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Free-living Amoebae in Carbonate Precipitating Microhabitats of Karst Caves and a New Vahlkampfiid Amoeba, *Allovahlkampfia spelaea* gen. nov., sp. nov.

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Summary. This is the first report on the diversity of small free-living amoebae (FLA) in carbonate precipitating habitats in karst caves. Of 11 samples from nine different habitats in four Slovenian karst caves ten samples were positive for FLA, four strains were successfully isolated and transferred to clonal monoxenic cultures, including *Acanthamoeba castellanii* genotype T4, *Echinamoeba silvestris*, *Hartmannella vermiformis*, and a new vahlkampfiid amoeba *Allovahlkampfia spelaea* gen. nov., sp. nov. The latter was isolated from a stromatolitic stalagmite, a typical biogenic speleothem. *Echinamoeba silvestris* was identified from an aerophytic algal community and *Acanthamoeba* and *Hartmannella* were isolated from a cave pool with floating calcite rafts. The grazing of FLA on bacteria may help in creating conditions that enhance carbonate precipitation.

Key words: Cave, amoebae, *Allovahlkampfia spelaea* gen. nov., sp. nov.

INTRODUCTION

Although protozoa inhabiting caves have been an interesting object of studies since the 19th century, information on this topic is still scarce. Only a few comprehensive studies have been conducted, and most of them were focused on certain protozoan species (Coppellotti Krupa and Guidolin 2003, De Luca *et al*. 2005, Gittleson and Hoover 1969, Gittleson and Hoover 1970, Golemansky and Bonnet 1994; Landolt *et al*. 1992, Sudzuki and Hosoyama 1991). In caves, protozoa are usually isolated from the water column, guano or sediments. Another important group of barely studied group are protozoan epibionts and parasites on cave animals (Golemansky and Bonnet 1994, McAllister and Bursey 2004). While only a few studies have been performed on protozoa in caves, it is known that these organisms can be found in extreme habitats, e.g. extremely acidic sites (pH 0–1) (Macalady *et al*. 2007). In an Italian study of the diversity of protozoan communities in karst caves, more than 90 different taxa, mainly ciliates, were identified (Coppellotti Krupa and Guidolin 2003).

Defining the characteristics of protozoa adapted exclusively to cave habitats is not as easy as it is for

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higher animals (Pipan and Culver 2007). Protozoan species from caves can generally be found also in other environments such as forest litter, soils and mosses (De Luca *et al*. 2005, Gittleson and Hoover 1969). For example *Spathidium faurefremieti*, originally described from cave waters (Peştera Ialomicioara, Romania), was rediscovered in savannah soil in Kenya (Africa), and in floodplain soils of Brazil and Australia (Foissner 2003). Nevertheless, protozoa from caves are generally smaller and have more contractile vacuoles relative to protozoa from non-cavernicolous environments. (Coppellotti Krupa and Guidolin 2003). In a comprehensive study on cave protozoa the major groups of protozoa living in caves were in the following decreasing order of abundance: amoebae, ciliates and flagellates (Gittleson and Hoover 1969). Protozoa, especially ciliates are recognized as excellent bioindicators for water quality in different habitats and their characteristic give them a distinct advantage over higher organisms (Laybourn-Parry 1992, Foissner and Berger 1996). Similarly, cave protozoa can be used as indicator organisms for monitoring water quality due to their relative ease of culturing, short life cycle, their cosmopolitan distribution, and their sensitivity and quick response to environmental changes (Guidolin and Coppellotti Krupa 1999).

Small free-living amoebae (FLA) are ubiquitous protozoans that feed on bacteria, algae, fungi and detritus (Laybourn-Parry 1992, Sala and Güde 1999, van Hannen *et al*. 1999). They are widely distributed in terrestrial and aquatic habitats and may play a significant role at the base of food webs. The FLA help to maintain soil fertility by making nutrients available. Protozoan excretory products include dissolved phosphorus and nitrogen compounds and other organic molecules (Laybourn-Parry 1992). Most FLA genera are characterised by a biphasic life cycle consisting of a vegetative trophozoite stage and a persistent, physiologically static cyst stage that enables them to withstand adverse environmental conditions. Amoebae grazing on bacteria, fungi and other protozoans are important bio-control agents in agriculture and forestry (Hausmann *et al*. 2003). Protozoa and FLA have high specific metabolic rates, which results in a rapid transfer of energy and cycling of nutrients, and are highly depended on the temperature (Laybourn-Parry 1992). The purpose of this study was to evaluate the abundance and diversity of small FLA in cave habitats at cave temperatures where carbonate precipitates. These habitats were chosen for study since no comprehensive surveys have been conducted so far on small FLA from such environments. Previous studies of selected cave sites have shown that there exists potential prey (bacteria, fungi and algae) for small FLA and their presence is anticipated.

MATERIAL AND METHODS

Study sites

Altogether 11 samples from four Slovenian karst caves and 9 different habitats were aseptically taken and transferred to the laboratory (Fig. 1 and Table 1). From the cave Pečina v Borštu (566 m a.s.l., no. in cave register in Slovenia 935) formed in the Upper Cretaceous limestone (Zupan Hajna 2003) three samples were taken: calcite rafts, weathered limestone and droplets of condensed water on the wall near the entrance. The largest cave system in Slovenia with 21 km of cave passages is Postojnska jama (529 m a.s.l., cave register no. 747) was formed in Cretaceous limestones (Šebela 1998). In this cave two distinctive microhabitats were sampled: a trickle with constant dripping water and a pool with cave pearls beneath. From the ice cave Snežna jama na Raduhi (1556 m a.s.l., cave register no. 1254) with permanent ice at the entrance two samples were taken: calcite moonmilk and organic crust on sediment. Cave passages are developed in Upper Triassic limestone. In the cave fluvial sediments are presented in the form of laminated loams, sand and gravel of noncarbonate origin (Mihevc 2001a). The cave Škocjanske jame (cave register no. 735) was formed by the sinking river Reka at the contact of impermeable Eocene flysch and both Upper Cretaceous and Paleocene limestones (Mihevc 2001b). The entrance to the cave is at an altitude of 425 m above see level. Škocjanske jame is listed in the UNESCO World heritage list. Škocjanske jame is recognized the first underground karst wetland under the Ramsar Wetland Classification System. Two microhabitats in Škocjanske jame were screened for the presence of small FLA: stromatolitic stalagmites and rocky surfaces with developed algal aerophytic communities.

Description of habitats and sampling

Calcite rafts float on surface of a stagnant water body and are supported by the surface tension on a cave pool. In the cave Pečina v Borštu no active water flow is present. The pool which was sampled inside the cave is filled only with percolation water. The pool is occasionally dry and rafts accumulate on the bottom (sample no. 1; Table 1). Sometimes in caves zones of weathered limestone appear as a result of incomplete dissolution of carbonic acid (Zupan Hajna 2003). The thickness of the weathered limestone varies from a few millimetres up to several centimetres. A sample from weathered limestone which occasionally exhibits secondary carbonate precipitation in pores and on the surface was taken one cm beneath the surface (no. 2; Table 1). Condensates of water droplets are usually formed near the cave entrance where cold and warm air mix. Due to condensation corrosion beneath the droplets, an etched rocky surface is usually observed (Gams 2004). A sample was taken 25 m deep in the cave interior (no. 3; Table 1). This sample was taken to establish if air circulation can act as a vehicle of transport of FLA between habitats with carbonate precipitation within the same cave. In karst caves, in pools under a trickle with dripping water (no. 4, 6; Table 1), cave pearls are sometimes formed. Cave pearls are spheri-

Table 1. Free-living amoebae in different cave microhabitats.

Sample	Habitat	Cave	Position in cave (from entrance)	Temp. C°	pH	Free-living amoebae	
						Presence	Identification
1	Calcite rafts[w]	Pečina v Borštu	Interior, darkness (190 m)	10	7.8	+	*Acanthamoeba castellanii* genotype T4 *Hartmannella vermiformis*
2	Weathered limestone[a]	Pečina v Borštu	Interior, darkness (205 m)	10	8.1	+[d]	vahlkampfiids
3	Condensates[w]	Pečina v Borštu	Entrance, darkness (25 m)	10	7.6	–	
4	Trickle[b,w]	Postojnska jama	Interior, darkness (180 m)	9	7.8	–[d]	
5	Cave pearls[w]	Postojnska jama	Interior, darkness (180 m)	9	7.8	–[d]	
6	Trickle[c,w]	Postojnska jama	Interior, darkness (690 m)	9	7.8	–[d]	
7	Cave pearls[w]	Postojnska jama	Interior, darkness (690 m)	9	7.8	–[d]	
8	Moonmilk[a,w]	Snežna jama	Interior, darkness (540 m)	4	8.3	+[d]	vahlkampfiids
9	Organic crust[a]	Snežna jama	Interior, darkness (670 m)	4	–	–[d]	
10	Stromatolitic stalagmite[a]	Škocjanske jame	Entrance, sunlight (50 m)	12	–	+	*Allovahlkampfia spelaea*
11	Epilithic community[a]	Škocjanske jame	Entrance, sunlight (38 m)	12	–	+	*Echinamoeba silvestris*

[a] Aerophytic habitat.

[b] Discharge 0.30 mL s^{-1} when sampled 15 May 2006.

[c] Discharge 0.20 mL s^{-1} when sampled 15 May 2006.

[d] Cultures of amoebae lost due to rapid fungal overgrowth.

[w] Water habitat.

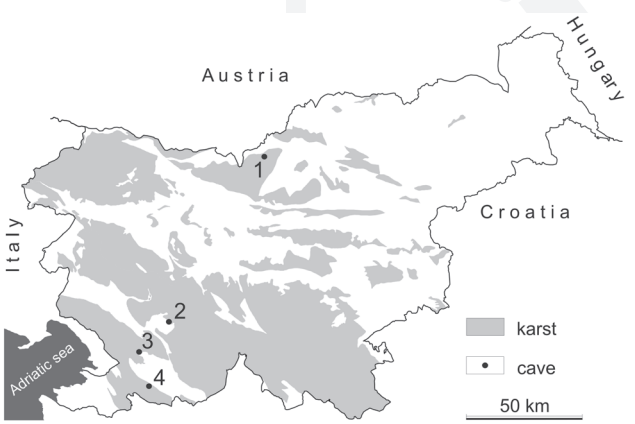

Fig. 1. Geographical location of studied karst cave in Slovenia. **1** – Snežna jama na Raduhi; **2** – Postojnska jama; **3** – Škocjanske jame; **4** – Pečina v Borštu.

cal concretions of calcite with an internal structure of concentric banding around a central grain. Pearls require rotation to coat the sand grain caused by dripping water in pools of saturated water (Lowe and Waltham 1995). Samples for the presence of FLA were taken from sites with a rich community of microcrustaceans (Pipan 2005). Approximate diameter of pearls is 1.5 cm (no. 5, 7; Table 1). Sites with cave pearls from where the samples were taken are not constantly under dripping water. Another interesting habitat in caves is calcite moonmilk. The cave deposit most commonly associated with biogenic calcite precipitation is moonmilk. This is a porous, plastic deposit normally consisting of calcite fibre crystals (Blyth and Frisia 2008). Moonmilk is precipitation of calcite crystals from water, and sometimes is a result of disintegration of bedrock or speleothems (Hill and Forti 1997). Moonmilk was sampled for the presence of FLA in a growing stalactite in the ice cave Snežna jama (no. 8; Table 1). In caves many times on different surfaces microbial communities are observed. For the presence of FLA from Snežna jama one such organic crust covering sediment was sampled (no. 9; Table 1).

In contrast to these habitats with relatively stable ecological conditions, the environmental parameters of stromatolitic stalagmites vary a lot. Stromatolitic stalagmites are special karst features resulting from abiotic carbonate deposits and biolithogenic activity of cyanoprokaryotes (Cox *et al*. 1989). Due to their location in the sun-illuminated cave entrance of Škocjanske jame these biogenic speleothems (no. 10; Table 1; Fig. 2f) are exposed to varying temperature, relative humidity and photon flux of direct and indirect sun illumination (Mulec *et al*. 2007). To compare this habitat with one of similar climate conditions in the same cave entrance, a nearby epilithic community of aerophytic algae was sampled for the presence of FLA as well (no. 11; Table 1).

In the case of solid specimens approximately 1 cm^3 were sampled (weathered limestone, organic crust, moonmilk, stromatolitic stalagmite and epilithic algal community). For liquid samples, whenever possible up to 50 mL of water with eventual accompany-

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ing solid particles were taken; condensed water, calcite rafts with water, trickle, cave pearls with water.

Amoeba culturing

All samples were cultured on 1.5 % non-nutrient agar plates precoated with a 48 h old culture of *Escherichia coli*. This is a standard method for culturing small FLA (Page 1991). Agar plating focusing on small FLA was the method of choice because the majority of habitats were "non-water" habitats and our aim was to compare a similar subset of amoebal communities from different cave habitats. Water samples were centrifuged 7 min. at 500 g, and a pellet was inoculated centrally onto an agar plate. Solid specimens were directly applied onto the middle of a plate. The inoculated plates were sealed with parafilm and incubated at RT (room temperature) up to four weeks. Growth of amoebae was examined daily by phase contrast microscopy (Nikon Eclipse E800). In order to obtain genetically uniform cultures isolates were cloned on a micromanipulator. Vahlkampfiid amoebae were subjected to the enflagellation test which was performed by flooding plates with uniform trophozoite cultures with 1 mL of Phosphate Buffered Saline (PBS), incubating them on a rocking platform and observing them every 30 min. for 8 hours, and then after 24, 48 and 72 hours. A second test series was conducted using amoeba saline (Page 1991) instead of PBS.

Identification and temperature tolerance test

All isolates were identified by their morphological and physiological characteristics according to the identification key of Page (Page 1991). Light microscopy was performed with a Nikon TMS microscope and a Nikon Labophot-2 with an attached Nikon Coolpix 950 were used for microphotography.

Relying on morphological features for identification is not a robust method in the case of *Acanthamoeba*. Until the species complex is resolved, it is becoming popular to genotype strains using molecular methods. Consequently, the two isolates found in the present study were genotyped. Subcultures of samples were incubated at 4°C, 12°C, RT, 30°C, 37°C and 42°C, respectively. After 48 hours of incubation of samples, amoebal growth was investigated by phase contrast microscopy (Nikon Eclipse E800).

DNA extraction

For molecular biological analyses actively growing amoebae (~10^6 cells) were harvested from culture plates with sterile cotton tipped applicators and washed three times in sterile 0.9 % NaCl by centrifugation 7 min. at 500 g. Whole-cell DNA was isolated by a modified UNSET-procedure (Hugo *et al*. 1992). The pellet was re-suspended in 500 µl of UNSET-lysis buffer, overlaid with 500 µl phenol-chloroform-isoamylalcohol (PCI) and shaken gently for 5 h at 300 RPM. DNA was isolated by multiple PCI-extractions, precipitated in ethanol, air dried and resuspended in 30 µl of sterile double distilled water.

PCR amplification and sequence analysis

In all PCRs 1, 3, and 6 µl whole-cell DNA, respectively, were used in 50 µl reaction volumes. For genotyping of the *Acanthamoeba* strain the JDP1 and JDP2 primers (Schroeder *et al.* 2001) were used and the following amplification program applied: 45 cycles; 95°C for 1 min., 60°C for 1 min., 72°C for 2 min. This PCR gives a 423–551 bp *Acanthamoeba* genotype-specific fragment of the 18S rRNA gene. The PCR amplicon was visualized by ethidium bromide in an agarose gel electrophoresis and compared to a lambda DNA length marker. The amplified fragment was sequenced by direct sequencing of the PCR products using the ABI PRISM® BigDye sequencing kit (PE Applied Biosystems, Langen, Germany). Sequencing was carried out in a 310 ABI PRISM® automated sequencer and sequences were obtained from both strands. The genotype was assessed after multiple sequence alignment with the model assumption of a < 5 % sequence dissimilarity within one genotype (Gast *et al*. 1996). Multiple sequence alignment was performed by pairwise alignments using CLUSTAL X application (Thompson *et al*. 1997). The alignments were assessed by eye and revised manually. The percentages of nucleotide dissimilarities were calculated using all sites of the gene.

For identification of the conspicuous vahlkampfiid amoeba strain SK1, the complete 18S rRNA gene was amplified using the SSU1 and SSU2 primers (Gast *et al*. 1996) and a standard PCR amplification program with 30 cycles of 1 min. 95°C, 2 min. 52°C and 3 min. 72°C. These are universal eukaryotic primers, complementary to the strongly conserved ends of the eukaryotic 18S rRNA genes. The amplicon was sequenced stepwise using the SSU1 and SSU2 primers and the internal P1-3 forward and reverse amoeba primers (Walochnik *et al*. 2004). The obtained fragments from both strands were combined to a consensus sequence using the GeneDoc programme (Nicholas *et al*. 1997). In order to find the closest match with published sequences BLAST search was performed. As BLAST search revealed equal sequence identities to most known vahlkampfiid genera, a second PCR that has been established for the identification of vahlkampfiid amoebae (De Jonckheere and Brown 2005a) was performed. This PCR amplifies the ITS1, 5.8S rDNA and ITS2 region of the vahlkampfiid amoebae and allows species identification after sequencing of the 5.8S rDNA. Thus, the PCR amplicon was visualized by ethidium bromide in an agarose gel electrophoresis and sequenced as described above.

Phylogenetic analysis

The 18S rDNA and the 5.8S rDNA of the vahlkampfiid amoeba were subjected to phylogenetic analysis. For analyses multiple sequence alignment was performed as described above but excluding primer sites, unique gaps, ambiguously aligned sites, and introns. Phylogenetic analyses used the PHYLIP package (Felsenstein 1989). The analyses were performed with different evolutionary models including maximum likelihood, neighbour joining, and maximum parsimony. For maximum likelihood analyses the datasets were analyzed beforehand with the MODELTEST software (Posada and Crandall 1998) in order to find the best fitting evolutionary model. Maximum likelihood was then performed with invariable sites and a gamma distribution of among-site rate variation (shape parameter $\alpha = 0.57$) with eight rate categories, neighbour joining with a Kimura two-parameter correction, and maximum parsimony employing heuristic search with a random sequence addition option and counting all steps. The 18S rDNA phylogenetic tree was rooted using the euglenozoans *Euglena gracilis* and *Diplonema ambulator* as an outgroup as the Euglenozoa are supposed to be the sister group of the Heterolobosea. The 5.8S rDNA phylogenetic tree was rooted with *Heteramoeba clara* as it was previously established (De Jonckheere and Brown 2005a). The confidence of the branching order was assessed by the generation of 100 bootstrap replicates, respectively.

Consensus trees were made from the resulting trees using CONSENSE and prepared as figure with the TREEVIEW (Page 1996) application. The sequences have been deposited in the GenBank nucleotide sequence database and are available under the following accession numbers: EU696948 (strain SK1, 18S rDNA sequence), EU696949 (strain SK1, ITS1, 5.8S rDNA, ITS2 sequence).

RESULTS

Habitats where carbonate precipitates contain diverse FLA, although culturing reflects only a subset of the total amoebal population. Of the 11 samples investigated from caves in the present study 10 were positive for small FLA. Altogether, four strains from four different genera were successfully isolated and transferred to clonal monoxenic culture: *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, and the strain SK1. Two more samples revealed vahlkampfiid amoebae, but were lost for further analysis due to rapid fungal overgrowth as was also the case for another five samples with lobosean amoebae. High abundance of fungi on plates is not surprising as these organisms colonize oligotrophic karst caves to a relative big extent (Cunningham *et al*. 1995, Rutherford and Huang 1994). The only sample that was negative for small FLA was sample no. 3, the condensed water (Table 1). All successfully isolated amoebae were able to grow at temperatures up to 30°C, including the isolates from the ice cave. No strain showed the ability to grow at 37°C or at 42°C.

Diagnosis of *Allovahlkampfia* n.g.

Since morphology does not allow identification to the genus level within the family Vahlkampfiidae, except for *Naegleria* (De Jonkheere and Brown 2005b), molecular identification was thus performed. Currently sequences of eight genera are available in the GenBank, including *Naegleria*, *Neovahlkampfia*, *Paravahlkampfia*, *Psalteriomonas*, *Sawyeria*, *Tetramitus*, *Vahlkampfia* and *Willaertia*. Whenever possible, two strains of each genus were included in the analysis. The 18S rDNA phylogenetic analysis displayed that this amoeba belongs to a group of recently isolated and not yet identified vahlkampfiid amoebae in the vicinity of the genera *Paravahlkampfia* and *Neovahlkampfia* (Fig. 3a). The strain SK1 showed between 89.97% and 96.9% sequence identity to these unidentified amoebae including the strain SO/1P that has recently been isolated from a keratitis patient (Ozkoc *et al*. 2008) and was excluded from the analysis as only a partial sequence of the 18S rRNA gene is available for this strain. SK1 displayed around 70% of sequence identity to all other vahlkampfiidae, except to *Psalteriomonas* and *Sawyeria* (~ 60%). The 5.8S rDNA analysis showed that SK1 clusters with *Paravahlkampfia* and *Neovahlkampfia* (Fig. 3b).

Diagnosis of *A. spelaea* n.sp.

Trophozoites (~20–40 μm) of the strain SK1 are usually mononucleate and have typical vahlkampfiid morphology (Fig. 2a). They are mostly monopodial and show markedly eruptive locomotion and prominent uroid filaments, which can be very long (>10 μm) (Fig. 2b–c). They grow well at temperatures below 30°C, with an optimum at RT. They can still grow at 4°C, however, they readily encyst when temperatures rise over 30°C. Trophozoites exposed to 42°C die. Trophozoites cannot be induced to transform into flagellates, neither in PBS nor in amoeba saline. The cysts (16–25 μm) are round and have no pores (Fig. 2d–e). The outer cyst wall is closely attached to the endocyst.

Type habitat and type material

The habitats of this amoeba are stromatolitic stalagmites, biogenic speleothems from the cave entrance (Fig. 2f). These speleothems are, due to their locality in the cave and periodic sun illumination in the entrance of Škocjanske jame, exposed to variable environmental parameters (Mulec *et al*. 2007). Nevertheless, amoebae have been previously found in the external surface of microstromatolites of hot springs (76°C) (Jones *et al*. 1997). In these structures beside amoebae also other organisms were observed: bacteria, cyanobacteria, and algae.

Etymology of the name

The new genus name *Allovahlkampfia* (gr.: *allo* – other) was chosen to express its vahlkampfiid nature and to follow the recently established genera *Paravahlkampfia* and *Neovahlkampfia*. The species name *spelaea* denotes its habitat (lat.: *spelaeum* – cave).

Differential diagnosis of *A. spelaea*

The results suggested a new genus, as SK1 exhibits only 70.19% sequence identity to *Neovahlkampfia* and between 57.23% and 59.12% to two *Paravahlkampfia* strains of which 5.8S rDNA sequence data are available. Identity between the two *Paravahlkampfia* strains is 96.86%. Unfortunately, no 5.8S rDNA sequence data are available for any of the "unidentified heterolobsean amoebae" that strain SK1 clustered with in the 18S rDNA analysis.

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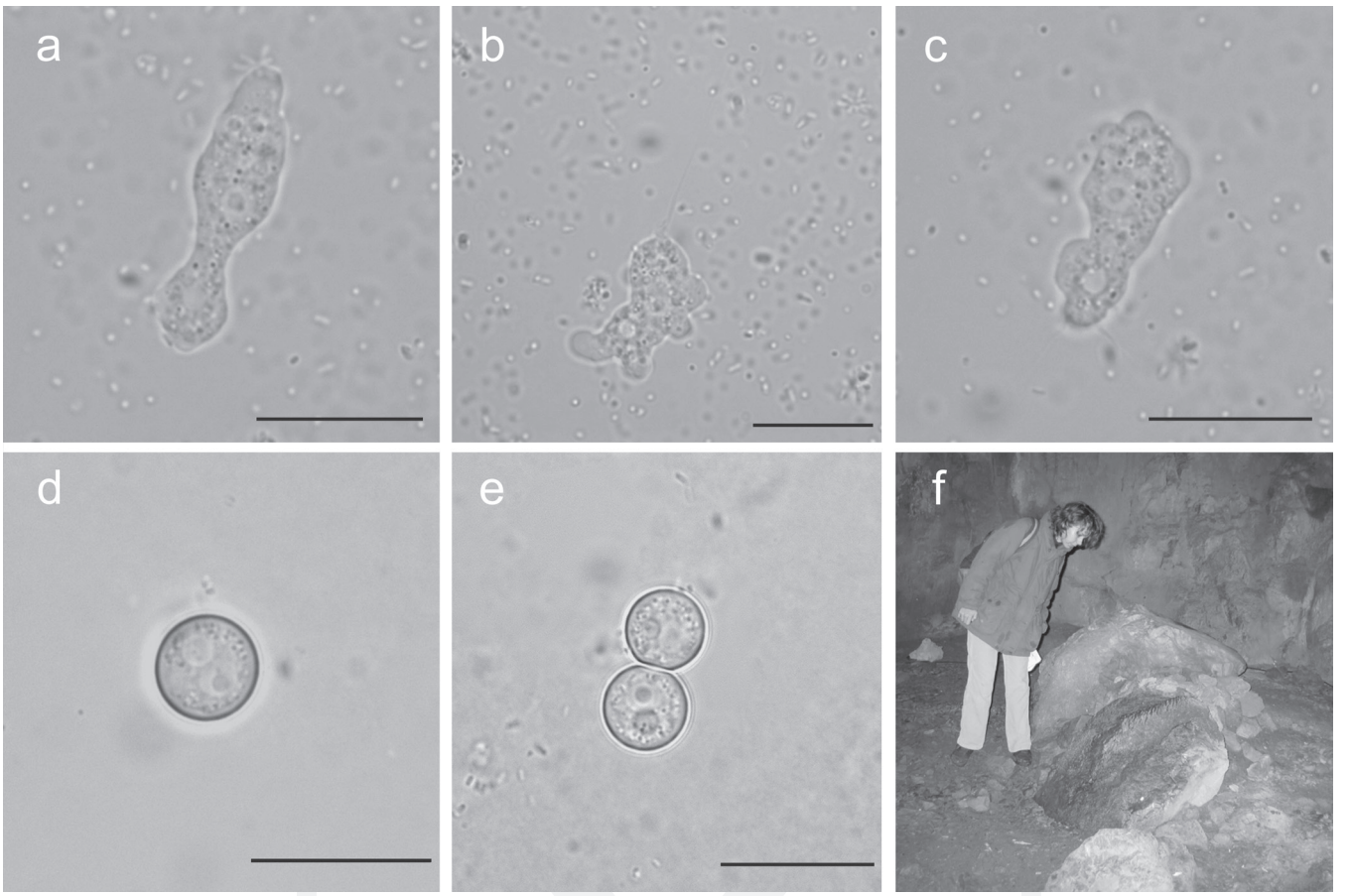

Fig. 2. Trophozoites, cysts and habitat of *Allovahlkampfia spelaea*. Trophozoite in stationary phase (**a**). Trophozoites during locomotion exhibit prominent uroid filaments (**b–c**). Cysts with characteristic nucleus showing a perinculear ring (**d–e**). *A. spelaea* was isolated from the surface of a stromatolitic stalagmite from the cave entrance in Škocjanske jame (**f**). Scale bars: 20 µm.

DISCUSSION

FLA can feed on microbes inhabiting the same niches. In the current study amoebae were typically found in habitats which offer sufficient food supply. In the pool with calcite rafts *Acanthamoeba castellanii* and *Hartmannella vermiformis* can graze at least on 2.5×10^4 cfu mL^{-1}, valkampfiids on 1.1×10^6 cfu g^{-1} in the weathered limestone and in moonmilk on 6.4×10^2 cfu g^{-1} (Mulec *et al*. 2002). Moreover, this cfu value for moonmilk still underestimates the total microbial population. Using molecular tools it was shown that moonmilk contains large microbial cell densities (Blyth and Frisia 2008). It was recently established that the microbial role in the formation of moonmilk is related to nucleation process, modification of the saturation state of the parent solution, change of the physicochemical precipitation which results in the creation of a variety of fibre crystal morphologies, sizes and fibre networks (Cañaveras *et al*. 2006, Blyth and Frisia 2008). In the case of stromatolitic stalagmites in the photic zone of a cave algae can represent a suitable food source for FLA (Mulec *et al*. 2007, Mulec *et al*. 2008). *Echinamoeba silvestris* was identified in the vicinity of these biogenic speleothems in the epilithic algal community. These amoebae are known to occur at extreme environments and have also been isolated from clinical specimens, although they are regarded as non-pathogenic (Baumgartner *et al*. 2003, Walochnik *et al*. 1999). In a recent study (Thomas *et al*. 2008) several different *Echinamoeba* strains were isolated from a drinking water treatment plant. Fungi and bacteria have been previously isolated from organic crust from Snežna jama (unpublished data J. Mulec,

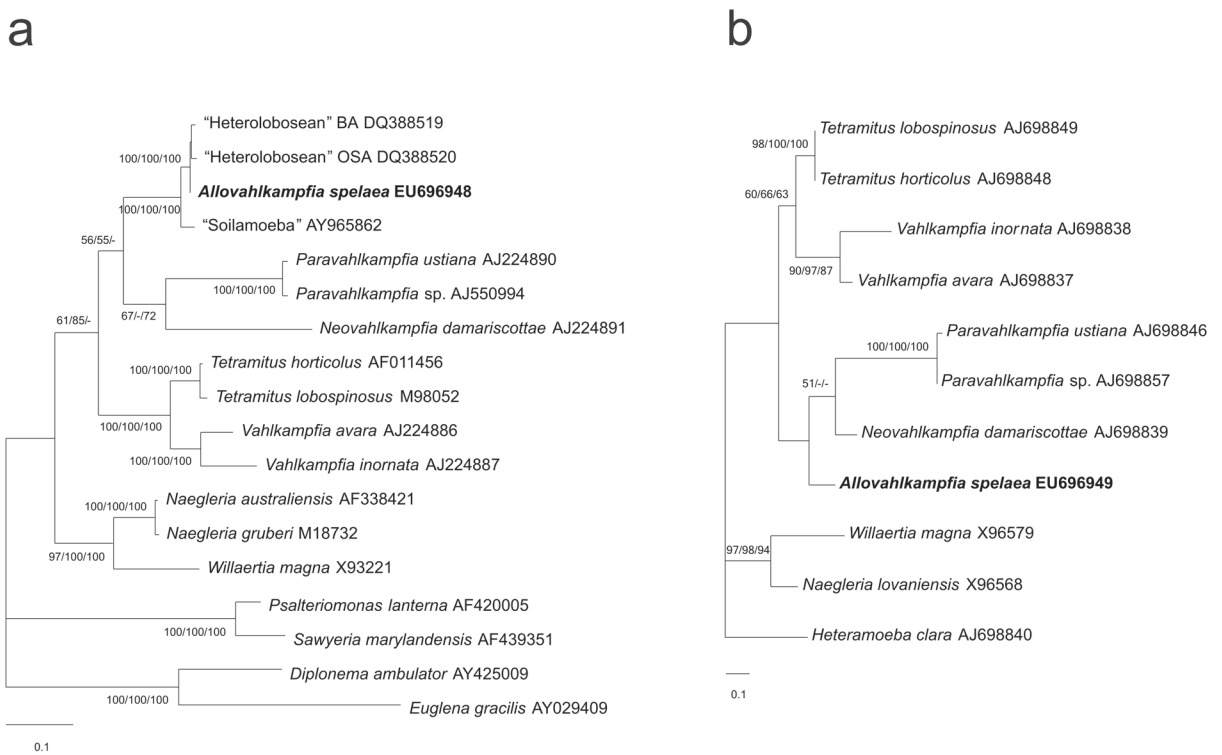

Fig. 3. Phylogenetic analyses for *Allovahlkampfia spelaea*. 18S rDNA maximum likelihood phylogenetic analysis using *Diplonema ambulator* (AY425009) and *Euglena gracilis* (AY029409) as an outgroup (**a**). 5.8S rDNA phylogenetic analysis using *Heteramoeba clara* (AJ698840) as an outgroup (**b**). Bootstrap values are based on 100 replicates and are given at the nodes (ML/NJ/MP). The minus indicates values below 50%. Whenever possible, same strains were used for both analyses. GenBank accession numbers are indicated following the name of the strain.

8 October 2001) and they can also serve as food for FLA. From the studied microhabitats only cave pearls and trickles were not previously screened for the presence of microbiota. However, the presence of rich community of metazoans from the same sites (Pipan 2005) indicates the existence of a food web based on microbes.

The majority of habitats are rather alkaline (Table 1). Alkaline conditions direct precipitation of carbonate. In some cases mineral precipitation is closely associated with bacterial cells when they produce an alkaline environment and nucleation site for mineral formation (Cacchio *et al*. 2003, Douglas and Beveridge 1998). As free-living protozoa represent an important part in a food chain of aquatic environments being the most important grazers on bacteria (Fenchel 1987), and consequently controlling bacterial abundance and community structure, it can be speculated that FLA indirectly create environments that enhance or diminish mineral formation. As demonstrated in the case of *Acanthamoeba castellanii* and *Hartmannella vermiformis* various bacterial species directly influence amoebal behaviour, e.g., growth rate or digestion of bacteria (Pickup *et al*. 2007).

It remains an open question, how and to what extent FLA colonized various cave habitats. No considerable anthropogenic interventions have been detected above the sampling sites. However, FLA can be introduced into a cave from the epikarst zone – above the cave the most upper layer of the rock beneath the soil. For invertebrates this has already been established as a biodiversity hotspot (Pipan 2005). Introduction of amoebae may also occur via ingestion-defecation of various animals (Frank and Bosch 1972, Simitzis-Le Flohic *et al*. 1982, Walochnik *et al*. 1999, Zaman *et al*. 1999). Bats are highly migratory animals that inhabit caves, and thus could be important in the spread of microbial

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propagules. In the vicinity of bat colonies the abundance of microbes in the air is usually elevated (Borda *et al*. 2004). Moreover, FLA, particularly the cysts can be easily spread by air and water currents. Habitats examined in this study are not populated by bats. Some representatives of FLA, e.g., *Acanthamoeba castellanii* genotype T4, are causative agents of human diseases (Martinez and Visvesvara 1997). Moreover, FLA can host various bacteria, including pathogenic ones, and thus function as trojan horses (Barker and Brown 1994). Slime moulds, also belonging to the amoebozoa, have repeatedly been isolated from caves (Landolt *et al*. 1992, Landolt *et al*. 2006, Orpurt *et al*. 1964). In contrast to the slime moulds, which seem to be distributed rather patchy in caves (Landolt *et al*. 2006), the distribution of FLA seems to be more ubiquitous. All 15 *Acanthamoeba* genotypes known to date are assumed to have worldwide distribution (Gast 2001, Gast *et al*. 1996, Hewett *et al*. 2003, Horn *et al*. 1999, Stothard *et al*. 1998). Acanthamoebae and hartmannellae are ubiquitously spread with a rather constant species composition and obviously karst caves are no exceptions. To confirm this statement further studies need to be conducted which will cover wider range of habitat types in caves.

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