. (2015). Because it did not fall in any established 18S rDNA lineage, no separate phylogeny was made for one of the limax-shaped strains. Sequences were aligned automatically as specified above and corrected manually. Parts that were too variable to align confidently were deleted from the alignment before phylogenetic analysis, as well as positions that were not covered (deletion or unknown base) by at least 50% of the sequences. The total number of differences and *p*-distances between sequences were calculated using MEGA 6.06 (Kumar *et al*. 1994, Tamura *et al*. 2013), using the complete sequences (most variable parts included) and pairwise exclusion of indels or missing data. The best-scoring model in jModeltest that was implemented in the phylogeny reconstruction program was chosen. For *Korotnevella*, *Angulamoeba* and *Vannella* the GTR+I+G model was selected, for *Hartmannella/Copromyxa* HKY+I+G (BI) and TrN+I+G (ML), for *Vexillifera* HKY+G and for *Cochliopodium* GTR+G. Phylogeny reconstruction was done as specified above.

RESULTS

Early screening

First, light microscopy observations were used to assign strains to the general amoeba morphotypes (based on Smirnov and Brown 2004). The locomotive cells of 10 strains were fan-shaped which is typical for the Vannellida, 7 strains had a dactylopodial morphology such as in *Korotnevella*, 3 strains had a limax shape resembling *Hartmannella*, 3 strains had a branched morphology as in *Angulamoeba*, 1 strain had a acanthopodial shape strongly resembling *Vexillifera* and the last one

Table 1. Overview and origin of the isolated strains of *Microcystis*-associated amoebae described in this paper. GenBank accession numbers of sequences obtained for this study are in bold. The last column indicates number of the specimens used to measure the species dimensions.

Species	Strain	Acc. no. GenBank	Acc. no. NIES MCC	Origin	Coordinates	Isolation date	Cloning	Type	N ind. measured
Angulamoeba microcystivorans	A1WVB	**KP719180** HM017143	3722	Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	23/07/2008	X	X	37
Angulamoeba microcystivorans	A2WVB	**KP719181** HM017144		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	23/07/2008			128
Angulamoeba microcystivorans	A4WVB	**KP719182**		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	23/07/2008	X		44
Angulamoeba microcystivorans	A7WVB	**KP719183**		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	23/07/2008			2
Korotnevella pelagolacustris	A8WVB	**KP719184**		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	25/07/2008			37
Korotnevella pelagolacustris	A16WVB	HM017145	3726	Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	25/07/2008		X	54
Korotnevella pelagolacustris	A21WVB	HM017146		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	25/07/2008			35
Korotnevella pelagolacustris	A54WVB	HM017147		Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	26/07/2008			4
Korotnevella pelagolacustris	A1LMS	JN568811		Lake Mälaren – Ekoln – Sweden	59° 44' 36" N – 17° 36' 24,8" E	17/09/2009			56
Korotnevella jeppesenii	A1JEPDK	**KP719185**		mesocosm – Silkeborg – Denmark	56° 9' 17" N – 9° 33' 47" E	30/07/2009			109
Korotnevella jeppesenii	A4DVDPB	**KP719186**	3725	fish pond – De Panne – Belgium	51° 4' 28" N – 2° 33' 45,9" E	12/09/2014	X	X	
Vexillifera westveldii	A18WVB	**KP719187**	3729	Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	25/07/2008	X	X	138
Copromyxa microcystidis	A2JEPDK	**KP719188**	3723	mesocosm – Silkeborg – Denmark	56° 9' 17" N – 9° 33' 47" E	30/07/2009		X	168
Copromyxa vandevyveri	A5DVDPB	**KP719189**	3724	fish pond – De Panne – Belgium	51° 4' 28" N – 2° 33' 45,9" E	12/09/2014	X	X	43
Schoutedamoeba minuta	A1P5ZHB	**KP719190**	3727	fish pond – Zonhoven – Belgium	50° 58' 48" N – 5° 19' 52,2" E	15/09/2009	X	X	43
Cochliopodium sp.n.	A1HEB	**KP719191**		fish pond – Herselt – Belgium	51° 1' 55" N – 4° 54' 2,8" E	1/10/2013	X		99
Vannella planctonica	A4P4ZHB	JN568810		fish pond – Zonhoven – Belgium	50° 59' 2" N – 5° 20' 20,5" E	15/09/2009	X		56
Vannella planctonica	A2SMB	**KP719192**		Schulensmeer – Lummen – Belgium	50° 57' 30" N – 5° 8' 40" E	15/09/2009	X		16
Vannella planctonica	A2FBB	**KP719193**	3728	Fort Bornem – Belgium	51° 4' 57" N – 4° 14' 6,5" E	24/08/2010		X	143
Vannella planctonica	A1DVDPB	**KP719194**		fish pond – De Panne – Belgium	51° 4' 28" N – 2° 33' 45,9" E	12/09/2014	X		18
Vannella planctonica	A2DVDPB	**KP719195**		fish pond – De Panne – Belgium	51° 4' 28" N – 2° 33' 45,9" E	12/09/2014	X		36
Vannella planctonica	A3DVDPB	**KP719196**	3765	fish pond – De Panne – Belgium	51° 4' 28" N – 2° 33' 45,9" E	12/09/2014	X		38
Vannella simplex	A17WVB	**KP719197**	3766	Westveld Pond – Gent – Belgium	51° 4' 5" N – 3° 46' 25,8" E	25/07/2008			63
Vannella simplex	A1SMB	**KP719198**		Schulensmeer – Lummen – Belgium	50° 57' 30" N – 5° 8' 40" E	15/09/2009	X		29
Vannella simplex	A6HEB	**KP719199**	3767	fish pond – Herselt – Belgium	51° 1' 55" N – 4° 54' 2,8" E	7/10/2013			77
Vannella simplex	A7HEB	**KP719200**		fish pond – Herselt – Belgium	51° 1' 55" N – 4° 54' 2,8" E	7/10/2013			35

had a lens-like morphology showing an easily visible tectum, closely resembling *Cochliopodium*.

Preliminary molecular identification based on BLAST searches of the 18S rDNA sequences in GenBank confirmed this, as the sequences were most similar to respectively *Vannella* (91–98% similarity), *Korotnevella* (91–93%), *Hartmannella*/*Copromyxa* (92–96%), *Angulamoeba* (87–99%), *Vexillifera* (96%) and *Cochliopodium* (85–86%). The only exception was the 18S rDNA sequence of one of the limax-shaped amoebae, that showed only a distant relationship with several variosean protosteloids. The amoebozoa-wide phylogenetic analysis confirmed the correct placement of all our strains (Suppl. Fig. 1).

Light microscopy observations of amoebae cultures and small-scale feeding experiments showed that all strains were able to feed on *Microcystis* cells, with as only exception the unknown limax-shaped amoeba. Some vannellids and the acanthopodial strain could also multiply when offered heterotrophic bacteria and/or pico-cyanobacteria as single food item. Amoebae could not grow when only green algae (*Acutodesmus*) were offered, although an ingested *Scenedesmus* coenobium was observed inside a food vacuole of a few trophozoites in a *Microcystis*/vannellid (strain A17WVB) co-culture contaminated with green algae.

Descriptive analyses

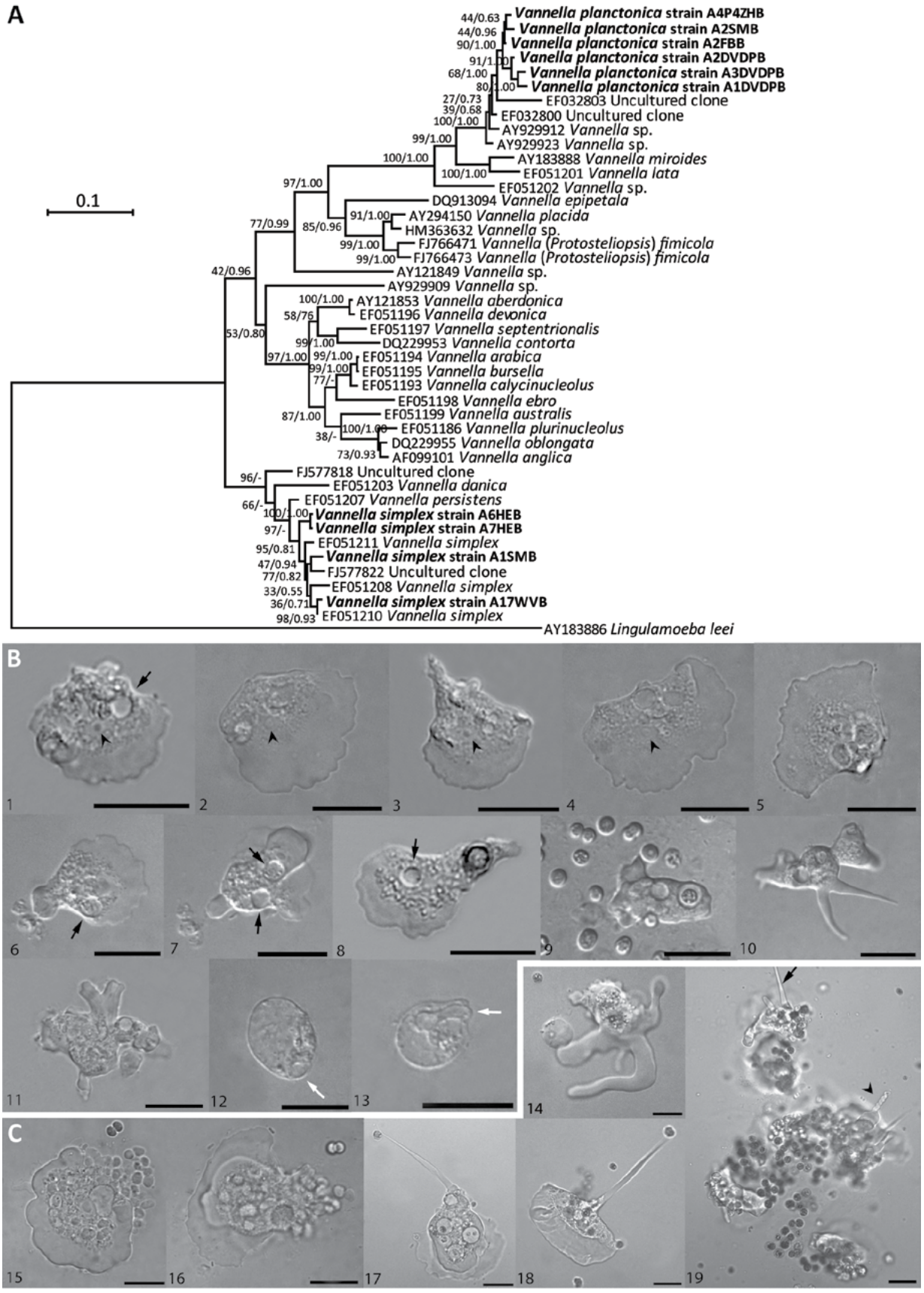
Vannella

The vannellid strains belonged to two lineages in different parts of the *Vannella* ML phylogeny (Fig. 1A). The first lineage had low bootstrap and moderate PP support and contained four *Microcystis*-associated strains interspersed between 3 *Vannella simplex* and 1 uncultured clone sequence (*p*-distances 0.3–3.5%, number of differing positions 4–63). Its sister taxon was *Vannella persistens* (2.0–3.6%, 24–68). The second lineage (upper part of the phylogeny) had moderate bootstrap and maximal PP support and consisted of six *Microcystis*-associated *Vannella* strains (*p*-distances 0.8–3.0%, 13–28 differing positions). Its closest relatives were unidentified *Vannella* strains or uncultured clones from natural samples (2.4–9.1%, 32–98).

The locomotive cells of the vannellid strains A17WVB, A1SMB, A6HEB and A7HEB, all closely related to *V. simplex* (Fig. 1C), shared a mainly flabellate to semicircular, rarely spatulate shape, a length/width ratio of 0.4–1.6 (mean 0.8) and an average coverage with hyaloplasm about half of the cell length anteriorly, but differed in average size. Locomotive forms of strains A17WVB, A1SMB and A6HEB/A7HEB were respectively 38–79 (mean 49), 20–56 (35) and 15–60 (31) µm long and respectively 45–83 (65), 20–61 (43) and 20–85 (41) µm wide. On several occasions, trophozoites of strain A17WVB were observed containing a flagellum-like pseudopodium, either very long and straight or contracted and helical in appearance (Fig. 1C 17–19). The trophozoites of strain A17WVB were always covered with fecal pellets. For the other 3 strains this feature disappeared after several months of cultivation, however fecal pellets of about 9 µm in diameter could always be observed freely dispersed in the culture medium.

Locomotive amoebae of strains A4P4ZHB, A2SMB, A2FBB, A1DVDPB, A2DVDPB, A3DVDPB, all belonging to the second vannellid lineage, were fan-shaped however with considerable variations. They could adopt a semicircular (Figs 1B 1–2), flabellate (Figs 1B 4–5) or spatulate shape containing a pronounced tail up to 21 µm long (Fig. 1B 3, 8). Feeding amoeba attached on *Microcystis* colonies (Fig. 1B 9) had a greatest linear dimension of 15–50 µm (mean 31). The hyaloplasm formed a broad margin around the cell and covered 13–71% (40%) of the cell anteriorly. When floating cells settled and became locomotive, the frontal hyaline area could display a wavy front edge (Figs 1B 2, 4, 6) and sometimes contained longitudinal ridges (Fig. 1B 7) as a result of transverse wave formation. The vesicular nucleus was 2–7 µm in diameter and contained a centrally located nucleolus of 1–5 µm in diameter (arrowheads in Figs 1B 1–4). Usually one small contractile vacuole was present posteriorly (black arrows in Figs 1B 1, 7, 8), with up to 3 vacuoles in some individuals. Floating amoebae were either radial shaped, 10–35 µm in diameter, symmetric, displaying radially 5–11 pointed and basally thickened (up to 6 µm in width) pseudopodia up to 50 µm in length, or sometimes irregularly shaped with only one to a few thick and round-tipped pseudopodia (Figs 1B 10–11) or in a few cases even without pseudopodia. Fecal pellets, varying in size between 5 and 13 µm, were present in the culture medium but did not tend to adhere posteriorly. Elliptical to ovoid cysts were observed a few times in old cultures. These had a thin wall (0.5 µm) and a diameter of 11–32 µm. Each had either a round opening of about 6 µm in diameter (white arrow in Fig. 1B 12) or a bone-shaped narrow slit of about 11 µm in length (white arrow in Fig. 1B 13).

Thirty *Vannella* species are currently recognized, 24 from marine and brackish, 4 from freshwater and


A
0.1
44/0.63 Vannella planctonica strain A4P4ZHB
44/0.96 Vannella planctonica strain A2SMB
90/1.00 Vannella planctonica strain A2FBB
91/1.00 Vanella planctonica strain A2DVDPB
68/1.00 Vannella planctonica strain A3DVDPB
80/1.00 Vannella planctonica strain A1DVDPB
27/0.73 EF032803 Uncultured clone
39/0.68 EF032800 Uncultured clone
100/1.00 AY929912 Vannella sp.
99/1.00 AY929923 Vannella sp.
100/1.00 AY183888 Vannella miroides
100/1.00 EF051201 Vannella lata
97/1.00 EF051202 Vannella sp.
DQ913094 Vannella epipetala
91/1.00 AY294150 Vannella placida
77/0.99 85/0.96 HM363632 Vannella sp.
99/1.00 FJ766471 Vannella (Protosteliopsis) fimicola
99/1.00 FJ766473 Vannella (Protosteliopsis) fimicola
42/0.96 AY121849 Vannella sp.
AY929909 Vannella sp.
100/1.00 AY121853 Vannella aberdonica
58/76 EF051196 Vannella devonica
EF051197 Vannella septentrionalis
53/0.80 99/1.00 DQ229953 Vannella contorta
97/1.00 99/1.00 EF051194 Vannella arabica
99/1.00 EF051195 Vannella bursella
77/- EF051193 Vannella calycinucleolus
EF051198 Vannella ebro
87/1.00 EF051199 Vannella australis
38/- 100/1.00 EF051186 Vannella plurinucleolus
DQ229955 Vannella oblongata
73/0.93 AF099101 Vannella anglica
96/- FJ577818 Uncultured clone
EF051203 Vannella danica
66/- EF051207 Vannella persistens
100/1.00 Vannella simplex strain A6HEB
97/- Vannella simplex strain A7HEB
95/0.81 EF051211 Vannella simplex
47/0.94 Vannella simplex strain A1SMB
77/0.82 FJ577822 Uncultured clone
33/0.55 EF051208 Vannella simplex
36/0.71 Vannella simplex strain A17WVB
98/0.93 EF051210 Vannella simplex
AY183886 Lingulamoeba leei
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2 from soil environments (Smirnov *et al*. 2007). Our phylogenetic analysis placed 4 of our vannellid strains in a clade containing several strains of *Vannella simplex*, a species with a wide geographically distribution, isolated from both freshwater and brackish habitats and genetically rather variable (Smirnov *et al*. 2002, Nassonova *et al*. 2010). Since our strains also share the relatively large cell size and the coverage by fecal pellets, both typical for *V. simplex* isolates (Smirnov *et al*. 2002), we believe they indeed belong to this species. Our remaining vannellid strains were much smaller in size, lacked the coverage by fecal pellets and formed a separate clade with uncultured clones or unidentified *Vannella*'s as their closest relatives. They were clearly separated from two other freshwater species, *V. miroides* and *V. lata*. No sequence information is available for *V. cirifera*, the only other freshwater *Vannella* species known, however this species was announced a *nomen dubium* by Smirnov *et al*. (2002, 2007). For this reason and because of its specific ecology we declared the strains of our second lineage as belonging to a new species, *Vannella planctonica*.

Korotnevella

All the dactylopodial strains were placed in the genus *Korotnevella*, which appeared monophyletic with good bootstrap and posterior probability support (Fig. 2A). The *Microcystis*-associated *Korotnevella* strains fell into two lineages, containing two and five strains respectively, without representatives of known species. *P*-distances were 4.9–5.9% (75–99 differing positions) between both lineages and 0.0–1.7% (0–23) within a lineage.

Locomotive amoebae belonging to the first lineage, strains A4DVDPB and A1JEPDK, were triangular, elongated or irregularly shaped (Fig. 2B). No differentiated uroidal structures were observed. Feeding amoebae (on *Microcystis* colonies) were spherical to cylindrical without dactylopodia (Fig. 2B 7) but sometimes with a very long and thin sub-pseudopodium when maintained in starved conditions. Length of the locomotive form was 16–41 µm (mean 28), width 6–20 µm (11) and length/width ratio 1.2–6.0 (2.6). One vesicular globular nucleus about 2–3 µm in diameter was present containing one slightly eccentrically located globular nucleolus of about 1–2 µm, occasionally showing a highly refractive central pore-like area (lacuna) under phase-contrast illumination (arrow in Fig. 2B 7). Floating forms were spherical with a diameter of 10–16 µm (14) and displaying 4–10 long, hyaline, pointed pseudopodia of maximally 25 µm long. Large spherical planktonic aggregates of up to several hundred individuals were observed occasionally in older cultures. Cysts were never observed. The scales, covering the entire cell surface, consisted of an elliptical disc with upwards curved edges and a centrally located cone-like spine giving it a 'witches hat' appearance (Fig. 3a, c). The length of the scales observed at top view (Fig. 3a) varied from 193–327 nm (277), the width from 124–203 nm (172) and the L/W ratio from 1.2–2.1 (1.6). The spine length observed in cross-section (Fig. 3c) was 47–97 nm (76), width at the base 56–124 nm (80) and L/W ratio 0.5–1.5 (1.0).

The locomotive amoebae from the second lineage, strains A8WVB, A16WVB, A21WVB, A54WVB and A1LMS, were all triangular, elongated or irregularly shaped due to the formation of short to deeply inclined pseudopodia on one or both ends or all around the cell body (Figs 2C 3, 4, 8–12). No differentiated uroidal structures were observed. Amoebae during non-oriented movement were elongated (Figs 2C 2, 6) or irregularly shaped (Fig. 2C 1), containing several small, sometimes bifurcated, round-tipped pseudopodia. Feeding amoebae (on *Microcystis* colonies) were spherical to cylindrical without clear pseudopodia (Fig. 2C 5). Starved amoebae had a similar cell shape but sometimes contained a very long and thin sub-pseudopodium. Cell length during locomotion was 20–65 µm (mean 37), width 6–32 µm (14) and the length/

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Fig. 1. A – 18S rDNA maximum likelihood phylogeny of *Vannella*, including *Microcystis*–associated strains (in bold). ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B** – LM pictures of *V. planctonica* (strains A2FBB: 2, 4, 5, 9–13 and A4P4ZHB: 1, 3, 6–8) showing locomotive trophozoites (1–8), a grazing amoeba with a *Microcystis* cell inside a food vacuole (9), floating forms (10–11) and a cyst stage (12–13). **C** – LM pictures of *V. simplex* (strain A17WVB) showing the floating form (14), locomotive amoebae (15–18) with the presence of food vacuoles containing partly digested *Microcystis* cells (17), posteriorly adhered fecal pellets (15–16), a long flagellum-like pseudopodium encircling a *Microcystis* cell (17–18) and grazing amoebae on a colony of *Microcystis aeruginosa* with expanded (arrow) or contracted (arrowhead) flagellum-like pseudopodia visible in some of the trophozoites (19). The presence of a contracticle vacuole, a nucleus or a cyst opening is indicated by a black arrow, a black arrowhead or a white arrow respectively. Scale bars: 20 µm.

width ratio 1.2–6.0 (2.8). One vesicular globular nucleus about 6 µm in diameter was present containing one slightly eccentrically located globular nucleolus of about 3 µm (arrows in Figs 2C 8, 10, 12), occasionally showing a highly refractive central pore-like area (lacuna) under phase-contrast illumination (faintly visible in Fig. 2C 8). Floating forms (Fig. 2C 13) were of radial type, spherical, with a diameter of 10–25 µm (15) and displaying 1–20 (9) long, hyaline, pointed pseudopodia with a length of 20–100 µm (52). The entire cell surface was covered with scales, only visible with electron microscopy (Figs 3b, d). In cross-section (Fig. 3d), these were generally oriented in a roof-tile pattern (imbricated). The scales consisted of an elliptical disk with upwards curved edges and a centrally located cone-like spine giving it a 'witches hat' appearance. The length of the scales observed at top view was 260–391 nm (333), the width 153–206 nm (180) and the length/width ratio 1.5–2.6 (1.9). The spine length measured on scales observed in cross-section was 98–163 nm (121), the width measured at the base of the spine 61–131 nm (91) and the length/width ratio 0.8–2.2 (1.4). Starved amoebae tended to float and aggregate into large, spherical colonies of several tens to hundreds of individuals. A few times, a globular cyst-like body with a diameter of 15–20 µm was observed in the cultures (Fig. 2C 7).

Korotnevella is a genus of scale-bearing amoebae all characterized by the absence of parasomes (Smirnov *et al*. 2011). Currently 8 *Korotnevella* species are recognized – 4 from freshwater, 3 from marine habitats and 1 from terrestrial origin – that are mainly distinguished on the base of their scale morphology (O'Kelly *et al*. 2001, Udalov 2015). The *Korotnevella* species without 18S rDNA sequence data are *K. nivo*, a marine species with crown-like scales (Smirnov 1996/97), 3 freshwater species, being *K. bulla* with basket-like scales (Page 1981), *K. diskophora* with disc-like scales (Smirnov 1999) and *K. limbata* having both scale types (Udalov 2015) and the terrestrial *K. heteracantha* also bearing both scale types (Udalov 2015). TEM analysis of the *Microcystis*-associated *Korotnevella* strains from both lineages showed the presence of disc-like scales (Fig. 3) very similar to the scales of *K. diskophora*. Apart from some small differences in scale dimensions and spine length, our strains mainly differed from *K. diskophora*, by having a spherical floating form with radially many pointed pseudopodia (irregular with few pseudopodia in *K. diskophora*) and by their preference for *Microcystis* and thus planktonic occurrence (bacterivore and benthic lifestyle in *K. diskophora*). Apart from the phylogenetic divergence, both *Microcystis*-associated *Korotnevella* lineages also differ in their morphology since the size of the trophozoites and of the scales and spines is smaller in strains of the first lineage (A4DVDPB and A1JEPDK). Based on the above-mentioned differences between both *Microcystis*-associated *Korotnevella* lineages and with *K. diskophora,* we described both lineages as new species, *K. pelagolacustris* and *K. jeppesenii*.

Hartmannella/Copromyxa

The 18S rDNA sequences of the two strains most closely related to *Hartmannella*/*Copromyxa* were highly divergent (*p*-distance 9.4%, 113 differing positions). In the ML phylogeny (Fig. 4A) they were most closely related to and had a basal position in the lineage including *Copromyxa protea*, *Copromyxa*/*Hartmannella cantabrigiensis* and an unidentified strain ('Tubulinida sp.') (*p*-distances 9.7–15.2%, 117–197 differing positions).

The locomotive amoebae (mainly seen in starved cultures, see Figs 4B 1, 3, 7) of strain A2JEPDK were monotactic with anteriorly a short-lived but well pronounced hyaline cap (Figs 4B 7, 10) of about 3 µm long and 6 µm wide that became rapidly filled with granuloplasm prior to the formation of a new lateral lob-like pseudopodium (Figs 4B 2, 5, 6, 11). These alternating lateral sprouting lobes gave the locomotion a sort of eruptive-like appearance and with any change of direction transformed monopodial movement swiftly in a bipodial (Fig. 4B 4) or multipodial (several irregularly lo-

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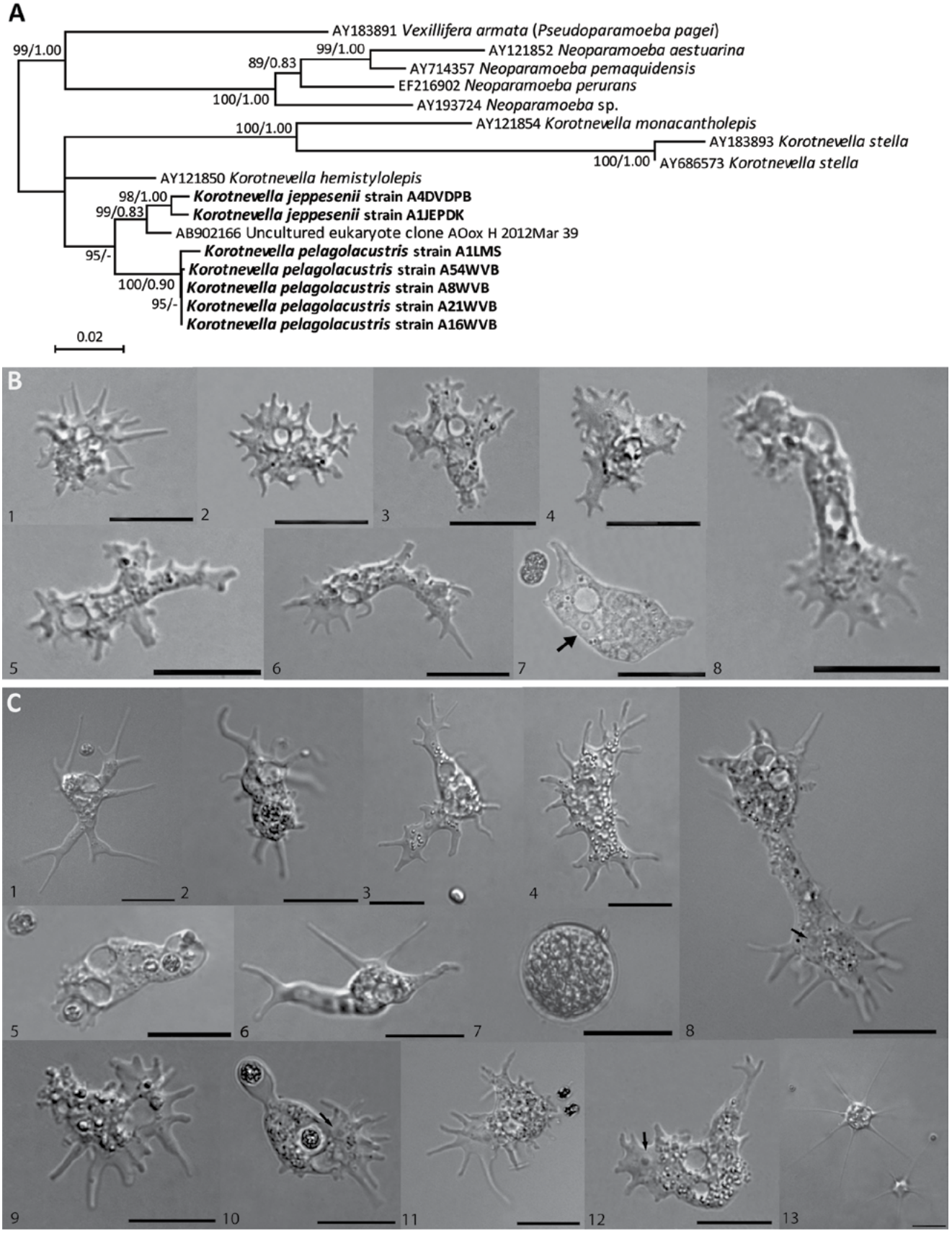
Fig. 2. A – 18S rDNA maximum likelihood phylogeny of *Korotnevella*, including *Microcystis*-associated strains (in bold). ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B** – LM pictures of *K. jeppesenii* (strain A1JEPDK) showing trophozoites in non-oriented movement (1–5), during locomotion (6, 8) and during grazing on *Microcystis aeruginosa* cells (7) with the arrow indicating a lacuna-containing nucleolus. **C** – LM pictures of *K. pelagolacustris* (strains A8WVB (7, 12), A16WVB (6, 8, 11), A21WVB (1, 13), A54WVB (3–5) and A1LMS (2, 9, 10)) showing trophozoites in different stages of non-oriented movement (1–4, 6), the locomotive form (8–12), grazing amoebae with *Microcystis* cells inside food vacuoles (5, 10), the cyst stage (7) and the floating form (13). Arrows indicate the nucleus with clearly visible nucleolus (in 8, 10, 12). Scale bars: 20 µm.


A
AY183891 Vexillifera armata (Pseudoparamoeba pagei)
99/1.00
99/1.00
AY121852 Neoparamoeba aestuarina
89/0.83
AY714357 Neoparamoeba pemaquidensis
EF216902 Neoparamoeba perurans
100/1.00
AY193724 Neoparamoeba sp.
100/1.00
AY121854 Korotnevella monacantholepis
AY183893 Korotnevella stella
100/1.00
AY686573 Korotnevella stella
AY121850 Korotnevella hemistylolepis
98/1.00
Korotnevella jeppesenii strain A4DVDPB
99/0.83
Korotnevella jeppesenii strain A1JEPDK
AB902166 Uncultured eukaryote clone AOox H 2012Mar 39
95/-
Korotnevella pelagolacustris strain A1LMS
Korotnevella pelagolacustris strain A54WVB
100/0.90
Korotnevella pelagolacustris strain A8WVB
95/-
Korotnevella pelagolacustris strain A21WVB
Korotnevella pelagolacustris strain A16WVB
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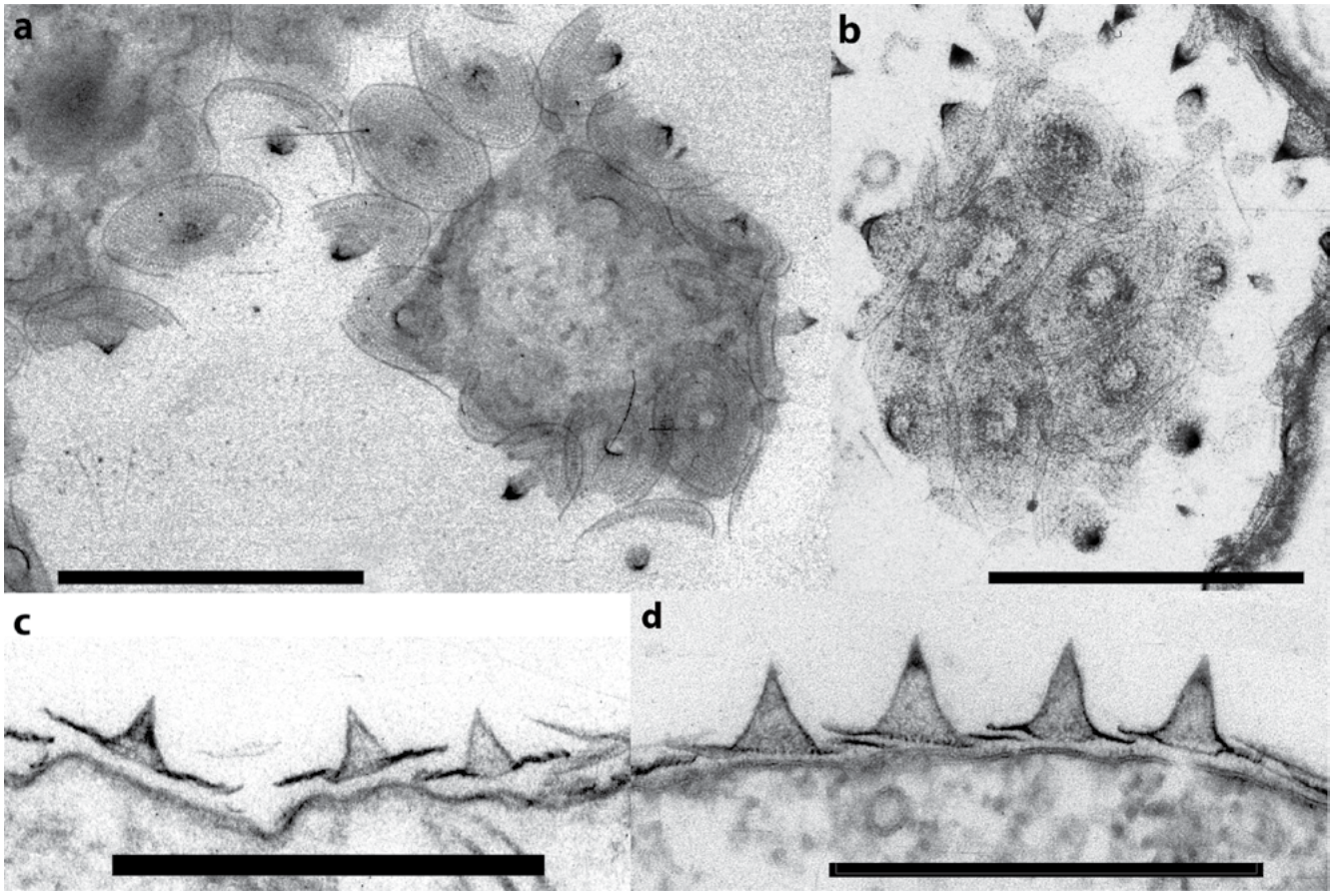

Fig. 3. TEM pictures of the scales on the cell surface of *Korotnevella jeppesenii* (a, c) and *K. pelagolacustris* (b, d) trophozoites viewed from top (a, b) and side (c, d). Scale bars: 500 nm.

cated pseudopodia, Fig. 4B 6) state. Pseudopodia were up to 13 µm long and 5 µm wide at the base. At times an eruptive lobe could be seen rippling down on one side of the amoeba (Figs 4B 8, 9). No differentiated uroidal structures were observed. Length of limax-shaped amoebae was 20–59 µm (mean 33 µm), width 7–17 µm (11) and length/width ratio 2–4.9 (3.1). The locomotion rate was estimated at 0.35–0.88 µm s^{-1}. Length during non-oriented movement was 10–43 µm (24), width 6–25 µm (13) and length/width ratio 1.0–4.3 (1.9). One vesicular oval nucleus of 5–10 µm long and 3–8 µm wide was present with one central to slightly eccentrically placed globular nucleolus of about 2–3 µm in diameter (Figs 4B 1, 5). Usually one small contractile vacuole posteriorly, rarely up to 3 vacuoles in very large individuals. The cytoplasm always contained 8–22 opaque crystals (e.g. arrow in Fig. 4B 1) with a length and width of respectively 1.1–7.2 µm (average 2.4)

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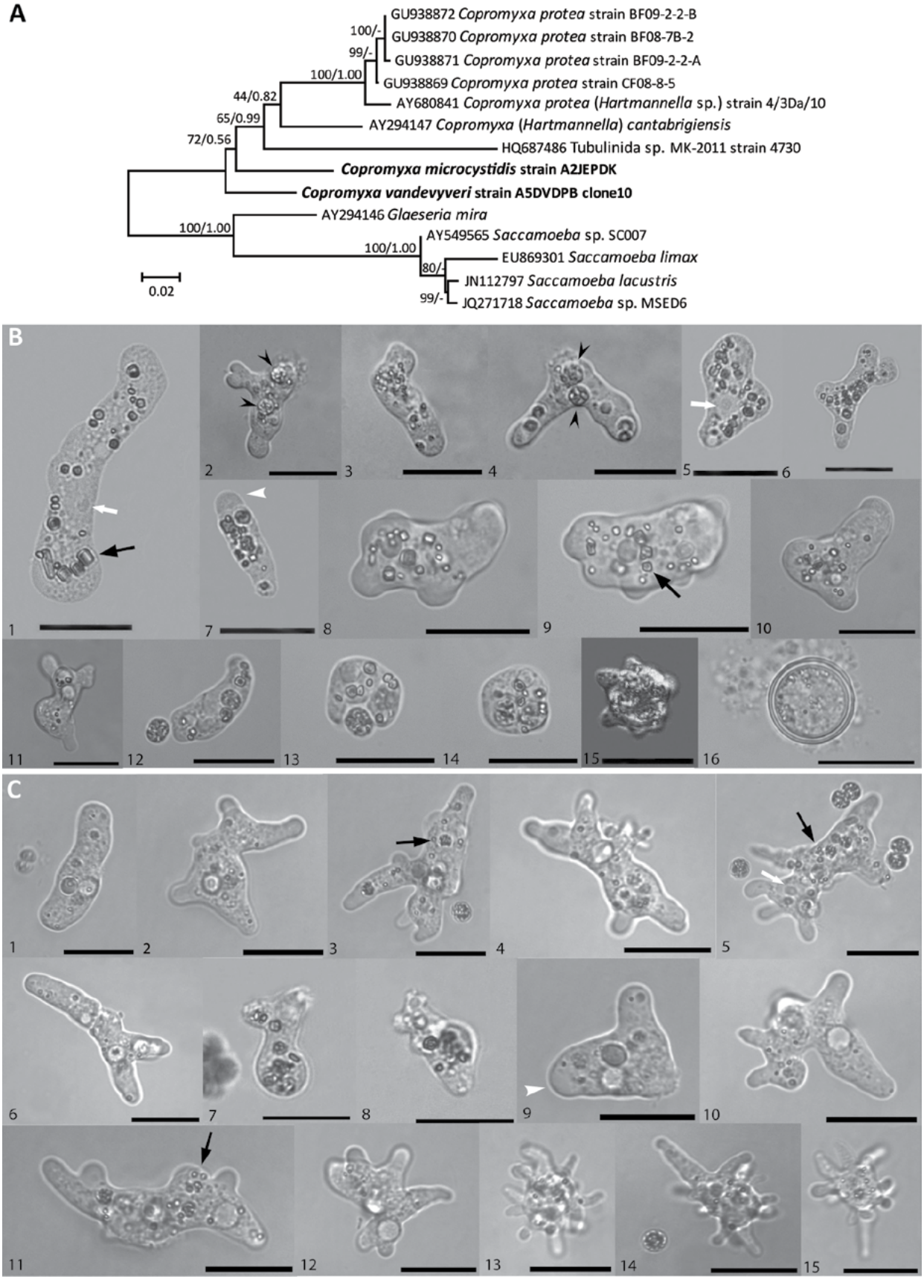
Fig. 4. A – 18S rDNA maximum likelihood phylogeny of the family Hartmannellidae, including *Microcystis*-associated strains of *Copromyxa* (in bold). ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B –** LM pictures of *C. microcystidis* showing locomotive trophozoites (1–7, 10) containing a pronounced hyaline cap (white arrowheads), a vesicular nucleus with globular nucleolus (white arrows), big crystals (black arrows) and *Microcystis* cells inside food vacuoles (black arrowheads), non-oriented moving trophozoites (8–9, 11), a grazing amoeba capturing a *Microcystis* cell (12–14), the floating form (15) and a double-walled cyst stage (16). **C** – LM pictures of *C. vandevyveri* showing locomotive trophozoites (1, 6–9), trophozoites during non-oriented movement (2–5, 10–12) and floating forms (13–15). Black arrows indicate the presence of small, refractive crystals in the cytoplasm, the white arrow shows the vesicular nucleus with a globular lacuna-containing nucleolus, white arrowheads indicate a pronounced hyaline cap. Ingested *Microcystis* cells are visible in (9) and (11). Scale bars: 20 µm.


A
GU938872 Copromyxa protea strain BF09-2-2-B
GU938870 Copromyxa protea strain BF08-7B-2
GU938871 Copromyxa protea strain BF09-2-2-A
GU938869 Copromyxa protea strain CF08-8-5
AY680841 Copromyxa protea (Hartmannella sp.) strain 4/3Da/10
AY294147 Copromyxa (Hartmannella) cantabrigiensis
HQ687486 Tubulinida sp. MK-2011 strain 4730
Copromyxa microcystidis strain A2JEPDK
Copromyxa vandevyveri strain A5DVDPB clone10
AY294146 Glaeseria mira
AY549565 Saccamoeba sp. SC007
EU869301 Saccamoeba limax
JN112797 Saccamoeba lacustris
JQ271718 Saccamoeba sp. MSED6
100/-
99/-
100/1.00
44/0.82
65/0.99
72/0.56
100/1.00
100/1.00
80/-
99/-
0.02
B
C

and 0.7–3.4 µm (1.7), and several smaller, refractive spheres. The crystals could be single or paired and of irregular, truncated bi-pyramidal, cylindrical, centrally inclined cube-like or plate-like (sometimes divided in 4 equal squares) shape. Spherical floating forms showed a high resemblance with those of *Saccamoeba limax* (Page 1988, p. 65). They were occasionally observed after re-inoculation and had a diameter of 15–20 µm and radially displayed 5–10 small hyaline lobes of about 3 µm in diameter (Fig. 4B 15). Cysts were spherical with a diameter of 9–20 µm, a cell wall of about 1 µm thickness and sometimes displayed an inner wall (Fig. 4B 16). These double-walled cysts are similar to so-called sphaerocysts observed in *Copromyxa protea* which may be part of a (para) sexual cycle (Brown *et al*. 2011). Empty cysts contained a round pore of about 5 µm in diameter (not shown).

Locomotive amoebae (mainly seen in starved cultures, Figs 4C 1, 6–9) of strain A5DVDPB were monotactic, displaying anteriorly a short-lived but well pronounced hyaline cap (Fig. 4C 9) about 5 µm long and 5 µm wide that was rapidly filled with granuloplasm just before a new lateral pseudopodium was formed. With any change of direction, monopodial movement was however easily transformed into a bipodial (Figs 4C 6, 9) and eventually a multipodial (several irregularly located pseudopodia) state due to these alternating sprouting (eruptive) lobes. Pseudopodia were up to 25 µm long and about 5 µm wide at their base, laterally harboring a few small, sprouting, short-living subpseudopodia (Figs 4C 3, 11) of which one eventually became the new leading pseudopodium. Limax-shaped amoebae were 20–55 µm long (mean 38) and 8–25 µm wide (13) with a length/width ratio of 1.4–6.7 (3.1). Amoebae during non-oriented movement (e.g. when feeding on *Microcystis* cells) were more irregularly shaped, 15–25 µm in diameter, with radially 5–12 small blunted, round-tipped pseudopodia (Figs 4C 3–5,10–12). No differentiated uroidal structures were observed apart of a few adhesive uroidal filaments occasionally (slightly visible in Fig. 4C 9). One vesicular oval nucleus of 4–8 µm long and 4–7 µm wide with one centrally to slightly eccentrically located globular nucleolus of about 3 µm in diameter (arrow in Fig. 4C 5) was present. The nucleolus may contain a highly refractive central pore-like area (lacuna). Usually one contractile vacuole of about 6 µm in diameter present posteriorly (e.g. Figs 4C 9–12). The cytoplasm contained 10–20 small, spherical to irregular shaped, dark-colored, refractive, single or paired crystals of about 0.5–1.6 µm (1.0) in diameter (e.g. Figs 4C 5, 7, 11). Floating forms were occasionally observed after re-inoculation. They were spherical to slightly elongated, with a diameter of 15–20 µm, radially displaying 5–12 small hyaline lobes with a maximal length of 15 µm (Figs 4C 13–15), generally resembling those of *Nolandella hibernica* (Page 1980, Fig. 4). Spherical cysts about 20 µm in diameter were occasionally present in starved cultures.

Together with *Saccamoeba*, *Glaeseria*, *Cashia* and *Copromyxella* (for the last two, 18S rDNA sequences are not available), *Hartmannella* and *Copromyxa* belong to the family Hartmannellidae (Smirnov *et al*. 2011). Currently, *Hartmannella* contains only three species (Smirnov *et al*. 2011). Two of these are marine – *H. lobifera* and *H. vacuolata* – and one is from freshwater, *Hartmannella cantabrigiensis* (Smirnov 1996/97, Anderson *et al*. 1997). *Copromyxa* contains only one species, *C. protea*, however there is ongoing discussion as to whether *H. cantabrigiensis* should be included in *Copromyxa* (Brown *et al*. 2011, Smirnov *et al*. 2011). The trophozoites of our strains were characterized by their aquatic lifestyle and absence of fruiting-body formation in contrast to *Copromyxella* and *Copromyxa protea* that are terrestrial, dung-inhabiting, aggregative, fruiting amoebae (slime molds) (Raper *et al*. 1978, Brown *et al*. 2011); their clearly visible and rather extended hyaline cap in contrast to *Glaeseria, Cashia* and *Saccamoeba* (Page 1988); their absence of a differentiated uroidal structure in contrast to *Saccamoeba* (Page 1988, Anderson *et al*. 1997, Corsaro *et al*. 2010); and their generally eruptive-like movement which is usually non-eruptive in all other members of the family, including *Hartmannella* (Page 1988, Smirnov 1996/97, Anderson *et al*. 1997). The last character is however not very reliable since Raper *et al*. (1978) observed potentially 'explosive' pseudopodia in trophozoites of all described *Copromyxella* species, Page (1980) mentioned uncharacteristic eruptive activity in some *Nolandella* species, Smirnov *et al*. (2011) observed occasional eruptions in a *Hartmannella/Copromyxa* strain and Watson *et al*. (2014) mentioned semi-eruptive movement in the recently established hartmannellid *Ptolemeba.*

Brown *et al*. (2011) emended the generic diagnosis of *Copromyxa* by also including aquatic, potentially non-fruiting limax-shaped amoebae (*H. cantabrigiensis, Hartmannella* strain 4/3Da/10) similar in morphology to our strains. For this reason and because we also observed a double-walled cyst stage very similar to the sphaerocysts described for *C. protea* in one of our cultures, we placed our strains within the genus *Copro-*

myxa. The phylogenetic distance between our strains and the other taxa within this clade warranted the description of two new species (based on the large divergence in 18S rDNA sequence), *C. microcystidis* and *C. vandevyveri*.

Unknown limax-shaped strain

BLAST search in GenBank and the Amoebozoa phylogeny both showed the closest relationship of the limax-shaped strain A1P5ZHB with variosean amoebae. While BLAST search revealed the protosteloid varioseans *Nematostelium ovatum, Schizoplasmodium cavostelioides* and *Ceratomyxella tahitiensis* as closest relatives (*p*-distance 12.4–13.1%, 228–243 differing positions), the Amoebozoa phylogeny placed our strain as sister of the ramose/reticulose varioseans *Darbyshirella* + *Ischnamoeba*, however without bootstrap support (Suppl. Fig. 1).

Locomotive amoebae of strain A1P5ZHB were about 12–22 µm (mean 16 µm) long and 4–7 µm (6) wide, had a length/width ratio of 1.9–3.8 (2.8) and anteriorly displayed a short-lived small hyaline cap (arrowheads in Figs 5f, i). Stationary amoebae were roundish, elongated or irregularly shaped, 6–16 µm (11) long and 3–10 µm (7) wide with radially displaying 2–5 small, erupting and short-lived hyaline lobes (Figs 5a–f). No differentiated uroidal structures were seen apart of small adhesive uroidal filaments about 2 µm long (arrow Figs 5g, h) that were occasionally present. The vesicular globular nucleus was about 2 µm long and contained one centrally located globular nucleolus of about 1 µm in diameter. Usually one small contractile vacuole of about 2 µm was visible posteriorly (Fig. 5h). The cytoplasm always contained several small, refractive granules of 0.5–1 µm in diameter (e.g. arrow Fig. 5i). Spherical floating forms, occasionally visible after reinoculation, were about 10 µm in diameter and radially displayed about 5 small hyaline lobes (as in Fig. 5a). No cyst stage or aggregation was observed.

The trophozoites of the phylogenetically most closely related taxa are all reticulose or highly branched which in the case of the protosteloid amoebae belonging to the schizoplasmodiid clade also aggregate into fruiting bodies (Shadwick *et al*. 2009, Berney *et al*. 2015). Older cultures of our strain contained a slightly yellowish-colored mucilage matrix on the bottom of the culture wells (cfr. the variosean *Angulamoeba microcystivorans*, Berney *et al*. 2015). It was unclear if this mucus originated from the amoebae or from the *Microcystis* culture that in this case also harbored the epiphytic cyanobacterium *Pseudoanabaena mucicola*. The secondary structure of the 18S rDNA sequence of our strain however contained a characteristic expansion of helix 43 in the variable region V7, a unique feature that unites all variosean sequences (Berney *et al*. 2015).

Given the morphological and phylogenetic divergence between our strain and all known varioseans, we erected a new genus *Schoutedamoeba* gen. n. represent-

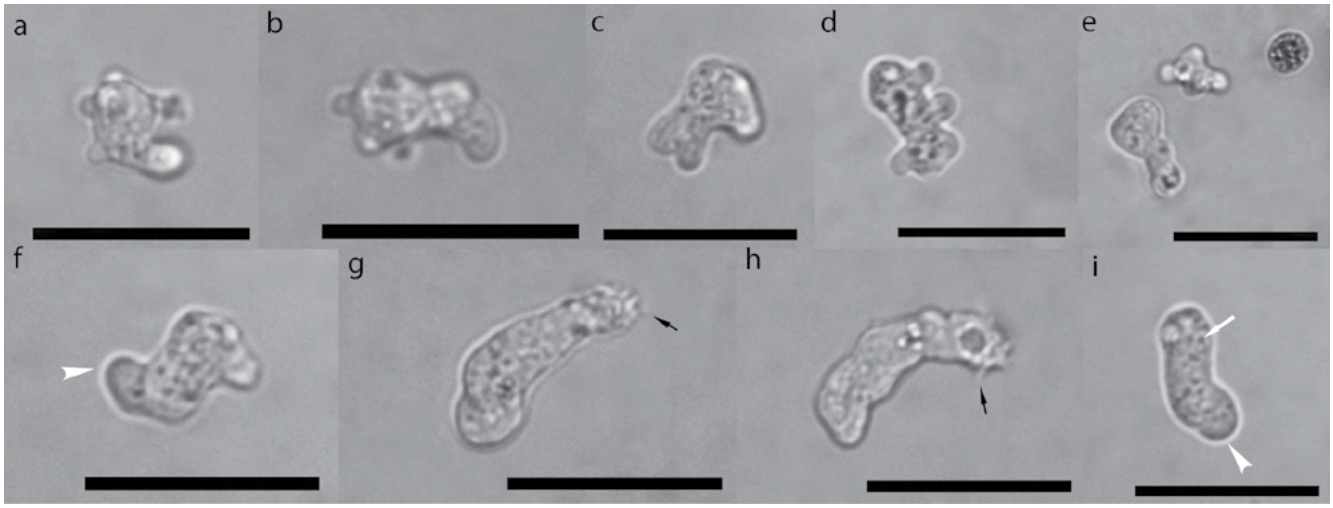


Fig. 5. LM pictures of *Schoutedamoeba minuta* showing trophozoites in non-oriented movement (a–f) and the limax-shaped locomotive form (g–i). The presence of a pronounced hyaline cap, small adhesive uroidal filaments and tiny granules in the cytoplasm are indicated with white arrowheads, black arrows and a white arrow respectively. Scale bars: 20 µm.

ing a new variosean lineage, the first with a hartmannellid-like morphology, with *S. minuta* its single representative so far.

Vexillifera

The acanthopodial strain A18WVB was placed in the monophyletic genus *Vexillifera* with *V. multispinosa* its closest relative (Fig. 6A), however the *p*-distance between these two strains was rather large (15.9%, 264 differing positions).

Locomotive amoebae of strain A18WVB were highly variable. Their shape was either triangular, spherical, slightly elongated or irregular and pseudopodia were often dactylopodial-like (e.g. Figs 6B 3, 7). Length without subpseudopodia was 13–43 µm (mean 22) and width 5–24 µm (11). About 9–22 (15) round-tipped subpseudopodia of maximally 11 µm long were present predominantly at the broader anterior side. A temporal, uroid-like, hyaline, bulbous or fasciculate structure (3–5 µm long) was sometimes seen posteriorly during locomotion (arrow in Fig. 6B 5). Usually one, rarely 2–3, small contractile vacuole(s) were visible posteriorly. The globular to elliptical vesicular nucleus was 2–5 µm in diameter and contained one slightly eccentrically located globular nucleolus of 1–2 µm. The cytoplasm always contained 10–40 small (1–2 µm), refractive spheroid granules. Floating amoebae (Figs 6B 8–11) were slightly elongated, about 15 µm long and 10 µm wide, displaying 5–17 sharply pointed pseudopodia that reached maximally 24 µm in length. No cyst stage was observed.

Vexillifera currently contains 24 species of which two were most probably misclassified (*V. expectata* belongs to *Hartmannella/Copromyxa* and *V. armata* is probably *Pseudoparamoeba pagei*, see Dyková *et al*. 2011). Six of the remaining species were isolated from marine habitats, 1 from brackish waters and 4 from freshwater fish tissues (amphizoic species). The other species are all free-living amoebae from freshwater habitats, however none of these are represented in GenBank since it only contains the free-living marine species *V. minutissima* (Bovee 1985) and the amphizoic *Vexillifera* species on fish (Dyková *et al*. 2011). Our *Microcystis*-associated strain showed some morphological similarities with *V. telma* and *V. displacata,* described from eutrophied stagnant freshwaters respectively muddy bottoms of slow-running streams in the US (Bovee 1985), however it differed in having an irregular floating form with long radiating pseudopodia (in contrast to the globular one of *V. telma* with only very small radiating pseudopodia), in being much larger (in contrast to *V. displacata* that is 7–12 µm long and 6–8 µm wide) and in its specific ecology (planktonic *Microcystis* colonies where it feeds on *Microcystis* cells and associated biota). These differences warranted its description as a new species, *Vexillifera westveldii*.

Cochliopodium

The lens-shaped strain A1HEB was placed in the monophyletic genus *Cochliopodium* (Fig. 7A), but the 18S rDNA sequence differed from other representatives of the genus by no less than 19.7–23.5% (143–301 bp).

Locomotive amoebae of strain A1HEB were round, oval or triangular (with the base directed anteriorly), had a length of 15–40 µm (mean 26 µm), a width of 15–54 µm (30) and a L/W ratio 0.7–1.2 (0.9) (Fig. 7B). The central mass of granuloplasm was always entirely surrounded by a hyaline sheet that covered 12–40% (23) of the body length and was often much narrower posteriorly (e.g. Figs 7B 3, 5). Locomotive amoebae contained 1–14 pointed subpseudopodia of maximally 10 µm long, that were never furcated and mostly situated at the lateral and anterior hyaloplasmic margin (Figs 7B 1–4). Uroidal structures were rarely visible posteriorly, either consisting of one to a few trailing adhesive filaments of maximally 10 µm long (Figs 7B 4, 5) or rarely plicate with a length of maximal 13 µm (Figs 7B 8, 9). The slightly ovoid vesicular nucleus was 3–5 µm in diameter and contained one globular nucleolus of 1–2 µm. Only one, centrally located contractile vacuole was visible in each trophozoite. The granuloplasm contained many, 1–2 µm long, opaque granules and up to 20 irregular, sometimes plate-like shaped crystals of 1–6 µm long. With bright field illumination easily visible micro-scales were about 0.9 µm long and 0.6 µm wide. These entirely covered the tectum, forming a regular punctuation of the hyaloplasm up to the cell's margin (Fig.7B 8). The trophozoites could also adopt

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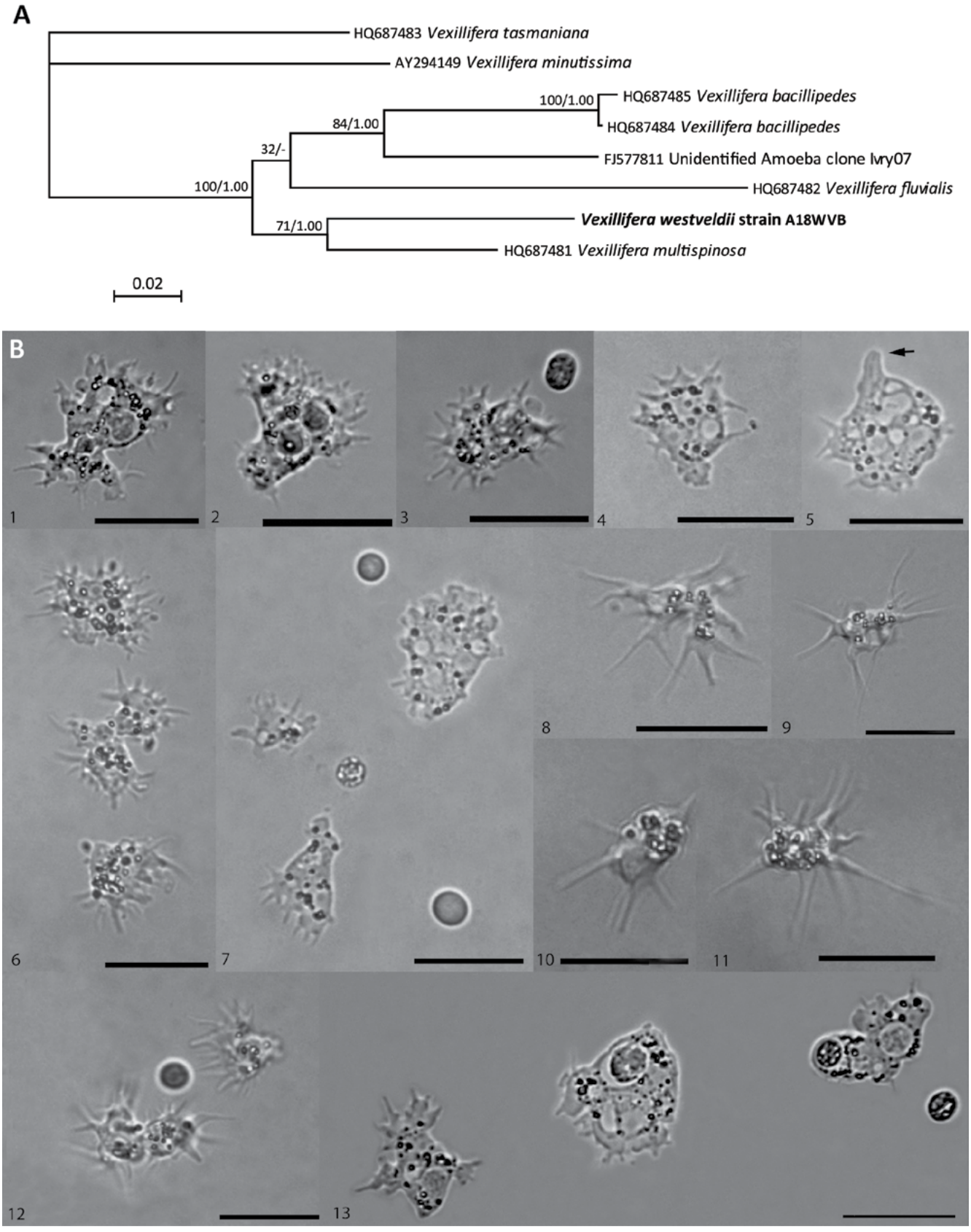
Fig. 6. A – 18S rDNA maximum likelihood phylogeny of *Vexillifera*, including the *Microcystis*-associated strain (in bold). ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B** – LM pictures of locomotive (1–7, 12–13) and floating (8–11) amoebae. *Microcystis* cells inside food vacuoles are visible in (1–2) and (13). The arrow in (5) points to a temporal, uroid-like structure sometimes visible during locomotion. Scale bars: 20 µm.


A
HQ687483 Vexillifera tasmaniana
AY294149 Vexillifera minutissima
100/1.00
HQ687485 Vexillifera bacillipedes
84/1.00
HQ687484 Vexillifera bacillipedes
32/-
FJ577811 Unidentified Amoeba clone Ivry07
100/1.00
HQ687482 Vexillifera fluvialis
Vexillifera westveldii strain A18WVB
71/1.00
HQ687481 Vexillifera multispinosa
0.02
B
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13

a globular to ovoid bell-shaped form that had a length of 8–30 µm (13) and a width of 8–22 µm (11). These were predominantly seen attached when feeding on *Microcystis* colonies (Fig. 7B 11). The clear peripheral hyaloplasmic sheet around the cell was then completely retracted with the exception of a small zone posteriorly where the hyaloplasm was clearly folded towards the substratum (for instance a *Microcystis* cell). When a potential food item was detected, this fringe of folded hyaloplasm did produce some small hyaline subpseudopodia that started surrounding the object of interest (Figs 7B 6, 7). Under a coverslip, this bell-shaped form could swiftly transform into a locomotive form with the re-formation of the peripheral hyaloplasmic sheet. The posterior fringe of folded hyaloplasm was then still visible for a while when it slowly retracted (Figs 7B 8, 9). Floating amoebae were globular to ovoid with a diameter of 8–20 µm (13), containing 2–8 long thin pseudopodia of maximal 36 µm long (Fig. 7B 12, 13). No cyst stage was observed. Although this strain initially grew well with *Microcystis* offered as food it suddenly died after several weeks. Due to this early loss, no TEM analysis could be carried out to retrieve detailed information on the scale structure.

Around 20 mainly morphologically defined *Cochliopodium* species currently exist (Geisen *et al*. 2014), 8 isolated from freshwater, 2 from fresh to brackish water, 3 from brackish, 4 from marine and 3 from terrestrial habitats. Of the 3 freshwater strains without molecular data, *C. minutum* was assigned a *nomen dubium* (Kudryavtsev 2006), *C. vestitum* is more than twice as large (L: 39–70 µm, mean 59; W: 48–74, mean 65) and has with LM clearly visible spines on the scales (Kudryavtsev 2005) and *C. granulatum* (Penard 1902), apart from also being much larger (generally > 50 µm), has many large subpseudopodia during locomotion and seems to preferably graze on diatoms. *C. minutoidum,* isolated from terrestrial habitats (Kudryavtsev 2006), is only half as large (L: 8–20 µm (mean 14), W: 9–24 (17)). Given the molecular difference, the absence of the above-mentioned morphological features (Fig. 7B) and its specific ecology, our *Microcystis*-associated strain is undoubtedly a new species of *Cochliopodium.* However, since we lacked detailed information on the scale structure, we opted not to give this taxon an official name.

Angulamoeba

In the *Angulamoeba* ML phylogeny (Fig. 8A), the additional reticulate strains (A4WVB, A7WVB) and sequences (A1WVB, A2WVB) were situated in the same lineage as the already published *Angulamoeba microcystivorans* sequences (Van Wichelen *et al*. 2010, Berney *et al*. 2015). *A. microcystivorans* sequences differed by 0.0–0.3% (0–3 bp) from each other. The nearest other sequence, obtained from a natural sample, differed by 6.6–7.5% (65–111 bp) from *A. microcystivorans*.

The trophozoites of all our strains were elongated, deeply inclined, sometimes seemingly connected with filopodia, and became aggregated into a plasmodium-like mucilage matrix at high population densities. They had an amoeboflagellate (1–3 flagella) and a cyst stage (Fig. 8B) and fed voraciously on *Microcystis*, resulting in the clearance of *Microcystis* cultures in a matter of days.

The morphology and 18S rDNA sequence of our strains were similar to our previously studied strain A1WVB isolated from the same bloom sample and newly described as *Angulamoeba microcystivorans* (Berney *et al*. 2015). We thus concluded they belonged to this species as well.

DISCUSSION

Isolation of planktonic amoebae associated with *Microcystis* bloom samples revealed a diverse array of free-living amoebae of which the majority appeared to be new to science. Our study not only increased the species diversity of several already known genera of naked amoebae (*Korotnevella, Vannella, Copromyxa, Vexillifera, Cochliopodium*) but also resulted in the description of a new variosean genus (*Schoutedamoeba*). We also noticed a few other, unknown amoebae on *Microcystis* colonies in our samples which we failed to cultivate. At

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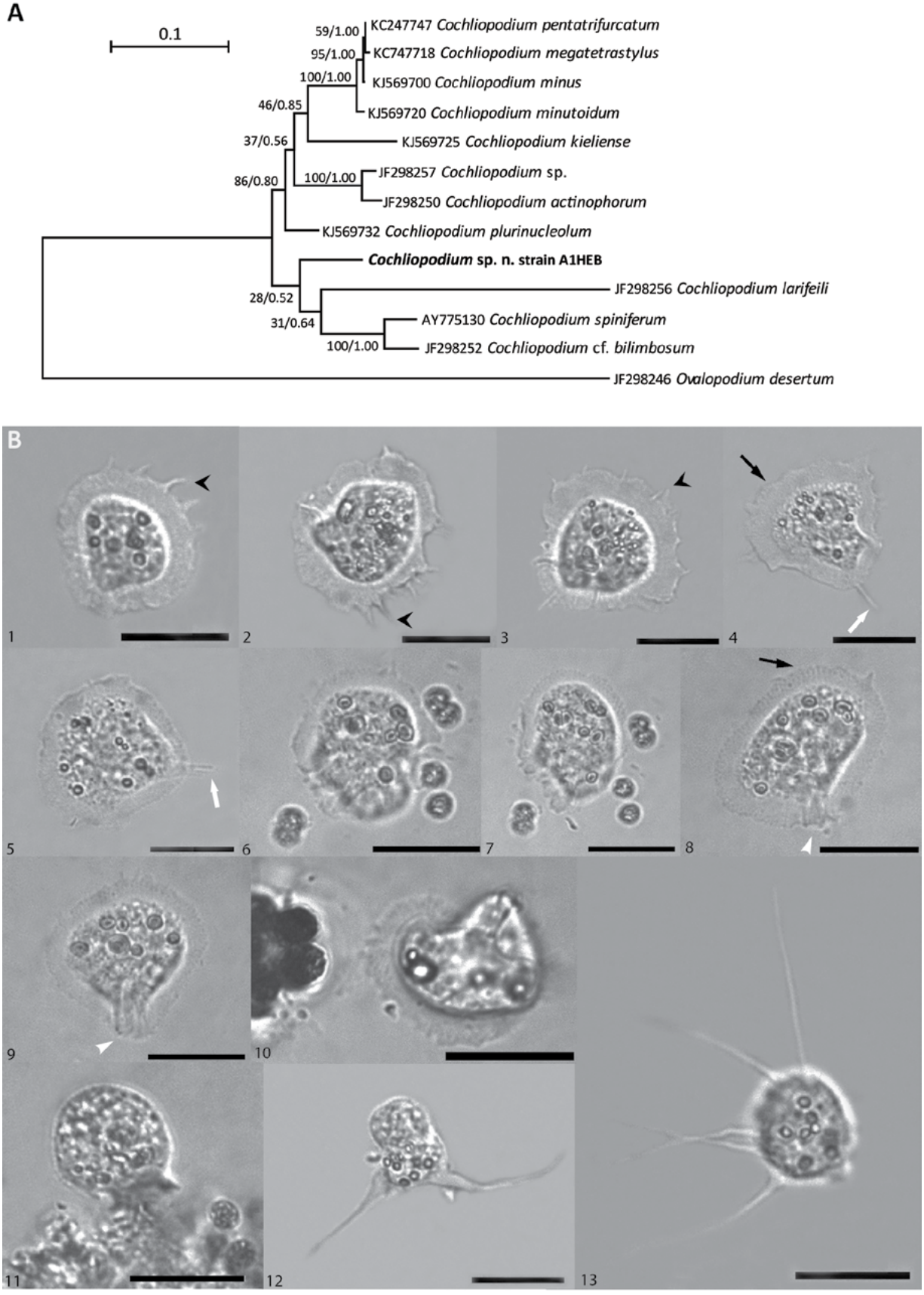
Fig. 7. A – 18S rDNA maximum likelihood phylogeny of *Cochliopodium*, including the *Microcystis*-associated strain (in bold). ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B** – LM pictures of locomotive amoebae clearly showing the surrounding hyaline sheet (black arrows) punctuated with microscales (best visible in 8), a few small subpseudopodia (black arrowheads) and trailing adhesive uroidal filaments (white arrows) (1–5), a trophozoite during and just after grazing on *Microcystis* cells showing a prominent fringe of folded hyaloplasm (white arrowheads) (6–9), the bell-shaped form on colonies of *Microcystis aeruginosa* (10–11) and floating amoebae (12–13). Scale bars: 20 µm.


A
0.1
59/1.00
KC247747 Cochliopodium pentatrifurcatum
95/1.00
KC747718 Cochliopodium megatetrastylus
100/1.00
KJ569700 Cochliopodium minus
46/0.85
KJ569720 Cochliopodium minutoidum
37/0.56
KJ569725 Cochliopodium kieliense
86/0.80
100/1.00
JF298257 Cochliopodium sp.
JF298250 Cochliopodium actinophorum
KJ569732 Cochliopodium plurinucleolum
Cochliopodium sp. n. strain A1HEB
28/0.52
JF298256 Cochliopodium larifeili
31/0.64
AY775130 Cochliopodium spiniferum
100/1.00
JF298252 Cochliopodium cf. bilimbosum
JF298246 Ovalopodium desertum
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least two additional amoeboid taxa not studied here are known to graze on *Microcystis*, the nucleariid amoeba *Nuclearia* cf. *simplex* (Opisthokonta) and the testate amoeba *Penardochlamys* sp. (Amoebozoa), both isolated from eutrophic Japanese lakes (Yamamoto 1981, Yamamoto and Suzuki 1984, Nishibe *et al*. 2004, Manage 2009, Mizuta *et al*. 2011). It seems therefore reasonable to assume that even with the 8 new species described in this study, several MAA remain to be described. Based on the current data, it appears that the ability to feed on (toxic) *Microcystis* is scattered over the entire phylogeny of amoeboid protists since the 12 currently known *Microcystis*-associated amoebae cover 7 genera belonging to 3 classes of Amoebozoa and 1 genus belonging to the Opisthokonta. Especially for specialist grazers this is indicative for a repeated evolution of specialization on *Microcystis*. Apart from *Microcystis*, also other planktonic colony-forming cyanobacteria in eutrophied freshwater bodies are prone to amoeba infestations (Cook and Ahearn 1976, Wright *et al*. 1981, Laybourn-Parry *et al*. 1987, Becares and Romo 1994). The fact that only one, very specific habitat from a rather restricted geographical area (*Microcystis* blooms in eutrophied ponds in western Europe) revealed so many new species, indicates not only that the diversity of planktonic naked amoebae is seriously underestimated, but also that only a tiny fraction of the total diversity of naked amoebae is currently known (about 200 species are described, see also Smirnov *et al*. 2011).

The degree to which MAA are restricted to *Microcystis* colonies is unknown. The strong food preference for *Microcystis* cells as shown by light microscopy observations and small-scale feeding experiments (Van Wichelen, unpublished data) suggests that most of the amoebae are highly specialized (*Angulamoeba microcystivorans, Vannella planctonica, Korotnevella pelagolacustris, Copromyxa microcystidis*). Others were found to be less preferential (*Vexillifera westveldii*) or unable to graze on *Microcystis* cells (*Schoutedamoeba minuta*) and thus may rather be more generalistic bacterivorous aggregate-dwelling amoebae scavenging the mucilage matrix containing a diverse community of heterotrophic bacteria (Worm and Søndergaard 1998, Brunberg 1999, Shi *et al*. 2012, Cai *et al*. 2014). A better understanding of the ecology and degree of specialization of these amoebae could be gained by investigating amoeba population dynamics in ponds throughout the year in both the benthic and planktonic environment together with (switches in) their feeding habits. In any case, it was shown earlier that MAA can have strong effects on bloom biomass and genotypic turnover in natural *Microcystis* populations (Van Wichelen *et al*. 2010). Their grazing activities also might be important for the biodegradation of cyanotoxins as was shown for *Microcystis*-associated bacteria and flagellates (Maruyama *et al*. 2003, Zhang *et al*. 2008).

Species delimitation comes down to the identification of separately evolving metapopulation lineages (De Queiroz 2007), which, as a consequence of their independent evolution, may have evolved differences in morphology, ecology, intrinsic reproductive barriers and the DNA sequence of various molecular markers. Each of these properties and the congruence between them can be used as a line of evidence for assessing species limits. The more specimens and data sources are investigated the more accurate (and thus stable) species delimitation should be (e.g. Foissner *et al*. 2011, Reid and Carstens 2012, Xu *et al*. 2012), even though good results can be obtained using only one data source (e.g. Agatha 2004, Douglas *et al*. 2011, Heger *et al*. 2013). The limited number of amoeba species described, of available strains per species, of strains for which DNA sequences are available, and of routinely used molecular markers (only the 18S rDNA) renders species discovery in naked amoebae challenging, and precludes more objective assessments of species limits such as automated phylogenetic species delimitation (Leliaert *et al*. 2014). Also in this study, only one to two strains were isolated for most of the new species described. However, given the often large differences in 18S rDNA sequence with the most closely related other species and the often clear differences in morphology and ecology with known species, there is little doubt

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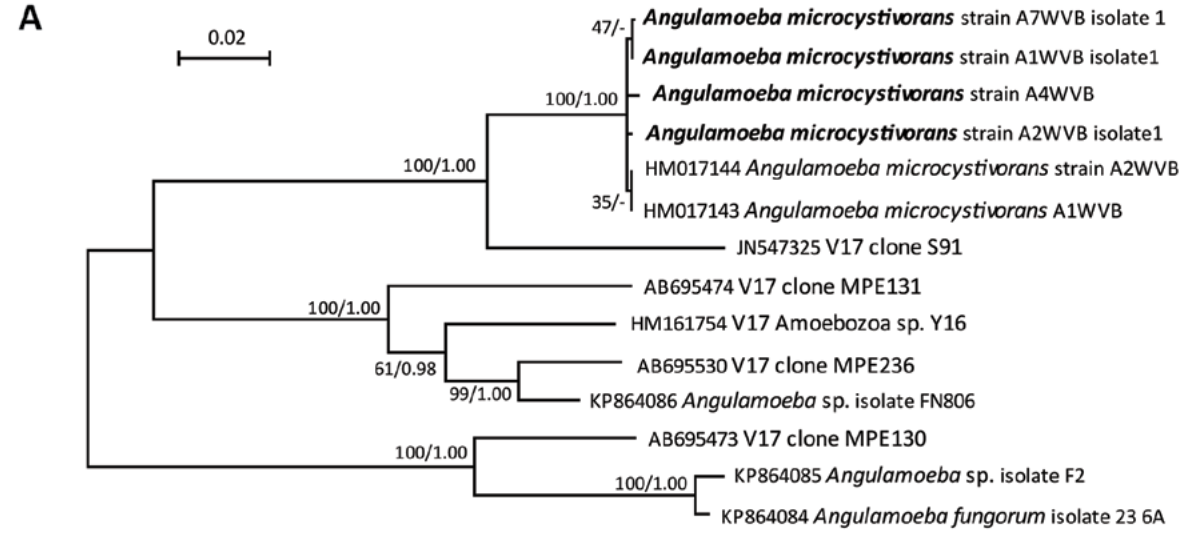
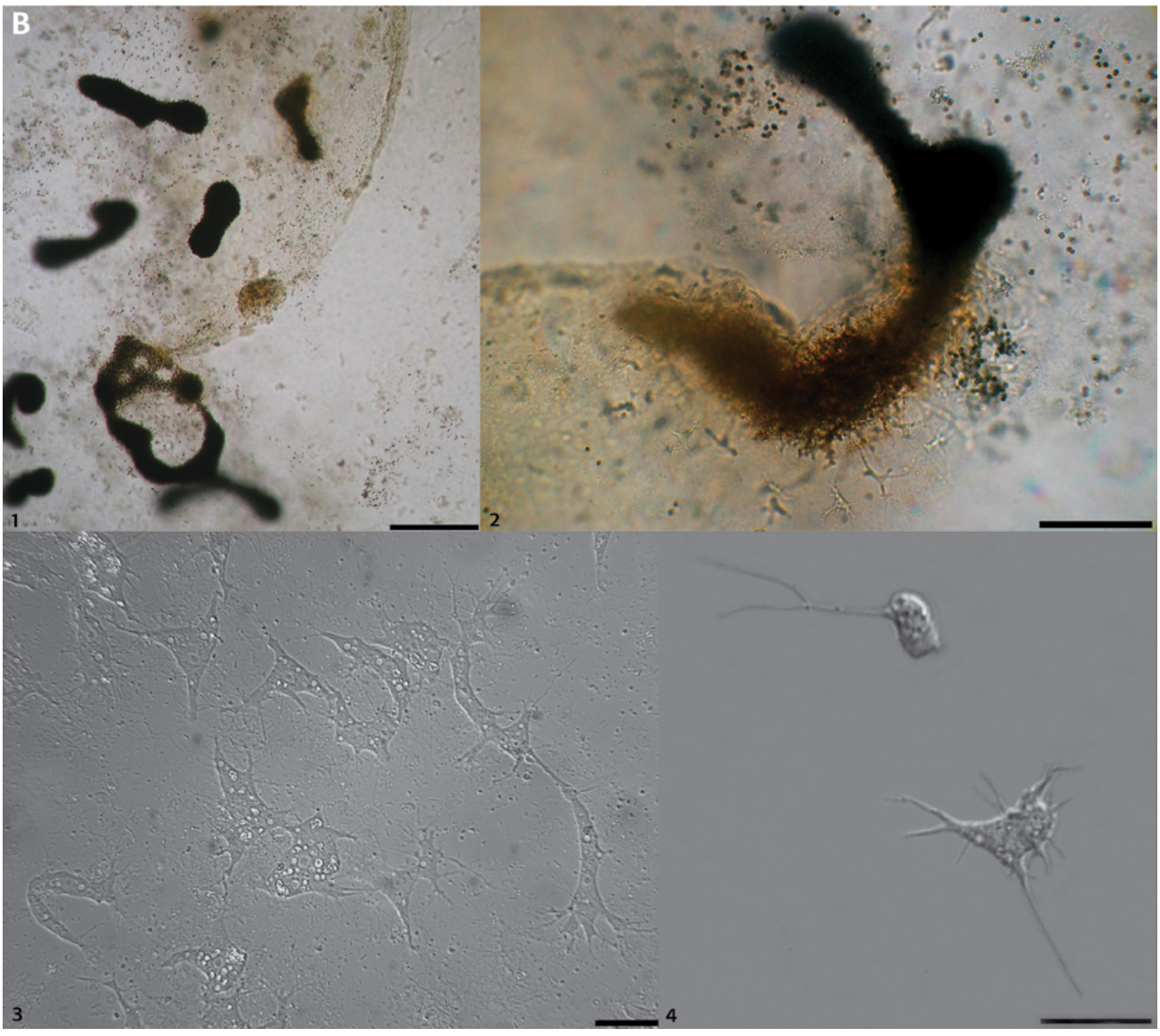
Fig. 8. A – 18S rDNA maximum likelihood phylogeny of *Angulamoeba*. Sequences new for this study are indicated in bold. ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names. **B** – LM pictures of *Angulamoeba microcystivorans* (strains A1WVB (1, 2, 4) and A4WVB (3)) showing the characteristic rusty-colored plasmodium-like mucilage matrix with embedded trophozoites devouring a culture of *Microcystis aeruginosa* colonies (1–2), multi-vacuolated trophozoites that are seemingly connected by their filopodia (3) and amoeba-flagellates in different stages of transition (4). Scale bars: 200 μm (1), 100 μm (2), 20 μm (3, 4).


A
0.02
47/-
Angulamoeba microcystivorans strain A7WVB isolate 1
Angulamoeba microcystivorans strain A1WVB isolate1
100/1.00
Angulamoeba microcystivorans strain A4WVB
Angulamoeba microcystivorans strain A2WVB isolate1
100/1.00
HM017144 Angulamoeba microcystivorans strain A2WVB
35/-
HM017143 Angulamoeba microcystivorans A1WVB
JN547325 V17 clone S91
AB695474 V17 clone MPE131
100/1.00
HM161754 V17 Amoebozoa sp. Y16
61/0.98
AB695530 V17 clone MPE236
99/1.00
KP864086 Angulamoeba sp. isolate FN806
AB695473 V17 clone MPE130
100/1.00
100/1.00
KP864085 Angulamoeba sp. isolate F2
KP864084 Angulamoeba fungorum isolate 23 6A


B
1
2
3
4

that they indeed belong to new species. The inclusion of more strains, species and molecular markers (such as the COI; Nassonova *et al*. 2010, Tekle 2014) will in the future allow for critical assessments of species limits. The sometimes large differences in 18S rDNA sequence observed here within (*V. planctonica*, *V. simplex*, *K. jeppesenii*) and between (*Copromyxa*, *Vexillifera*, *Cochliopodium*) amoeba species are remarkable, given that in most organisms this is a very conserved marker unsuitable for distinguishing closely related species (Piganeau *et al*. 2011). The addition of more strains and comparison with other molecular markers and inclusion of molecular clocks can reveal to what extent (1) the often very large differences between sister species in the 18S rDNA phylogenies in comparison with other groups are a consequence of the small fraction of amoeba diversity sequenced and (2) the 18S rDNA evolves faster than in other groups, and is therefore suitable for distinguishing even closely related species, as is the case in foraminifers (Darling *et al*. 2004, Darling and Wade 2008). The latter seems reasonable given the sometimes large intragenomic variation in 18S rDNA in Amoebozoa (e.g. up to 3.2% in some *Vannella simplex* strains: Smirnov *et al*. 2007, Nassonova *et al*. 2010).

Most of the amoeba species described here are difficult or impossible to distinguish from related species solely based on morphology, a common problem for naked amoebae (e.g. Smirnov and Goodkov 1999, Sims *et al*. 2002). An incomplete knowledge of the life cycle and especially the formation of fruiting bodies, considered to be an important taxonomic criterion, complicates matters even more and pleads for more extensive growth tests under different culture conditions. This would help interpreting the association of seemingly non-aggregating MAA with groups of fruiting amoebae (*Schoutedamoeba minuta*, *Copromyxa microcystidis, C. vandevyveri*, *C. cantabrigiensis*). It could also shed light on the occasional aggregation of trophozoites into large, floating clusters as was observed in this study for *Korotnevella pelagolacustris* and *K. jeppesenii*, or the 'atypical' aggregation and cellular fusion observed in some *Cochliopodium* species (Tekle *et al*. 2013, Anderson and Tekle 2013) which is supposed to play a role in parasexual activity (Tekle *et al*. 2014). Inversely, protosteloid amoebae appear scattered over the entire amoebozoan phylogeny (Shadwick *et al*. 2009). One example is the placement of strains of the protosteloid amoeba *Protosteliopsis fimicola* within *Vannella* based on an 18S rDNA phylogeny (Fig. 1A). Problems with routine species identifications because of incomplete life cycle information and lack of morphological characters can be avoided using DNA barcoding for identification (Hebert *et al*. 2003, Savolainen *et al*. 2005). Similar to other protist groups, building a DNA barcode database useful for species identifications will require continuous taxonomic efforts (Mann *et al*. 2010, Pawlowski *et al*. 2012). This will help identify the flow of eukaryotic sequence data produced by next-generation sequencing of environmental DNA, which is quickly increasing in microbial eukaryote community ecology (e.g. Jumpponen *et al*. 2010, Medinger *et al*. 2010, Li *et al*. 2011, Kermarrec *et al*. 2013).

Taxonomic conclusions

Based on the molecular and morphological characterization of our *Microcystis*-associated amoeba strains, we describe the following new taxa:

Vannella planctonica Van Wichelen & Vanormelingen sp. n.

Diagnosis: Locomotive form fan-shaped. Cell length during locomotion 15–57 µm (mean 35), width 13–64 µm (33) and length/width ratio 0.4–2 (1.1). Vesicular nucleus 2–7 µm in diameter with one centrally located nucleolus. Floating form usually radial and symmetric with 5–11 pointed pseudopodia that are basally thickened. Cysts elliptical to ovoid, up to 32 µm in diameter.

Feeding behavior: Feeds voraciously on *Microcystis aeruginosa* cells (see Van Wichelen *et al*. 2012) although growth is also sustained on heterotrophic bacteria and pico-cyanobacteria (Van Wichelen, unpublished).

Type material: Strain A2FBB is designated as type strain. It is available from the NIES Microbial Culture Collection (NIES-3728). The 18S rDNA GenBank accession number is KP719193. Other strains are A4P-4ZHB, A2SMB, A1DVDPB, A2DVDPB and A3D-VDPB (NIES-3765).

Type locality: Strain A2FBB was isolated from a *Microcystis* bloom sample from a ditch surrounding a moat (Fort Bornem, Belgium) (Table1). Other strains were isolated from *Microcystis* blooms in fish ponds in Zonhoven and in De Panne and in a recreational lake (Schulensmeer, Lummen), all located in the Flemish part of Belgium.

Etymology: *planctonica* referring to its planktonic life style.

Differential diagnosis: *V. lata* cells are always flabellate and the hyaline area occupies a larger proportion

of the cell (⅔) than in *V. planctonica* (40%). *V. miroides* is smaller (< 35 µm), contains crystals in its cytoplasm and has a more extended hyaline area (> ½ body length) surrounding the whole cell (Page 1988, Smirnov *et al*. 2007). *V. simplex* is much larger in size (on average 42–52 µm) and has usually fecal pellets adhered to the cell (Page 1988, Smirnov *et al*. 2007), a feature never observed in *V. planctonica*. The 18S rDNA sequence distinguishes *V. planctonica* from all other *Vannella* species currently present in GenBank.

***Korotnovella pelagolacustris* Van Wichelen & Vanormelingen sp. n.**

Diagnosis: Locomotive form dactylopodial with a length of 20–65 µm and a width of 6–32 µm. Vesicular nucleus about 6 µm in diameter with one slightly eccentrically located nucleolus. Floating form radial, maximally 25 µm in diameter, with up to 20 pointed pseudopodia. Scales elliptical, disk-shaped with a length of 260–391 nm and a width of 153–206 nm carrying a central spine 98–163 nm in length. Cyst formation rarely observed. Amoebae occasionally aggregate during cultivation.

Feeding behavior: It feeds voraciously on *Microcystis aeruginosa*. No growth was observed when offering other *Microcystis* morphotypes (*M. viridis*, *M. wesenbergii*), heterotrophic bacteria and green algae (*Acutodesmus*) (Van Wichelen *et al*. 2012, Van Wichelen, unpublished).

Type material: Strain A16WVB is designated as the type strain. It is available from the NIES Microbial Culture Collection (NIES-3726). The 18S rDNA GenBank accession number is HM017145 (see Van Wichelen *et al*. 2010). Other strains are A8WVB, A21WVB, A54WVB and A1LMS.

Type locality: Strain A16WVB was isolated from a *Microcystis* bloom sample from a small, eutrophied urban pond in Westveldpark, Sint-Amandsberg (Gent, Belgium). Other strains were isolated from the same locality and from a *Microcystis* bloom in Lake Mälaren (Ekoln, Sweden) (Table 1). Very high densities of a morphological similar amoeba were found in *Microcystis* bloom samples (06/07/2015) from a fishpond in Zandhoven (Belgium).

Etymology: *pélagos* Gr. sea, pelagic; *lacus* Lat. pond, lake: referring to its habitat.

Differential diagnosis: *K. pelagolacustris* closely resembles *K. diskophora* (Smirnov 1999), the only described *Korotnevella* species so far with disc-like scales on its cell surface (basket or crown-like shaped or more than 1 scale type in all other *Korotnevella* spp.). However, *K. diskophora* is smaller in size (16–34 µm, mean 23 µm) and has smaller spines (about 80 nm) on its scales. Moreover, the floating form of *K. pelagolacustris* is globular with up to 20 radiating thin pseudopodia while the one described for *K. diskophora* is irregular with 1–4 long thin, tapering pseudopodia. Both species also differ in their ecology. *K. diskophora* was isolated from bottom sediments of the small freshwater Lake Leshevoe in North-Western Russia where it feeds on bacteria while *K. pelagolacustris* is isolated from *Microcystis* blooms in eutrophied water bodies where it specifically feeds on the cells of certain *Microcystis* colonies (Van Wichelen *et al*. 2010, Van Wichelen unpublished). *K. jeppesenii,* the other newly described species with the same ecology as *K. pelagolacustris,* is clearly smaller in size (max. 41 µm), has smaller (max. 327 nm), relatively wider (mean L/W ratio 1.6) disc-like scales with shorter spines (max. 97 nm) and differs in 18S rDNA sequence.

***Korotnovella jeppesenii* Van Wichelen & Vanormelingen sp. n.**

Diagnosis: Locomotive form dactylopodial with a length of 16–41 µm and a width of 6–20 µm. Vesicular nucleus about 2–3 µm in diameter with one slightly eccentrically located nucleolus. Floating form radial, maximally 16 µm long, containing up to 10 long, pointed pseudopodia. Scales elliptical, disk-shaped with a length of 193–327 nm, a width of 124–203 nm, carrying a central spine of 47–97 nm in length. Amoebae occasionally form aggregates during cultivation. Cysts never observed.

Feeding behavior: Presumably highly specific grazer of *Microcystis*, similar to *K. pelagolacustris*.

Type material: Strain A4DVDPB is designated as the type strain. It is available from the NIES Microbial Culture Collection (NIES-3725). The 18S rDNA GenBank accession number is KP719186. Other strains: A1JEPDK.

Type locality: Strain A4DVDPB was isolated from a *Microcystis* bloom sample from a fish pond (Drie Vijvers) in De Panne (Belgium) (Table 1). Strain A1JEPDK was isolated from a *Microcystis* bloom sample originating from a mesocosm in Silkeborg (Denmark).

Etymology: Since this amoeba was first isolated from a mesocosm, being part of a long-term experiment estimating climate change effects on planktonic communities under supervision of Dr. Erik Jeppesen (Ozen *et al*.

2013), we named this amoeba in his honor. By doing so we also like to express our gratitude for his pioneering and stimulating research on shallow lake ecology.

Differential diagnosis: *K. jeppesenii* closely resembles *K. diskophora* (Smirnov 1999) in its general morphology and size. However the length of the scales is smaller (mean 277) than in *K. diskophora* (about 350). Its globular floating form with radiating pseudopodia also differs from *K. diskophora*'s atypical irregular one. Moreover, *K. jeppesenii* occurs in *Microcystis* blooms where it specifically feeds on *Microcystis* cells whereas *K. diskophora* is a bacterivorous bottom-dweller (Smirnov 1999). *K. pelagolacustris* is larger in size (max. 65 µm), has larger scales (max. 391 nm) with larger spines (max. 163 nm) and differs in 18S rDNA sequence.

Copromyxa microcystidis Van Wichelen & Vanormelingen sp. n.

Diagnosis: Locomotive amoebae limax-shaped, 20–59 µm long and 7–17 µm wide with anteriorly a well pronounced hyaline cap. Monopodial movement eruptive-like by means of alternating lateral sprouting lobes regularly adopting a bipodial or multipodial state. Vesicular oval nucleus of 5–10 µm in diameter with one central to slightly eccentrically placed globular nucleolus. Usually one, rarely up to 3 small contractile vacuoles present posteriorly. The cytoplasm always contains up to 22 opaque crystals and several smaller, refractive spheres. Floating form spherical with a diameter of 15–20 µm and radially displaying up to 10 small hyaline lobes. Cysts spherical with a diameter of maximally 20 µm.

Feeding behavior: It feeds voraciously on *Microcystis aeruginosa*. Other *Microcystis* morphotypes (*M. viridis*, *M. wesenbergii*), heterotrophic bacteria and green algae (*Acutodesmus*) were not ingested (Van Wichelen, unpublished).

Type material: Strain A2JEPDK is available from the NIES Microbial Culture Collection (NIES-3723). The 18S rDNA GenBank accession number is KP719188.

Type locality: Isolated from a *Microcystis* bloom sample originating from a mesocosm in Silkeborg (Denmark) (Table 1).

Etymology: *microcystidis* referring to *Microcystis*, its main food.

Differential diagnosis: Morphologically, *C. microcystidis* differs slightly from the next species (*C. vandevyveri*) by its more eruptive-like pseudopodium formation, its smaller, more globular subpseudopodia during non-oriented movement and the presence of large crystals in the cytoplasm. Also its 18S rDNA sequence distinguishes it from *C. vandevyveri*.

Copromyxa vandevyveri Van Wichelen & Vanormelingen sp. n.

Diagnosis: Locomotive amoebae limax-shaped, 20–55 µm long and 8–25 µm wide, displaying anteriorly a well pronounced hyaline cap. Bipodial and multipodial movement common by means of alternating sprouting (eruptive) lobes. Pseudopodia often displaying a few small, sprouting, short-lived subpseudopodia laterally. Occasionally a few adhesive uroidal filaments present. Vesicular nucleus 4–8 µm long and 4–7 µm wide with one centrally to slightly eccentrically located globular nucleolus. Usually one contractile vacuole posteriorly. Cytoplasm contains up to 20 small crystals. Floating forms spherical to irregular, about 15–20 µm in diameter with radially up to 12 small hyaline lobes. Spherical cyst occasionally present.

Feeding behavior: Grazing on *Microcystis* cells was observed in the *Microcystis* bloom sample and in laboratory cultures.

Type material: Strain A5DVDPB is available from the NIES Microbial Culture Collection (NIES-3724). The 18S rDNA GenBank accession number is KP719189.

Type locality: Isolated from a *Microcystis* bloom sample from a fish pond (Drie Vijvers) in De Panne (Belgium) (Table 1).

Etymology: *vandevyveri* referring to the first author's beloved wife Evelien Van de Vyver but also refers to the habitat this amoeba occupies since in Dutch 'van de vijver' literally means 'from the lake'.

Differential diagnosis: Morphologically, *C. vandevyveri* differs slightly from *C. microcystidis* by its less eruptive-like pseudopodium formation, its longer, slender subpseudopodia during non-oriented movement (max. 25 µm long compared to 13 µm in *C. microcystidis*) and the absence of large crystals in the cytoplasm. Also its 18S rDNA sequence is clearly different from that of *C. microcystidis*.

Schoutedamoeba Van Wichelen & Vanormelingen gen. n.

Diagnosis: Very small (generally < 20 µm), uninucleate limax amoeba characterized by alternating eruptive-like pseudopodia formation. Occasional presence of small adhesive uroidal filaments. Posteriorly one small contractile vacuole. Cytoplasm contains several small refractive spheres. Globular floating form with

radially several small round, bulb-like pseudopodia. No aggregation or cysts observed. Its 18S rDNA shows a secondary structure (expansion of helix 43 in the variable region V7) typical for the Variosea.

Differential diagnosis: Morphologically very similar to small Hartmannellidae and Vahlkampfiidae. Probably only distinguishable by its 18S rDNA sequence.

Etymology: The genus is named in honor of Prof. Dr. Henri Schouteden, a prominent Belgian zoologist who was the first to study and describe amoebae from Belgian localities (Schouteden 1905a, b; Van Oye 1956).

Schoutedamoeba minuta Van Wichelen & Vanormelingen sp. n.

Diagnosis: Locomotive amoebae limax-shaped, 12–22 µm long and 4–7 µm (6) wide anteriorly displaying a short-lived small hyaline cap. A few small adhesive uroidal filaments present occasionally. Vesicular nucleus about 2 µm in diameter with one centrally located nucleolus. Usually one small contractile vacuole posteriorly. The cytoplasm always contain several small, refractive granules. Floating form spherical, about 10 µm in diameter with radially several small hyaline lobes. No cyst stage or aggregation observed.

Feeding behavior: *Microcystis* cells never observed inside food vacuoles. It probably feeds on bacteria inside the mucilage matrix of *Microcystis* colonies.

Type material: Strain A1P5ZHB is available from the NIES Microbial Culture Collection (NIES-3727). The 18S rDNA GenBank accession number is KP719190.

Type locality: Isolated from a *Microcystis* bloom sample from a fishpond in Zonhoven (Belgium) (Table 1). Morphological similar amoebae were found occasionally in other *Microcystis* bloom samples, e.g. from an urban pond in Brugge (08/09/2015).

Etymology: *minuta* Lat., minute, referring to its small cell size.

Differential diagnosis: Morphologically probably indistinguishable from small members of the Hartmannellidae and due to its eruptive-like movements also from small Vahlkampfiidae (Heterolobosea).

Vexillifera westveldii Van Wichelen & Vanormelingen sp. n.

Diagnosis: Locomotive amoebae acanthopodial with a triangular, spherical or slightly elongated shape. Pseudopodia often dactylopodial-like. Length without subpseudopodia 13–43 µm and width 5–24 µm. Usually one, rarely 2–3, small contractile vacuole(s) posteriorly. Vesicular nucleus 2–5 µm in diameter with one globular nucleolus. The cytoplasm always contains small, refractive spheroid granules. Floating amoebae slightly elongated, about 15 µm long with up to 17 sharply pointed pseudopodia. No cyst stage observed.

Feeding behavior: *Microcystis* cells were observed inside food vacuoles, however the amoebae seem to grow better when only pico-cyanobacteria or heterotrophic bacteria were offered as food. No growth was observed with *Acutodesmus* as food source (Van Wichelen, unpublished data).

Type material: Strain A18WVB is available from the NIES Microbial Culture Collection (NIES-3729). The 18S rDNA GenBank accession number is KP719187.

Type locality: Strain A18WVB was isolated from a *Microcystis* bloom sample from a small, eutrophied urban pond in Westveldpark, Sint-Amandsberg (Gent, Belgium) (Tabel 1). High densities of morphological very similar amoebae were found on *Microcystis*-colonies in samples (07/09/2013) from an eutrophied moat in Bazel (Belgium).

Etymology: *westveld* refers to the pond (Westveldpark) from where the strain was isolated.

Differential diagnosis: Locomotive trophozoites of *V. westveldii* morphologically show strong similarities with *V. multispinosa*, *V. bacillipedes, V. telma* and *V. displacata* (Bovee 1985, Page 1988, Dyková *et al*. 2011). However, *V. westveldii* is slightly to much larger in size compared to *V. telma* (20–32 µm), *V. multispinosa* (10–20 µm), *V. bacillipedes* (8–24 µm) and *V. displacata* (7–12 µm). *V. westveldii* is distinguishable from *V. telma* by its irregular floating form with long radiating pseudopodia in contrast to the globular one of *V. telma* with only very small radiating pseudopodia. Moreover, *V. westveldii* is isolated from *Microcystis* blooms in contrast to the other species that were isolated from flowing water (*V. displacata*, *V. bacillipedes*) or fish organs (*V. multispinosa*, *V. bacillipedes*). The 18S rDNA sequence also distinguish *V. westveldii* from all other *Vexillifera* species currently present in GenBank.

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Supplementary Fig. 1. 18S rDNA maximum likelihood phylogeny of Amoebozoa, with in red, lineages to which our *Microcystis*-associated strains (in bold) belong. ML bootstrap values respectively posterior probabilities are shown at the nodes. GenBank accession numbers are given together with the species names.