Protist 173 (2022) 125870
http://www.elsevier.de/protis
Published online date

Protist

ORIGINAL PAPER

Five Groups in the Genus *Allovahlkampfia* and the Description of the New Species *Vahlkampfia bulbosis* n.sp.



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Submitted December 11, 2020; Accepted March 18, 2022
Monitoring Editor: Alastair Simpson

Heterolobosea is one of the major protist groups in soils. While an increasing number of soil heterolobosean species has been described, we have likely only scratched the surface of heterolobosean diversity in soils. Here, we expand this knowledge by morphologically and molecularly classifying four novel strains. One was identified as *Naegleria clarki*, while the remaining three strains had no identical Blast hit against GenBank and could only be reliably identified to the genus level: two strains as *Allovahlkampfia* spp. and one strain as *Vahlkampfia* sp. One *Allovahlkampfia* strain was most closely affiliated with *Allovahlkampfia* sp. NI64 and the other strain was affiliated with '*Solumitrus*' *palustris*, which is now named *Allovahlkampfia palustris* comb.nov. As there are only two valid species described within *Allovahlkampfia*, we combined all published sequences related to *Allovahlkampfia* and propose five new groups within this genus. The last strain was most closely related, but clearly distinct from, *Vahlkampfia orchilla,* based on DNA barcoding. As such, we propose this amoeba as a new species named *Vahlkampfia bulbosis* n.sp. Together, our study extends the described diversity of soil heteroloboseans through the description of a new *Vahlkampfia* species and by revising the morphologically and phylogenetically diverse genus *Allovahlkampfia.*
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Key words: *Allovahlkampfia*; *Vahlkampfia bulbosis* n.sp.; Heterolobosea; ITS region; 18S rRNA gene.

Introduction

Protists within the Heterolobosea have a global distribution ranging from tropical to polar regions, including extreme environments (Larsen and Patterson 1990; Park and Simpson 2011; Reeder et al. 2015; Tyml et al. 2016). Heteroloboseans are morphologically diverse, including amoebae,

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https://doi.org/10.1016/j.protis.2022.125870
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flagellates, amoeboflagellates and acrasid "slime molds" (Brown et al. 2012; Brugerolle and Simpson 2004; Harding et al. 2013; Pánek et al. 2017; Yubuki and Leander 2008). During locomotion, most heterolobosean amoebae adopt a cylindrical limax shape with eruptive bulges (Page and Blanton 1985). Some heterolobosean species, including several *Naegleria* species (the most well-studied heterolobosean genus due to the particular attention paid to the human pathogenic *Naegleria fowleri*), can shift between an amoeba and a flagellate stage (De Jonckheere 2002, 2011, 2012; Visvesvara et al. 2007).

Classical identification of heterolobosean species has relied on morphological characters (Page 1967). However, molecular tools have revealed that morphological characters can be ambiguous, with limited ability to distinguish between species (De Jonckheere and Brown 2005). Molecular sequencing using barcoding regions such as the 18S rRNA gene and the ITS (internal transcribed spacer) region has substantially increased the taxonomic resolution within Heterolobosea and corrected the often erroneous taxon relatedness previously proposed based on morphological characters (Brown and De Jonckheere 1999; De Jonckheere and Brown 2005). For example, the genus *Vahlkampfia* has been shown to be genuinely polyphyletic, leading to its subsequent division into several new genera including *Neovahlkampfia*, *Paravahlkampfia,* and reassignment of other species to *Tetramitus* (Brown and De Jonckheere 1999).

To date, approximately 150 heterolobosean species, including *ca.* 26 soil species, have been described (Anderson et al. 2011; Brown and De Jonckheere 2004; De Jonckheere 2002; De Jonckheere et al. 2011; De Obeso Fernadez Del Valle and Maciver 2017; Geisen et al. 2015; Murase et al. 2010; Sandon 1927). Soil isolates are underrepresented in the current phylogenies, with soil species described for seven out of the 35 known heterolobosean genera: *Naegleria*, *Allovahlkampfia, Fumarolamoeba, Parafumarolamoeba, Paravahlkampfia, Tetramitus* and *Vrihiamoeba* (Pánek et al. 2017).

As explained above, due to its potential pathogenicity, *Naegleria* spp. have received a disproportionate amount of attention in research focused on (soil) Heterolobosea, which has led to a thorough molecular species definition within the genus *Naegleria* (De Jonckheere 2004). However, much less is known about other heterolobosean genera. Among *Allovahlkampfia* species, only two, *A. spelaea* (Walochnik and Mulec 2009) and *A. minuta* (De Obeso Fernadez Del Valle and Maciver 2017) have been formally described. A third species, which was originally described as '*Solumitrus*' *palustris* (Anderson et al. 2011), has also repeatedly been shown to be placed within *Allovahlkampfia* (Brown et al. 2012; Geisen et al. 2015).

The aim of the current study was to increase the existing knowledge concerning the diversity and phylogeny of soil heterolobosean amoebae. To this end, we isolated four distinct heterolobosean amoebae from soils of different origins, examined each strain's morphology and tested their ranges of thermotolerance. We also sequenced the 18S rRNA gene and the ITS region, including the 5.8S rRNA gene, to allow for robust phylogenetic analysis of each strain. We could show that one strain was identical to *Naegleria clarki,* while two strains were affiliated to new species/strains of *Allovahlkampfia* and one strain represented a new species within *Vahlkampfia.*

Results

Cultivation and Morphological Identification

Seven wells containing potential heterolobosean amoebae were observed in 96-well plates. After purifying each strain, we isolated four heterolobosean strains. All strains exhibited a typical heterolobosean eruptive locomotion and showed an irregular shape with eruptive pseudopods projecting in various directions during the non-locomotive stage (Fig. 1). All strains formed cysts (Fig. 1).

No specific floating forms, flagellate stages, or formation of fruiting bodies could be observed across a range of test conditions for any of the strains examined.

Strain NL10 mostly adopted an elongated limax locomotive form, but occasionally showed an elongated flabellate shape (Fig. 1a). A clear and large hyaline part was observed in the locomotive form. Trophozoites (length range: 13.7–21.2 μm, width range: 5.6–10.0 μm based on 11 different cells) of NL10 were uninucleate with one contractile vacuole being observed. One nucleus was observed that was not centrally located in the trophozoites (nucleus diameter range: 2.6–4.7 μm). The nucleolus was centrally located in the nucleus (diameter range: 1.7–2.3 μm). Cysts were spherical but profoundly differed in size, ranging from 6.0 to

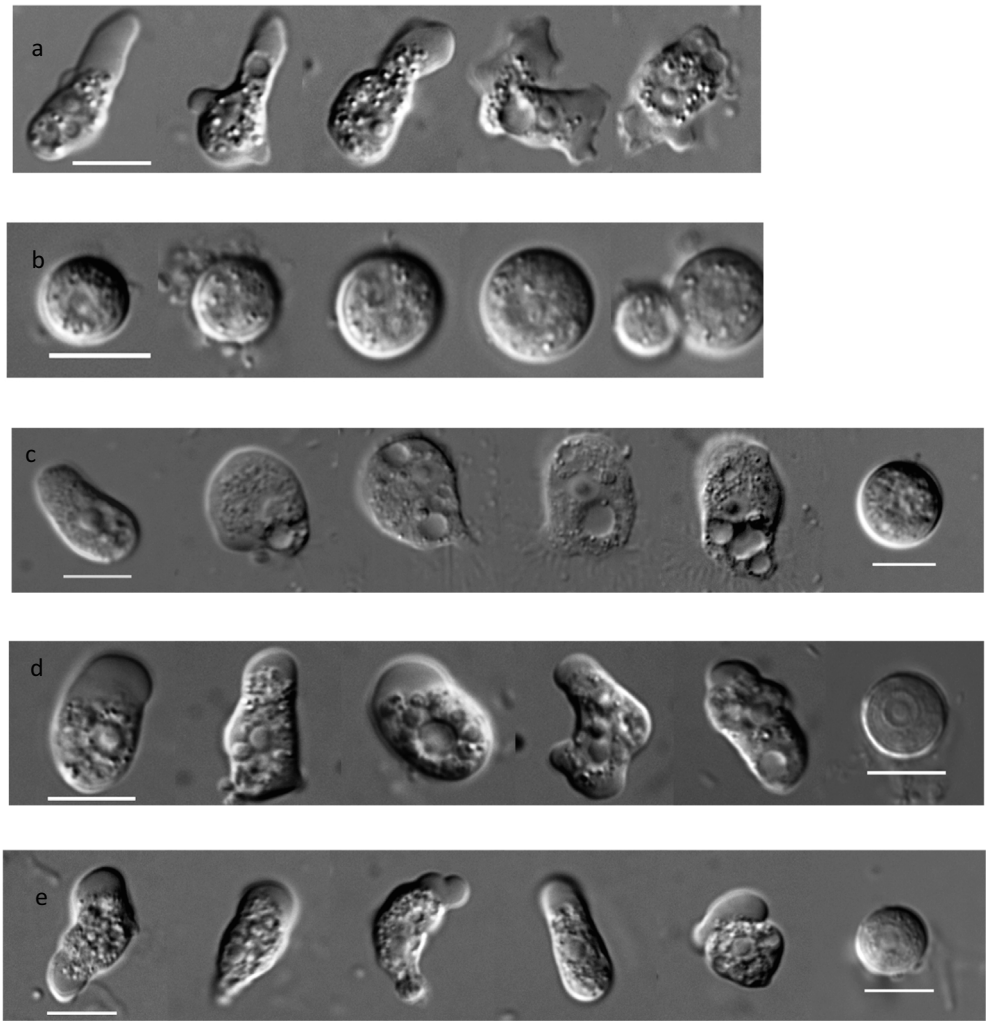


Figure 1. Differential interference contrast (DIC) images showing trophozoites and cysts of all strains described in this study. (**a**) NL10 (*Allovahlkampfia* sp.); (**b**) cysts of NL10, note the size difference in cysts; (**c**) NL28 (*Allovahlkampfia* sp.) and the last image shows the cyst of NL28; (**d**) NL81 (*Naegleria clarki*) and the last image shows the cyst of NL81; (**e**) CN7 (*Vahlkampfia bulbosis* n.sp.) and the last image shows the cyst of CN7; Scale bar: 10 μm.

11.1 μm (Fig. 1b). NL10 remained active at temperatures of up to 37 °C (Table 1).

Strain NL28 occasionally moved in a limax shape, but more often moved in a flabellate shape with an enlarged anterior with a small hyaline area (Fig. 1c). At the posterior end, strain NL28 showed a villous uroid (Fig. 1c). One to three contractile vacuoles could be observed in trophozoites (length range: 16.0–32.5 μm, width range: 5.3–19.0 μm based on 28 different cells) of strain NL28. One nucleus (diameter range: 3.0–6.4 μm, not centrally located in the trophozoite) could be observed. The nucleolus was 1.7–3.4 μm in diameter. Cysts were spherical. Strain NL28 could only tolerate temperatures up to 20 °C, as not even cysts were formed above that temperature (Table 1).

Strain NL81 mostly showed a limax shape during locomotion. The locomotive trophozoite was 14.5–42.3 μm in length and 7.9–21.5 μm in width (based on 20 different cells) and formed a clear hyaline cap at the anterior end (Fig. 1d, Table 1). One nucleus with a single nucleolus located in the center was observed in NL81 (nucleus diameter range: 2.3–4.6 μm, nucleolus diameter range: 1.7–3.5 μm). One large contractile vacuole was observed in the granular cytoplasm at the posterior end. Cysts of NL81 were spherical (diameter range:

Table 1. List of strains with their origins and morphological characters.

Strain	Origin	GPS	Max Temp (°C)	Locomotive from	Trophozoite length range/ average (μm)	Trophozoite width range/ average (μm)	Cyst diameter range/ average (μm)	Nucleus diameter range/ average (μm)	Nucleolus diameter range/average (μm)
CN7	China	32° 03′09″, 118° 55′36″	37	Limax	13.7–24.2/ 18.7 ± 0.7	4.4–11.4/ 7.3 ± 0.4	7.2–12.2/ 9.6 ± 0.2	2.3–3.8/ 3.3 ± 0.1	2.1–3.1/ 2.4 ± 0.1
NL10	Netherlands	51° 51′60″; 5°53′34″	37	Mostly limax / Occasionally flabellate	13.7–21.2/ 17.5 ± 0.8	5.6–10.0/ 7.6 ± 0.4	6.0–11.1/ 8.5 ± 0.2	2.6–4.7/ 3.3 ± 0.2	1.7–2.3/ 1.9 ± 0.03
NL81	Netherlands	51° 55′32″; 4°29′39″	20	Limax	14.5–42.3/ 21.1 ± 1.4	7.9–21.5/ 14.0 ± 0.8	6.7–13.7/ 10.3 ± 0.2	2.3–4.6/ 3.3 ± 0.1	1.7–3.5/ 2.2 ± 0.1
NL28	Netherlands	51° 56′60″; 6°10′59″	20	Occasionally limax	16.0–32.5/ 22.8 ± 1.1	5.3–19.0/ 9.8 ± 0.8	8.4–13.3/ 10.2 ± 0.2	3.0–6.4/ 4.1 ± 0.2	1.7–3.4/ 2.6 ± 0.1

6.7–13.7 μm), with a clear nucleus and a perinuclear layer of granules, surrounded by a smooth and separated cyst wall (Fig. 1d). The trophozoites and cysts of strain NL81 had a thermotolerance to only 20 °C (Table 1).

CN7 showed a limax shape during locomotion, the hyaline area became larger and formed asymmetrical bulges pointing in different directions when the amoebae stopped moving (Fig. 1e). Amoebae were 13.7–24.2 μm long and 4.4–11.4 μm wide during locomotion (based on 20 different cells). The anterior end of CN7 was usually broader than the posterior end, where sometimes a bulbous uroid was formed (Fig. 1e). No contractile vacuole was found in CN7. Cysts (diameter range: 7.2–12.2 μm) were spherical with a smooth wall and were uninucleate with a centered nucleolus (diameter: 2.1–3.1 μm). Cysts contain granular cytoplasm, cyst pore was not found. CN7 trophozoite had thermotolerance of up to 37 °C (Table 1).

Phylogenetic Analyses

We sequenced nearly the complete 18S rRNA gene in all strains (Table 2) except NL81. Only half of the full length 18S rRNA gene sequence could be obtained for NL81, possibly because of primer limitations. The lengths of the ITS region, including the 5.8S rRNA gene, were variable with especially NL81 showing a longer ITS region than the other strains (Table 2). The 5.8S rRNA gene was 161 bp long in NL10 and strain NL28, while the 5.8S rRNA gene was longer (175 bp) in NL81 and shorter (156 bp) in CN7 (Table 2).

The 18S rRNA gene sequence of NL81 was 100 % identical to the top blast hit, *Naegleria clarki*. Phylogenetic analyses on the 18S rRNA gene confirmed that NL81 is closely related to *Naegleria clarki* (Fig. 2 Multigene, Supplementary Material Figs S1, S2 18S rRNA, 5.8S rRNA). NL28 and NL10 were placed within the genus *Allovahlkampfia* based on Blast searches and phylogenetic analyses on the 18S rRNA gene. NL28 showed the closest affiliation with *Allovahlkampfia spelaea* (Walochnik and Mulec 2009), and NL10 with *Allovahlkampfia sp.* Nl64 (Geisen et al. 2015) (Fig. 2). The BLAST search of the ITS region also revealed the same affiliation of NL10 to *Allovahlkampfia sp.* Nl64. In contrast, the Blast search of the ITS region suggested that strain NL28 more closely resembled '*Solumitrus*' *palustris* (99% query cover with 89.9% identity), and a close relationship was confirmed

Table 2. Sequence length (bp) of the 18S rRNA gene and ITS region of all strains.

Strains	18S rRNA gene	ITS1	5.8S	ITS2
CN7	1883	167	156	456
NL10	2051	186	161	147
NL81	1318 (partial ength)	441	175	481
NL28	2103	305	161	152

by phylogenetic analyses (Fig. 2 Multigene, Supplementary Material Fig. S2 5.8S rRNA). CN7 most closely matched *Vahlkampfia inornata* when Blasting the 18S rRNA gene, while Blasting the ITS region suggested *Vahlkampfia orchilla* as the closest relative.

As NL81 was identical in 18S rRNA gene sequence to *Naegleria clarki* strain 4564/IV (Dyková et al. 2006), we focused our further analyses on the remaining three strains. For the two strains that are affiliated with *Allovahlkampfia* spp., we constructed phylogenetic trees containing all published sequences resembling *Allovahlkampfia* strains and further compared their sequence similarities (Fig. 3 ITS region, Table 3). This revealed that NL10 is most closely affiliated with *Allovahlkampfia. sp*. Nl64 (Fig. 3 ITS region). Strain NL28 showed the highest similarity to '*Solumitrus*' *palustris* based on the 18S rRNA (99.3%) and 5.8S rRNA genes (99.4%) (Table 3).

To resolve the identity of CN7, we performed phylogenetic analyses based on the ITS region sequences with published sequences resembling *Vahlkampfia* strains (Fig. 4). CN7 formed a distinct, well-supported clan with *Vahlkampfia orchilla* and *Vahlkampfia* sp. S10, with posterior probability of 0.997 and maximum likelihood, 98%. Sequence similarity of all strains in the closely related branches showed that CN7 had 99.0 % similarity with closest related species, *Vahlkampfia inornata* (18S rRNA gene), while showing 99.3 % similarity with *Vahlkampfia orchilla* (5.8S rRNA gene sequences; Table 4).

Phylogenetic analysis strongly supported heterogeneity in the genus *Allovahlkampfia* as previously addressed by Geisen et al. (2015) (Fig. 3). By closely comparing all strains in the genus *Allovahlkampfia* (Table 5, 6), we propose to divide this genus into five groups (Fig. 4, Tables 5,6). The first

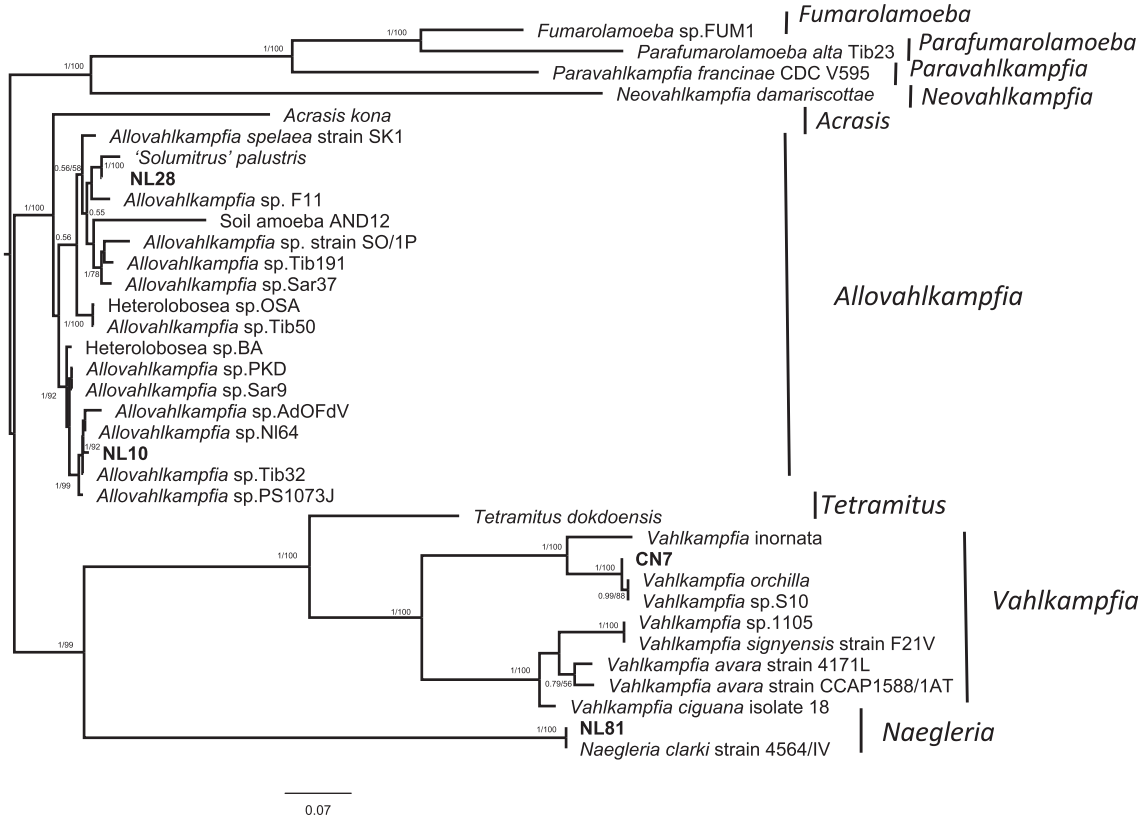


Figure 2. Unrooted maximum-likelihood (ML) tree based on combined 18S rRNA gene and ITS sequences. Support value at each node show posterior probability (pp) of Bayesian Inference (left) and ML bootstrap value (BS) inferred using RaxML (right). Support values of pp < 0.5 and BS < 50% are not shown.

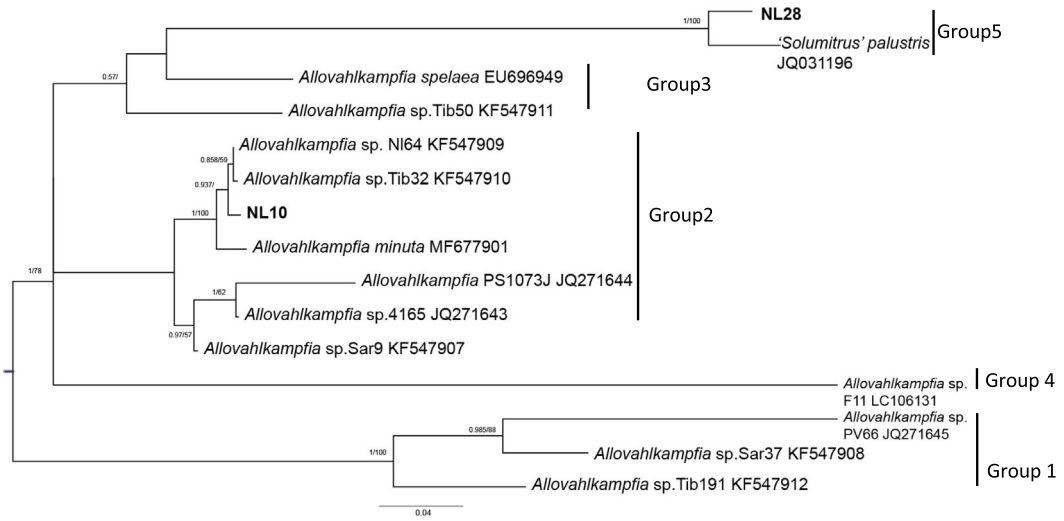


Figure 3. Unrooted maximum-likelihood tree based on the entire ITS region in *Allovahlkampfia* strains sequences. Support value at each node show posterior probability (pp) of Bayesian Inference (left) and ML bootstrap value (BS) of RaxML (right). Support values of pp < 0.5 and BS < 50% are not shown. GenBank accession numbers of the ITS sequences used in the analysis are listed next to the taxon names.

group contains Tib191, Sar37 (Geisen et al. 2015) and PV66 (Dyková and Kostka 2013) (JQ271645), as these species show a limax locomotive form and showed a similarity within the ITS region of 85.3–89.2%. All strains in this first group had a 162–163 bp long 5.8S rRNA gene, which was 1–2 bp longer than that of other groups (161 bp) (Table 5). The second group consists of seven strains (Sar9, 4165, Tib32, NL10, Nl64, *A. minuta*, PS1073J) (Geisen et al., 2015; De Obeso Fernadez Del Valle and Maciver 2017). These seven strains grouped as a branch in the genus *Allovahlkampfia* with 92.9–99.8% similarity across the entire ITS region (Fig. 4, Table 6). Within the second group, Tib32, NL10 and Nl64 showed the highest degree of similarity (99.0–99.8 %) with 1–5 bp differences. Despite the highest similarity, these three species show slight morphological differences, specifically with NL10 showing a mostly limax locomotive form compared to the other two strains that mostly moved in a flabellate form. The third group consists of *A. spelaea* (Walochnik and Mulec 2009) and Tib50 (Geisen et al. 2015) with higher ITS similarity (89.3%) to each other as compared to with other strains. The fourth group consists of the single strain F11, which has a low similarity (68.3–79.3%) with all other described strains in the genus *Allovahlkampfia.* The last group consists of NL28 and '*Solumitrus*' *palustris,* which show 95.0 % similarity across the entire ITS region (Table 6).

Discussion

In this study, we isolated four soil strains of Heterolobosea, one of which we describe as the new species, *Vahlkampfia bulbosis* n.sp. By adding NL10 and NL28 to the genus *Allovahlkampfia*, we also delimitate the genus, proposing that it should contain (at least) five separate groups.

New Vahlkampfia Species

For CN7, which we eventually named *Vahlkampfia bulbosis* n.sp., we found that it was most closely related to *Vahlkampfia inornata* based on the 18S rRNA gene, but to *Vahlkampfia orchilla* (*V. orchilla* type strain AJ973127 and *V. orchilla* strain 10 (EU154997) based on the ITS region (De Jonckheere 2006a; Yera et al. 2008). We attribute this lack of congruence to the absence of a published 18S rRNA gene sequence for *V. orchilla,* and, based on the 5.8S rRNA gene comparison between reported species in the genus *Vahlkampfia* (Table 4), we believe that *V. orchilla* is a sister species of *V. bulbosis* n.sp. Future additions of sequences of *Vahlkampfia* spp. will improve our understanding of patterns of relatedness within this still poorly sampled genus. We justify the creation of a new species not only based on profound molecular differences, but also based on distinct morphological features. In particular, CN7 is 8 μm smaller

Table 3. Percentage identity matrix based on manually modified alignments of the 18S rRNA gene (below the diagonal, in bold) and the 5.8S rRNA gene (above the diagonal, in bold) among *Allovahlkampfia* strains and Soil amoeba AND 12 as outgroup. NA, no value due to lack of a reference sequence.

	NL10	NL28	*A. spelaea*	Nl64	*A. palustris*	SO/1P	BA	Sar9	PS1073J	Tib32	OSA	Tib50	Tib191	Sar37	F11	*A. minuta*	AND12
NL10	**100**	91.9	98.7	100	92.9	NA	NA	99.4	100	99.4	NA	98.7	95.5	94.2	98.1	100	NA
NL28	96.1	**100**	94.8	93.5	99.4	NA	NA	94.2	93.5	94.2	NA	93.5	93.5	93.5	94.2	93.5	NA
Allovahlkampfia. spelaea	96.0	97.6	**100**	98.7	94.2	NA	NA	99.4	98.7	99.4	NA	98.7	96.8	95.5	99.4	98.7	NA
Allovahlkampfia sp. Nl64	99.4	96.1	96.1	**100**	92.9	NA	NA	99.4	100	99.4	NA	98.7	95.5	94.2	98.1	100	NA
Allovahlkampfia palustris	92.5	99.3	94.2	92.4	**100**	NA	NA	93.5	92.9	93.5	NA	92.9	92.9	92.9	93.5	92.9	NA
Allovahlkampfia sp. SO/1P	95.5	96.6	96.4	95.2	93.7	**100**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Heterolobosea sp. BA	98.0	96.3	96.4	98.3	92.7	95.4	**100**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Allovahlkampfia sp. Sar9	98.2	96.7	96.7	98.6	93.9	95.6	99.1	**100**	99.4	100	NA	98.1	96.1	94.8	98.7	99.4	NA
Allovahlkampfia sp. PS1073J	98.8	96.0	95.9	99.3	92.4	95.2	98.3	98.5	**100**	99.4	NA	98.7	95.5	94.2	98.1	100	NA
Allovahlkampfia sp. Tib32	99.4	96.0	96.0	100	92.4	95.1	98.3	98.5	99.3	**100**	NA	98.1	96.1	94.8	98.7	99.4	NA
Heterolobosea sp. OSA	96.2	97.5	97.6	96.5	94.9	96.2	96.5	96.8	96.3	96.5	**100**	NA	NA	NA	NA	NA	NA
Allovahlkampfia sp. Tib50	96.3	97.5	97.6	96.4	95.0	96.2	96.5	96.8	96.3	96.5	99.9	**100**	95.5	94.2	98.1	98.7	NA
Allovahlkampfia sp. Tib191	95.8	97.3	97.4	95.9	94.9	97.2	96.0	96.4	95.7	95.8	96.7	96.7	**100**	98.7	96.1	95.5	NA
Allovahlkampfia sp. Sar37	95.4	97.6	97.1	95.6	95.0	97.3	95.7	95.9	95.4	95.8	96.5	96.6	98.3	**100**	94.8	94.2	NA
Allovahlkampfia sp. F11	95.8	98.0	97.2	96.0	96.2	96.4	96.2	96.5	95.9	95.9	97.0	97.0	96.7	96.7	**100**	98.1	NA
Allovahlkampfia. minuta	98.7	95.6	95.6	99.3	92.3	94.5	97.6	97.9	98.8	99.6	95.8	95.9	95.2	95.1	95.9	**100**	NA
Soil amoeba AND12	90.7	91.0	90.5	90.8	87.2	90.6	90.6	91.0	90.8	90.7	90.6	90.5	91.0	90.8	90.7	90.0	**100**

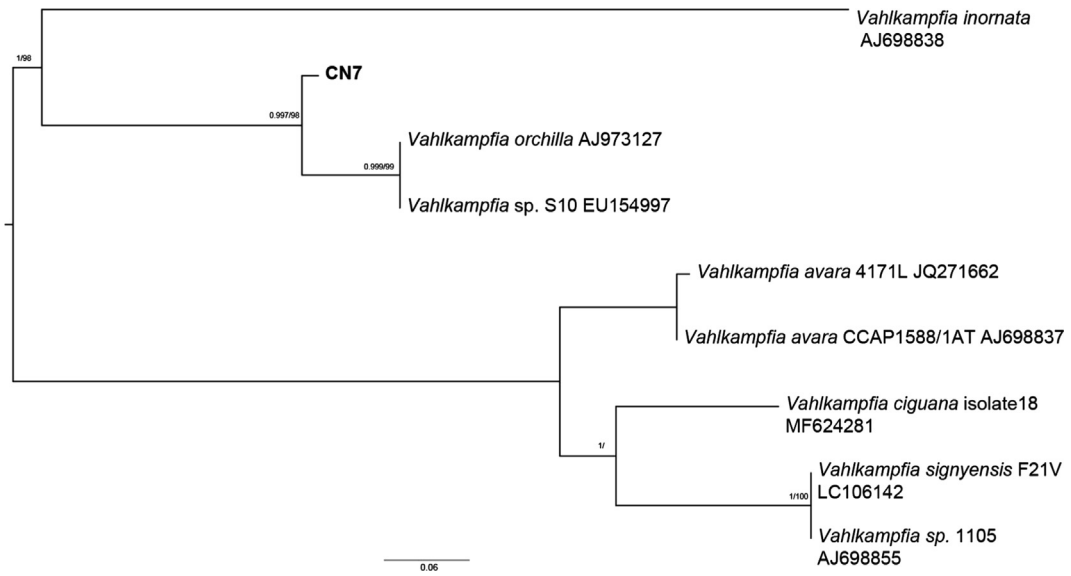


Figure 4. Unrooted maximum-likelihood tree based on the entire ITS region in *Vahlkampfia* strains sequences. Support value at each node show posterior probability (pp) of Bayesian Inference (left) and ML bootstrap value (BS) of RaxML (right). Support values of pp < 0.5 and BS < 50% are not shown. GenBank accession numbers of the ITS sequences used in the analysis are listed next to the taxon names.

in length than *V. inornata* and has a bulbous instead of a gelated uroidal region as in *V. inornata*. In fact, CN7 is now by far the smallest member of the genus *Vahlkampfia* (Supplementary Material Table S2). A major morphological difference between CN7 and *V. orchilla* is the absence of a pink color after centrifugation in CN7 (De Jonckheere 2006a). We acknowledge that this feature might not represent a stable morphological feature as the pink colour in *V. orchilla* could have originated from endosymbiotic bacteria that might just not have been present in our culture condition (Supplementary Material Table S2).

So far, all reported species, whose placement in the genus *Vahlkampfia* has been confirmed using molecular data, were isolated from aquatic ecosystems or from human hosts (Supplementary Material Table S2). The only possible exception is *Vahlkampfia signyensis* strain 1105 that was found in soils from the South Orkney Islands near Antarctica, but this could potentially also represent a marine species (Garstecki et al. 2005). Moreover, *Vahlkampfia* species were reported from soil (Rahdar et al. 2016), but there were only identified using an outdated morphological classification system lacking molecular characterization. As such, *V. bulbosis* n.sp. represents the first soil *Vahlkampfia* species.

Traditional identification of *Vahlkampfia* species was mainly based on morphological features (Page 1983, 1988). Molecular methods induced profound changes in the genus, as several species considered to belong to *Vahlkampfia* were transferred to other genera. These include, for example, the now called *Tetramitus aberdonica* and *Tetramitus enterica* (Brown and De Jonckheere 1999). A few *Vahlkampfia* species, such as *V. dumnonica,* are still based on morphological features only (Page 1983). This shortcoming should be solved to more accurately reflect on the real diversity within *Vahlkampfia.* For that, molecular information for those taxa with missing molecular information, including the 18S rRNA gene and the ITS region, are urgently needed.

New Strains in *Naegleria* and *Allovahlkampfia*

We identified NL81 as a strain of *Naegleria clarki* (Dyková et al. 2006). Interestingly, our strain showed a much lower thermotolerance than that previously reported (De Jonckheere 2014), especially considering that *Naegleria* species commonly have a high thermotolerance. This could be related to different origins of the strains, as the previously described *Naegleria clarki* strain was isolated from fish organs with potentially higher body temperature

Table 4. Percentage identity matrix obtained with manually modified alignments of the 18S rRNA (below the diagonal, in bold) and 5.8S rRNA (above the diagonal, in bold) among *Vahlkampfia* strains. NA, no value due to lack of a reference sequence.

	CN7	*V.orchilla*	S10	*V. avara* 4171L	*V. avara* CCAP1588/1A	*V. ciguana* 18	F21V	1105	*V. inornata*
CN7	**100**	99.3	99.3	75.3	75.3	74.0	73.3	73.3	81.6
Vahlkampfia orchilla	NA	**100**	100	74.7	74.7	74.7	72.6	72.6	81.0
Vahlkampfia sp.S10	NA	NA	**100**	74.7	74.7	74.7	72.6	72.6	81.0
Vahlkampfia avara 4171L	93.9	NA	NA	**100**	100	94.0	92.7	92.7	74.8
Vahlkampfia avara CCAP1588/1A	94.4	NA	NA	99.7	**100**	94.0	92.7	92.7	74.8
Vahlkampfia ciguana isolate18	NA	NA	NA	NA	NA	**100**	91.4	91.4	76.2
Vahlkampfia signyensis F21V	NA	NA	NA	NA	NA	NA	**100**	91.4	76.2
Vahlkampfia sp. 1105	NA	NA	NA	NA	NA	NA	NA	**100**	72.8
Vahlkampfia inornata	99.0	NA	NA	94.6	94.1	NA	NA	NA	**100**

(Dyková et al. 2001) than in temperate soil from which we isolated our strain. No flagellate stages were observed in NL81, which is in contrast to previous studies reporting flagellate stages in *Naegleria clarki* (De Jonckheere 2014).

In our study, NL10 was affiliated with the genus *Allovahlkampfia* and was most related to Nl64 and Tib32 based on the 18S rRNA gene sequences - note that these two sequences were nearly identical except for the group I intron (Geisen et al. 2015). However, further analysis showed that NL10 was only closely related to Nl64 (Table 3), due to the slight 0.6 % dissimilarity between Nl64 and Tib32 in the 5.8 S rRNA gene reported by Geisen et al. (2015). Our study also showed that NL10 differs from Nl64 at one position less than Tib32 based on the ITS region (Table 6). Considerable variation in morphology could also be found, which was consistent with previous *Allovahlkampfia* studies (Geisen et al. 2015; Walochnik and Mulec 2009). NL10 occasionally adopted more of an elongate flabellate locomotive form, which is different from its closest relative Nl64, which exhibits mostly a flabellate locomotive form (Geisen et al. 2015). The size of our strain was most strikingly different from Nl64, as trophozoite lengths of NL10 were half the size of Nl64. Interestingly, the mean cyst diameter of NL10 was larger than that of Nl64 (Fig. 1b, Table 1). Thermotolerance also varied considerably between Nl64 (below 30) and NL10 (37 °C), the latter being the same as the closely related Tib32 (Geisen et al. 2015). To date, NL10 is the *Allovahlkampfia* sp. with highest thermotolerance (37 °C).

Allovahlkampfia palustris comb.nov. *(*Basionym *Solumitrus palustris)*

Sequence errors in '*S*'. *palustris* likely caused differences between 18S rRNA gene and ITS region read Blasting results (Brown et al. 2012; Geisen et al. 2015; Harding et al. 2013). After manual modification of the alignments, our phylogenetic analyses revealed that NL28 was most closely related to '*S*'. *palustris,* followed by other *Allovahlkampfia* spp. (Figs. 2,3, Table 3). Our results were in line with other studies that identify '*S*'. *palustris* as an *Allovahlkampfia* species and highlight the need to trim the erroneous ends of the sequences of '*S*'. *palustris* to achieve robust phylogenetic results (Brown et al. 2012; Geisen et al. 2015). Therefore, *Solumitrus* is considered to be a subjective junior synonym of

Table 5. Information of groups in the genus *Allovahlkampfia*. NA, no available information.

	Strains	Source	Origins	Elevation	Max Temp (°C)	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	Locomotive form
Group 1	Tib 191	Soil	Tibet, China	4149	<37	129	163	162	Limax
	Sar37	Soil	Sardinia, Italy	181	<37	117	163	130	Limax
	PV66	Beer bottle	Czech Republic	NA	NA	NA	162	NA	NA
Group 2	Sar 9	Soil	Sardinia, Italy	181	<37	163	161	128	Mostly limax
	4165	Liver	Czech Republic	NA	NA	NA	161	NA	NA
	Tib32	Soil	Tibet, China	4149	<37	154	161	127	Mostly flabellate
	NL10	Soil	Netherlands	NA	37	186	161	147	Mostly limax / Occasionally flabellate
	Nl64	Soil	Netherlands	57	<30	155	161	127	Mostly flabellate
	A. minuta	Lakeside	United Kingdom	NA	<28	NA	161	NA	flabellate
	PS1073J	Liver	NA	NA	NA	NA	161	NA	NA
Group 3	*A.spelaea*	Stromatolitic stalagmites	Slovenia	NA	<42	NA	161	NA	Mostly limax
	Tib50	Soil	Tibet, China	4149	<37	153	161	125	Limax or Flabellate
Group 4	F11	Freshwater lake	Vega Island, Antarctica	NA	NA	110	161	105	Limax
Group 5	NL28	Soil	Netherlands	NA	20	305	161	152	Occasionally limax
	A. palustris	Freshwater marsh	USA	NA	NA	NA	161	NA	Limax

Table 6. Percentage identity matrix obtained with the entire ITS region of all reported strains in groups of genus *Allovahlkampfia* after modification. Note that below the diagonal (in blank) is percentage of similarity and above the diagonal (in blank) is the non- identical base pairs.

		Group 1			Group 2							Group 3		Group 4	Group 5	
		Tib191	Sar37	PV66	Sar9	4165	Tib32	NL10	Nl64	*A. minuta*	PS1073	*A. spelaea*	Tib50	F11	NL28	*A. palustris*
	Tib191		45	56	83	89	82	82	83	81	97	83	78	103	104	109
Group 1	Sar37	89.2		43	85	91	82	83	83	82	96	82	75	100	98	98
	PV66	85.3	88.4		85	77	82	83	83	75	77	74	74	89	101	100
	Sar9	80.6	79.7	77.7		10	16	17	17	19	34	61	61	109	108	115
	4165	77.2	76.2	78.0	97.8		24	26	25	19	23	65	67	91	99	106
	Tib32	80.7	80.3	78.3	96.7	94.6		5	1	9	28	65	62	107	108	115
Group 2	NL10	80.7	80.0	78.0	96.5	94.2	99.0		4	11	29	67	60	108	110	117
	Nl64	80.4	80.0	78.0	96.5	94.4	99.8	99.2		8	27	66	61	108	109	116
	A. minuta	76.9	76.1	76.1	95.4	95.4	97.8	97.3	98.0		18	64	60	93	94	102
	PS1073J	77.0	76.8	79.5	92.9	94.8	94.1	93.9	94.3	95.6		75	70	105	116	125
Group 3	*A. spelaea*	79.9	79.8	79.8	87.1	85.1	86.2	85.8	86.0	84.0	83.8		48	121	96	104
	Tib50	81.1	81.4	79.8	86.8	84.3	86.5	87.0	86.7	85.4	84.5	89.3		102	98	100
Group 4	F11	73.3	73.5	74.1	75.2	77.6	75.5	75.3	75.2	76.8	75.7	72.7	75.9		130	139
	NL28	75.3	76.3	73.3	77.5	77.7	77.3	76.9	77.1	76.7	75.3	79.3	78.6	70.1		24
Group 5	*A. palustris*	74.4	76.5	73.6	76.2	76.3	76.0	75.7	75.8	75.0	73.6	77.8	78.4	68.3	95.0	

Allovahlkampfia, and *Solumitrus palustris* is transferred as *Allovahlkampfia palustris* comb.nov.

A Subdivision of the Genus *Allovahlkampfia*

Based upon ITS sequence analyses that include two new *Allovahlkampfia* strains, we propose five groups of described *Allovahlkampfia* strains (Fig. 3). This proposal reflects previous observations of heterogeneity within the genus *Allovahlkampfia* (Geisen et al. 2015). Strains from the first group always harbored 5.8S rRNA genes that are 1–2 bp longer than those observed for the other groups (Table 6), and both of the two described strains (Tib191 and Sar37) were morphologically similar and displayed the limax locomotive form (Geisen et al. 2015).

The second group included seven strains, in which Nl64, NL10 and Tib32 showed only 1–5 bp length differences in the 5.8S rRNA gene. However, these three strains displayed slight differences in their locomotive form; Tib32 and Nl64 showed mostly a flabellate shape, while NL10 showed mostly a limax shape and occasionally flabellate and Sar 9 moved entirely in a limax shape (Fig. 1a, Table 5). NL10 might represent an intermediate morphotype between limax and flabellate.

The fourth group was composed of the single Antarctic strain, F11, that potentially is unique to extreme conditions and might represent a polar group such as found for the *Naegleria* polar cluster (De Jonckheere 2006b; Tyml et al. 2016). However additional polar *Allovahlkampfia* strains would be required before such a polar *Allovahlkampfia* group might be identified.

Allovahlkampfia palustris comb.nov. and NL28 are strains in the fifth group of the genus *Allovahlkampfia*, as these two strains showed the highest similarity with each other (Tables 5,6). We suggested the five groups mainly based on ITS sequences, however, species that lack reference ITS sequences were not included in this group. For example, our phylogenetic analysis also confirmed that Heterolobosea OSA had 94.9–97.6% similarity with other *Allovahlkampfia* species. Future studies utilizing new molecular data for identifying the groups within the genus *Allovahlkampfia* are needed to confirm the group definition.

Conclusions

We report strain CN7 as a new species, *Vahlkampfia bulbosis* n.sp. Furthermore, we identified two novel strains that allowed us to subdivide the genus *Allovahlkampfia* into 5 groups based on morphological and (mainly) molecular information. Together, our study extends the knowledge on soil Heterolobosea, but also suggests that we are still far from having captured a complete inventory of this group of soil protists. Future studies should prioritize the discovery of the unknown diversity among Heterolobosea, which seem especially abundant and diverse in soils. These should include the taxa described in pre-molecular times to infer more accurate phylogenies, while additionally describing novel taxa that seem to be widespread. Additional gene information to the ITS and 18S rRNA gene will also be needed (such as with single-cell transcriptomics (Kang et al. 2017; Tice et al. 2016)) to more deeply understand the evolutionary origin of this group and the relationships between members within Heterolobosea (Burki et al. 2020; Simpson et al. 2006).

Taxonomic Summary

Phylum: Heterolobosea.
Clade: Tetramitia.
Clade: Eutetramitia.
Family: Vahlkampfiidae.
Genus: Vahlkampfia.
Species: *Vahlkampfia bulbosis* n.sp.

Diagnosis: Trophozoites 13.7–24.2 μm (average 18.7 μm) in length and 4.4–11.4 μm (average 7.3 μm) in width; limax locomotion with highly eruptive pseudopodia; broader anterior than posterior end, where sometimes a bulbous uroid was formed; no contractile vacuole visible. Spherical cysts with a smooth wall and uninucleate with a centered nucleolus (nucleus diameter: 3.3 ± 0.1 μm, nucleolus diameter: 2.4 ± 0.1 μm); Thermotolerance of up to 37 °C.

Etymology: bulbosis referring to the bulbous uroid formed by CN7.

Type locality: Qilin, Jiangsu, China (32°03′09″, 118°55′36″).

Type material: A permanent microscopic slide of strain CN7 fixed by Bouin-Hollande's fluid following the method of Pánek et al. (2014), including trophozoites and cysts, has been deposited at Utrecht University, Department of Biology, Group of Ecology and Biodiversity (Accession number EB-P-001). This slide represents the name-bearing type of the species (an hapantotype). A culture of the strain CN7 has been deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and

Cell Cultures GmbH under the accession number DSM 113307.

Phylum: Heterolobosea.

Clade:Tetramitia.

Clade: Eutetramitia.

Order: Acrasida.

Family: Acrasidae.

Genus: *Allovahlkampfia* Walochnik and Mulec 2009.

Species: *Allovahlkampfia palustris* (Anderson et al. 2011) comb.nov. (basionym *Solumitrus palustris*).

Remarks: *Solumitrus* was suggested to subsumed to *Allovahlkampfia* in Brown et al. 2012 and Geisen et al. 2015 and it is nested in *Allovahlkampfia* in phylogenetic trees (Fig. 2). Therefore, *Solumitrus* is considered to be a subjective junior synonym of *Allovahlkampfia.* Species now assigned to *Allovahlkampfia* are: *Allovahlkampfia spelaea* (Walochnik and Mulec 2009); *Allovahlkampfia palustris* (Anderson et al. 2011; basionym *Solumitrus palustris); Allovahlkampfia minuta (*De Obeso Fernadez Del Valle and Maciver 2017*);*

Strains included: Group1: Tib 191, Sar 37 (Geisen et al. 2015), PV66 (Dyková and Kostka 2013) (JQ271645); Group2: Sar9, Tib32, Nl64 (Geisen et al. 2015), NL10, 4165 (JQ271643), PS1073J (JQ271644); Group3: Tib50 (Geisen et al. 2015); Group4: F11 (Tyml et al. 2016); Group5: NL28.

Methods

Isolation and cultivation: Four strains were isolated from three different soils; NL81 was isolated from a green house in Rotterdam, the Netherlands (51°55′32″; 4°29′39″); CN7 was isolated from the rhizosphere of tomato in Qilin town, Jiangsu province, China (32° 03′09″, 118°55′36″); NL28 was isolated from a grassland soil in the province Friesland, the Netherlands (51°56′60″; 6°10′59″); NL10 was isolated from rhizosphere soil of *Centaurea stoebe* in the Netherlands (51°51′60″; 5°53′34″). Isolations used one gram of soil sample suspended in 20 mL PAS (Page's Amoeba Saline). Soil suspensions were gently shaken for 30 min at a Laboshake (Gerhardt GmbH & Co. KG, Königswinter, Germany), and one microliter of the mixed soil suspension was pipetted into each well of 96-well plates (Costar, Corning, New York, USA) containing *Escherichia coli* OP50 (*E.coli*) as food source. After several days of incubation at 15 °C, we screened each well to select protists under an inverted microscope Nikon Eclipse TS100-F (NIKON, Tokyo, Japan). Wells containing potentially pure protist strains were further diluted several times in order to purify a single protist strain. Note that this isolation method is dependent on many factors, for instance, dilution, medium and site characteristics (such as fungal load). All protists strains were maintained at 15 °C and regularly transferred to new medium with *E.coli*.

In order to examine thermotolerance, we grew each protist species with *E. coli* in 96-well plates and incubated them at 15, 20, 25, 28, 32, 37 °C. Each temperature assay was replicated five-fold. We then tracked the number of active protists cells each day until encystment or extinction. This took 7–10 days, depending on the species. We defined thermotolerance as the maximum temperature at which presence of protists was observed.

Morphological characterization: All four strains were identified under the locomotion, stationary and cyst stages. Ten microliters of each active protist or protist cysts were deposited on a glass slide, immediately covered by the glass slip, and the edges were sealed using nail polish. The images of each protist trophozoite and cyst were acquired using a Nikon Eclipse Ti-E inverted microscope (NIKON, Tokyo, Japan) with Differential interference contrast (DIC) using the Plan Fluor 40x 1.30 N.A. oil objective (Nikon) and a CoolSNAP HQ2 camera (Photometrics). The 16-bit images were projected onto the CCD chip at a magnification of 107.5 nm/pixel with intermediate magnification 1.5X (Nikon). The images were captured with 50 ms exposure time using MicroManager (v.1.4.22. ImageJ 1.48v). Time-lapse movies were generated with a time interval 1 second to determine the locomotive form. Photos of each strain were also taken under inverted microscope Nikon Eclipse TS100-F (NIKON, Tokyo, Japan). Length and width were measured for trophozoites or cysts per strain in ImageJ (1.48v). NL10 measurement was based on 11 different cells, NL28 measurement was based on 28 different cells, NL81 measurement was based on 20 different cells, CN7 measurement was based on 20 different cells. In short, the scale was set by the image scale (1 pixel = 107.5 nm), and we further measured the length or width by drawing a line from the top to bottom of the protist trophozoite. The presence of floating and flagellate forms of each protist were investigated. The ability to form flagellates was examined by adding strains to distilled water for *ca.* 90 mins. The formation of fruiting bodies was investigated by following the method in (Brown et al. 2012), in short, autoclave-sterilized *Quercus alba* bark together with sterile DI H_2O slurry of *Rhodotorula mucilaginosa* were added to the agar dishes containing actively growing strains. A permanent microscopic slide of strain CN7 was prepared by fixation using Bouin-Hollande's fluid, following the method of Pánek et al. (2014).

DNA extraction and amplification: Protist DNA was extracted from 100 μL of protist culture using the E.Z.N.A Bacterial DNA extraction Kit (Omega, Bio-Tek Inc., Georgia, USA) and DNeasy Blood & Tissue Kit (QIAGEN N.V., Maryland, USA) following the manufacturer's instructions, with an additional 2 min bead-beating step at maximum speed to improve the DNA yield. The extracted DNAs were stored at -20 °C before use in polymerase chain reaction (PCR).

The 18S rRNA gene was amplified using various pairs of general eukaryotic primers as shown in Supplementary Material Table S1. The general PCR procedure utilized an initial 3 min 95 °C denaturation step followed by 38 cycles of 95 °C for 30 s, a strain-specific annealing temperature (Supplementary Material Table S1) for 30 s, 72 for 90 s, and a final extension step of 5 minutes.

The ITS region, including the 5.8S rRNA gene, was amplified using the primers JITS-F and JITS-R (De Jonckheere and Brown, 2005), PCR amplifications were run in a Veriti 96-well thermal cycler (Applied Biosystems, California, USA) with the following program: 95 °C for 5 min, followed by 30 cycles with 95 °C 30 s, 50 °C for 60 s, and ending with 72 °C for 120 s.

PCR products (25 μL) were subjected to gel electrophoresis in 1% agarose dissolved in Tris-borate buffer (2.5 mM disodium EDTA, 89 mM Tris base, and 8.9 mM boric acid). The band containing the PCR product of interest was then excised from the gel and purified using the QIAquick Gel Extraction Kit (QIAGEN N.V., Maryland, USA). The cleaned PCR products were then sequenced (BaseClear B.V., Leiden, the Netherlands) using appropriate primers.

Phylogenetic analysis: The taxonomic affiliation of each strain was first determined by comparison against the NCBI database using a BLAST search. To obtain resolved phylogenetic affiliations of our

strains, the sequences of closely affiliated species were also downloaded for further analysis. In total, we generated the following five datasets, one including 30 18S rRNA gene sequences; one including the ITS regions for the same set of 30 species; one multigene matrix including both 18S rRNA gene and ITS regions that assembled using SequenceMatrix-Window-1.7.8 (Vaidya et al. 2011), one including the ITS region for 18 species in the genus *Allovahlkampfia*; and one including the same region for 9 species in the genus *Vahlkampfia*. All datasets were aligned within MAFFT (version 7) using the FFT-NS-2 method (Katoh et al. 2017) for the ITS and G-INS-i method for the 18S, and further manually adjusted in SEAVIEW (version 4.7) to remove any poorly aligned regions base pairs.

In order to assess the stability of the clades, phylogenetic analysis of all datasets was performed based on Bayesian analysis using MrBayes 3.2 (Huelsenbeck and Ronquist 2001) and maximum likelihood using RAxML (v0.9.0) (Kozlov et al. 2019). Bayesian analysis was conducted under 6 General Time Reversible (GTR) substitution types with assumptions of rate variations across sites according to gamma + invariable distribution. Markov chain Monte Carlo simulations were performed for 100,000 generations, sampled every 100 generations. The first 100 samples were discarded as burnin. Maximum likelihood analysis was also based on GTR + GAMMA + I model for all datasets with rapid bootstraps. All phylogenetic trees were illustrated in FigTree (V1.4.4).

We also compared similarities of published species sequences with the genera *Allovahlkampfia* and *Vahlkampfia* based on the manually modified alignments of the 18S rRNA and the 5.8S rRNA gene sequences. All sequences described in this study are available in GenBank, NL81 as MT739326, NL10 as MT739327, NL28 as MT739328, CN7as MT739329 for 18S rRNA gene; NL81 as MW031117, NL10 as MW031118, NL28 as MW031119, CN7 as MW031120 for the ITS region including the 5.8S rDNA. All strains are deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH under accession numbers DSM 113306 (NL81), DSM 113307 (CN7), DSM 113308 (NL28), DSM 113309 (NL10).

CRediT authorship contribution statement

Zhilei Gao: Methodology, Validation, Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing. **Alexandre Jousset:** Writing – review & editing, Supervision. **George A. Kowalchuk:** Supervision, Writing – review & editing. **Stefan Geisen:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision.

Acknowledgements

Z.G. was supported by Chinese Scholarship Council (CSC), S.G. by a NWO-VENI grant from the Netherlands Organization for Scientific Research (016.Veni.181.078). Moreover, we thank Xueyang Sun for the help of extracting DNA of protists strains and prof. Zhong Wei for providing the soil from China. We appreciate Dr. Xuankun Li (Gainesville) for comments and suggestions on the paper. We appreciate Dr. Ilya Grigoriev and Biology Imaging Center (Utrecht University, the Netherlands) for the help of taking protist images and videos.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.protis.2022.125870.

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