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A New Thermophilic Heterolobosean Amoeba, *Fumarolamoeba ceborucoi*, gen. nov., sp. nov., Isolated Near a Fumarole at a Volcano in Mexico

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Summary. An amoeba was isolated from a soil sample collected at the edge of a fumarole of the volcano Ceboruco in the state of Nayarit, Mexico. The trophozoites of this new isolate have eruptive pseudopodes and do not transform into flagellates. The strain forms cysts that have a double wall. This thermophilic amoeba grows at temperatures up to 50°C. Molecular phylogenetic analysis of the small subunit ribosomal DNA (SSU rDNA) places the amoeba into the Heterolobosea. The closest relatives are *Paravahlkampfia* spp. Like some other heterolobosean species, this new isolate has a group I intron in the SSU rDNA. Because of its position in the molecular phylogenetic tree, and because there is no species found in the literature with similar morphological and physiological characteristics, this isolate is described as a new genus and a new species, *Fumarolamoeba ceborucoi* gen. nov., sp. nov.

Key words: Group I intron, Heterolobosea, new genus, new species, SSU rDNA, thermophilic.

INTRODUCTION

The Heterolobosea are amoebae, which move with eruptive pseudopodia, have intranuclear promitosis, have mitochondrial cristae which are flattened, often disc-like, and in which stacked Golgi bodies are absent (Page and Blanton 1985). In the family Vahlkampfiidae within this class, the genus *Vahlkampfia* included all species in which the amoebae do not transform into flagellates (Page 1988). When it was discovered that one *Vahlkampfia* sp. did transform into flagellates it was transferred to a newly established genus, *Paratetramitus* (Darbyshire *et al.* 1976). Later, the small subunit ribosomal DNA (SSU rDNA) sequences proved that this new genus *Paratetramitus* actually belongs to the genus *Tetramitus* (Brown and De Jonckheere 1999). The molecular analyses also demonstrated that only two of the morphologically defined *Vahlkampfia* spp. are related closely enough to belong to the genus *Vahlkampfia*. All the others, except two, were found to belong to the genus *Tetramitus*. The two unrelated species were placed into newly established genera, *Paravahlkampfia* and *Neovahlkampfia*, respectively. As such the freshwater species *Vahlkampfia ustiana* became known as *Paravahlkampfia ustiana* and the marine species *V.*

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damariscottae became *Neovahlkampfia damariscottae* (Brown and De Jonckheere 1999). These conclusions were confirmed by the sequences of the internal transcribed spacers (ITS), including the 5.8S rDNA (De Jonckheere and Brown 2005).

Since that time no other *Neovahlkampfia* sp. and only two other *Paravahlkampfia* spp. have been described, *P. lenta* (Brown and De Jonckheere 2004) and *P. francinae* (Visvesvara *et al.* 2009). An isolate from the intestine of a lizard showed to have SSU rDNA sequences similar to *P. ustiana* but the differences were considered too small (0.2%) to describe the strain as a different species (Schuster *et al.* 2003). Another *Paravahlkampfia* strain was isolated from the eye of a keratitis patient but there was not enough information on this isolate to consider it as a different species (Ozkoc *et al.* 2008). In databases there are also different sequences (AY082995, AY394431, DQ388521) from uncultured amoebae, which show a close relationship to the sequences of the genus *Paravahlkampfia*, from samples taken from the acidic 'river of fire' in Spain (Zettler *et al.* 2002), from an acid mine drainage in the USA (Baker *et al.* 2004) and an unknown origin (Shutt and Gray, unpublished) respectively.

Recently, the new genus *Allovahlkampfia* was created for an isolate from a cave (Walochnik and Mulec 2009). In the phylogenetic tree this new genus forms a separate branch with some unidentified heterolobosean isolates near the *Paravahlkampfia* and *Neovahlkampfia* branches.

We here report the isolation of a heterolobosean amoeba, which belongs to a branch separate from the *Paravahlkampfia*, while the *Neovahlkampfia* and *Allovahlkampfia* branches are found to be totally unrelated to the former two. This new isolate from a fumarole near the volcano Ceboruco in Mexico is described herein as a new species and new genus, *Fumarolamoeba ceborucoi*.

MATERIALS AND METHODS

Soil samples showing algal growth were collected on December 6, 2008 at the edge of five different fumaroles of the volcano Ceboruco (21°125 N; 104°508 W), in the state of Nayarit, Mexico. The temperature of the vapor escaping from the fumarole has been recorded previously to be 83°C (Taran *et al.* 2002). The samples were transported at ambient temperature to the laboratory in Belgium and processed for isolation of amoebae 9 days later.

Soil samples were incubated on a solid medium (1.5% non-nutrient agar (NNA) spread with a lawn of *Escherichia coli*) and in a liquid medium (Page amoeba saline (PAS) with a suspension of *E. coli*) (Page 1988). The incubation temperature was 44°C.

Strains FUM1 and FUM4 were isolated from two samples incubated in the liquid medium PAS supplemented with *E. coli*. All further tests were performed with strain FUM1, as both strains appeared to be identical: they had the same ITS sequences (see results). Growth was tested at different temperatures at up to 52°C in the liquid PAS medium supplemented with *E. coli*.

DNA extraction, amplification of the SSU rRNA gene by PCR, and sequencing were carried out as described previously (Murase *et al.* 2010). The ITS, including the 5.8S rDNA, sequences were obtained as described by De Jonckheere and Brown (2005). The SSU rRNA sequence of strain FUM1 was placed into an alignment of selected heterolobosean SSU rRNA sequences obtained from the on-line comprehensive ribosomal RNA database Silva (<http://www.arb-silva.de/>http://www.arb-silva.de/; Pruesse *et al.* 2007). The SSU RNA sequence from strain FUM1, and of other heterolobosean sequences not available at the Silva database, were manually aligned to this set. The final dataset for tree construction comprised 39 other heterolobosean taxa (accession numbers): *Paravahlkampfia ustiana* (AJ224890), *P. francinae* (FJ169185), *Paravahlkampfia* sp. strain li3 (CDC: V453) (AJ550994), *Paravahlkampfia* sp. strain LA (DQ388521), *Neovahlkampfia damariscottae* (AJ224891), *Allovahlkampfia spelaea* (EU696948), *Tetramitus aberdonicus* (AJ224888), *T. jugosus* (M9805), *T. entericus* (AJ224889), *T. rostratus* (M98051), *T. lobospinosus* (M98052), *T. thermacidophilus* (AJ621575), *Vahlkampfia avara* (AJ 22488), *V. inornata* (AJ22488), *Naegleria fowleri* (U80059), *N. andersoni* (U80057), *Willaertia magna* (X93221, X93223 and AY266315), *P. flabellatum* (DQ979962), *Tulamoeba peronaphora* (FJ222603), *Acrasis rosea* (AF011458), *A. helenhemmesae* (GU437220), *Heteramoeba clara* (AF011460), '*Plaesiobystra hypersalinica*' (AF011459), *Psalteriomonas lanterna* (X94430), *Sawyeria marylandensis* (AF439351), *Monopylocystis visvesvarai* (AF011463), *Stachyamoeba* sp. ATCC 50324 (AF011461), *Percolomonas cosmopolitus* (AF519443 and AF011464), *Stephanopogon minuta* (AB365646), '*Macropharyngomonas halophila*' (AF011465), *Marinamoeba thermophila* (FM244741) , *Vrihiamoeba italica* (AB513360), *Oramoeba thermophila* (FN668558), the environmental heterolobosean isolates OSA (DQ388520), AND9 (AY965861) and AND12 (AY965862), and two uncultured heterolobosean clones, RT5in38 (AY082995) and WIM43 (AM114803). Names with quotation marks indicate that these names are not formally described. The sequences of the Euglenozoa *Euglena gracilis* (AY029409) and *Trypanoplasma borreli* (AY028454) were used as outgroups. A total of 645 of unambiguously aligned sites common to all sequences was retained for phylogenetic analysis. This alignment is available upon request. Phylogenetic trees were inferred by distance matrix neighbor-joining as implemented in ClustalX version 2 (Thompson *et al.* 1997), maximum likelihood (Felsenstein 1981) as implemented by the program PhyML version 2.4.5 (Guindon and Gascuel 2003), and by Bayesian analysis using MrBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001). The general time reversible (GTR) model of evolution (Tavaré 1986) was selected as the best model from 28 using the ModelFind program (http://www.hiv.lanl.gov/ content/sequence/findmodel/findmodel.html). The optimal parameters for the GTR model were estimated using the PhyML program. This model extended with gamma-shaped rate variation with four rate categories was used for both maximum likelihood (100 bootstrap samplings

in PhyML) and Baysian analysis (MrBayes). To estimate Bayesian posterior probabilities, Markov Chain Monte Carlo (MCMC) chains were run for 500,000 generations until convergence and sampled every 100 generations (burn-in: 1,000 generations).

A percentage identity matrix was obtained with alignments in ClustalX for genera closely related to the new isolate under investigation, for the SSU rDNA sequences (Table 1) and for the ITS, including the 5.8S rDNA, sequences (Table 2).

RESULTS

Amoebae were isolated from two out of the five samples incubated in liquid medium, not from those incubated on the agar medium. A free-swimming form was also observed in the cultures, which turned out to be a contaminating ciliate. The amoebae were made free of the contaminating ciliate by culturing on agar medium. On the agar medium the ciliate could not grow while the amoebae formed cysts. These cysts were transferred back to liquid medium, and as such the culture was free of the contaminating ciliate.

The amoeba grows at up to 50°C in liquid medium, but not at 52°C. At 51°C it does not multiply but just survives. At room temperature (22°C) the amoebae survive and keep moving. When tested at 44°C the strain does not grow on agar (NNE), and the amoebae rapidly encyst, except if PAS is added on top of the agar. The trophozoites rarely show the limax locomotion, but are mostly rounded, forming eruptive pseudopodes in all directions, rather than moving unidirectionally (Fig. 1a). The pseudopodes are continuously eruptive, whereby the hyaloplasm bulges outwards and spills around the periphery of the cell. No typical uroid or uroidal filaments are observed. In young cultures most trophozoites have two nuclei, but sometimes up to six nuclei are observed (Fig. 1c, d, e). As the culture ages

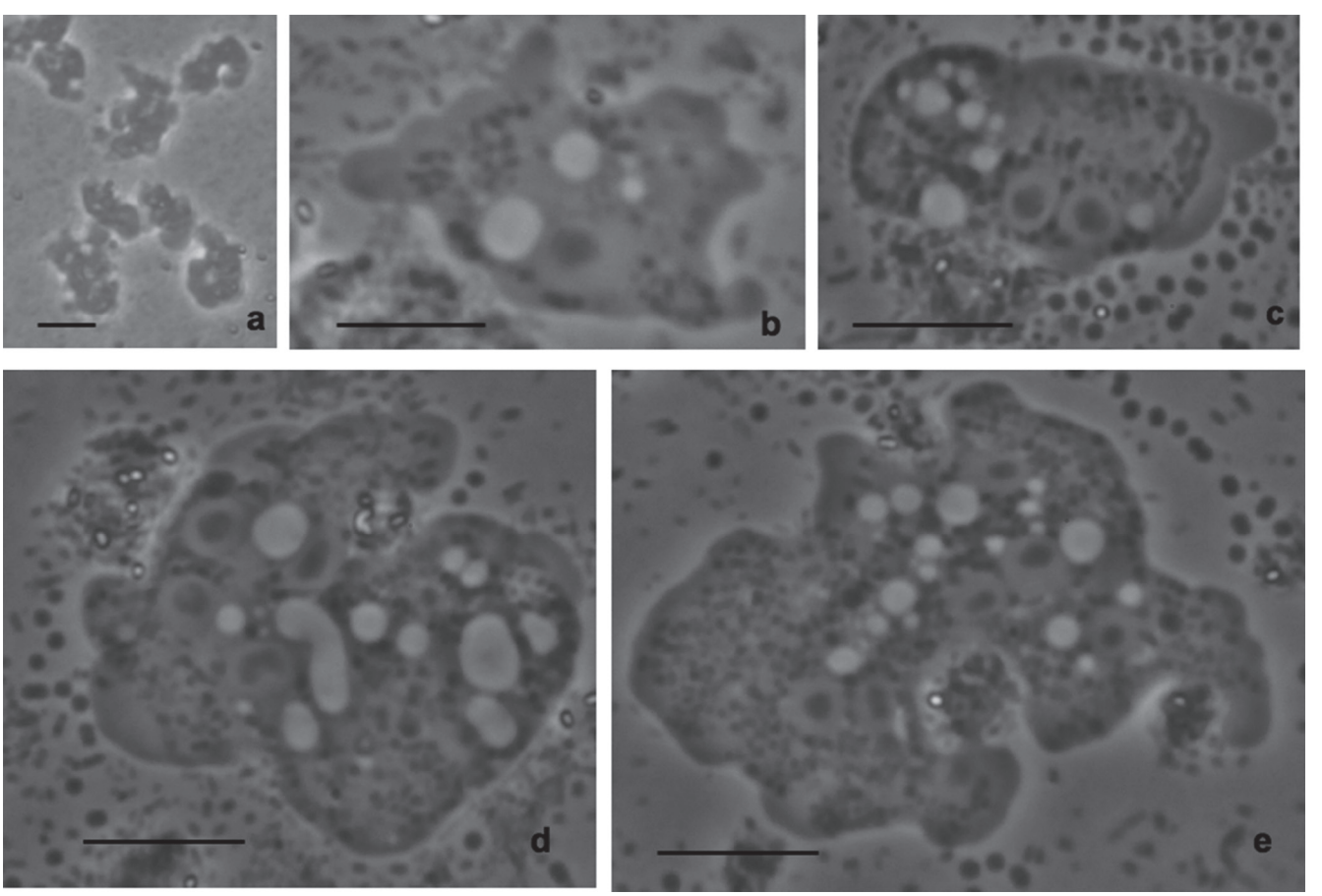

Fig.1. Trophozoites of *Fumarolamoeba ceborucoi,* gen. nov. sp. nov. with phase contrast. **a** – in PAS with inverted microscope (200X); **b**, **c**, **d**, **e** – on agarose covered with PAS (400X) with one, two, four and six nuclei, respectively.

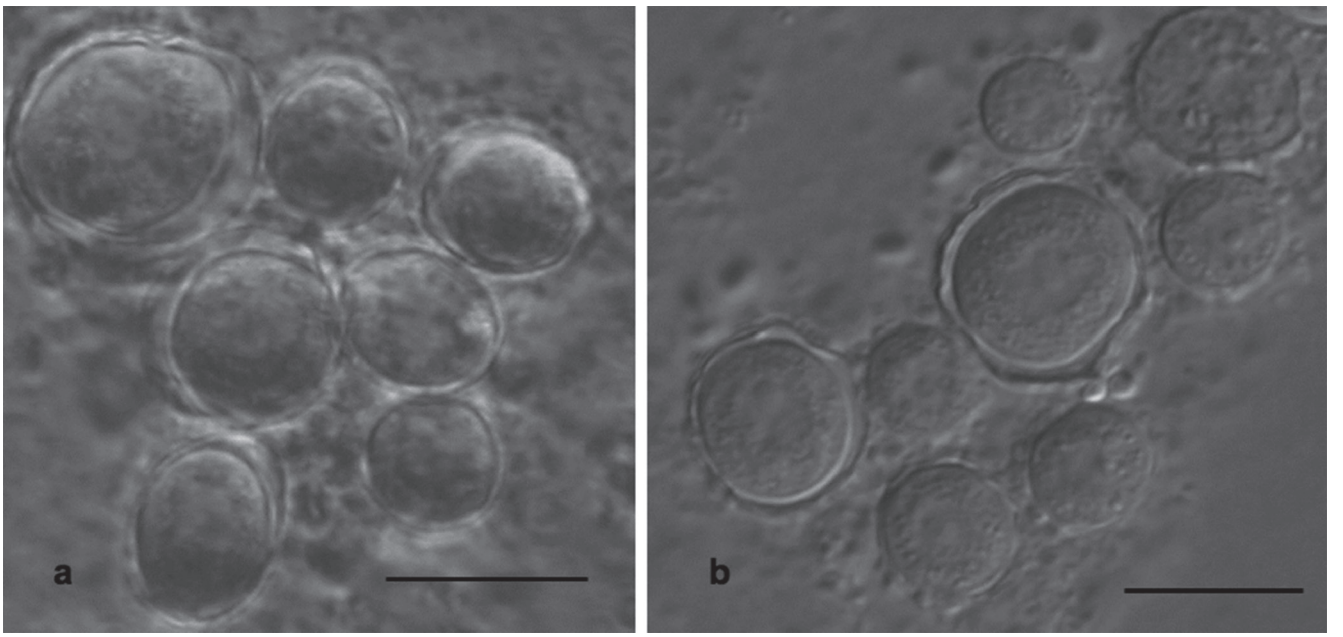

Fig. 2. Cysts *Fumarolamoeba ceborucoi,* gen. nov. sp. nov. with phase contrast on agarose (400X). **a** – phase contrast; **b** – Nomarski.

the number of nuclei diminishes to one per cell (Fig. 1b). The mean length and width of the amoeba grown on agar covered with PAS is 26.0 and 13.8 µm, ranging from 21.0 to 36.6 µm and 7.5 to 20.5 µm, respectively.

The amoeba does not transform into a flagellate stage but it forms cysts. The cysts have no pores but a double cyst wall, with the outer wall detached from the inner wall (Fig. 2). Cysts with two nuclei are frequently observed. The diameter of the cysts varies from 5.5 to 11.0 µm (mean 6.2 µm).

The SSU rDNA sequence is 2,371 bp long and contains a group I intron of 478 bp long (EBI accession N° FR719836). In a phylogenetic tree of the SSU rDNA sequence strain FUM1 was found to belong to a branch separate from the *Paravahlkampfia*, *Neovahlkampfia* and *Allovahlkampfia* clusters (Fig. 3). Its sequence clusters with the sequence of the uncultured heterolobosean clone RT5in38.

The SSU rDNA sequence of strain FUM1 shows 81% identity (Table 1) with *P. ustiana*, *P. francinae* (Visvesvara *et al.* 2009) and strain LA, an environmental isolate stated as being a *Paravahlkampfia* sp. in the database (DQ388521), and 75% identity to *A. spelaea* (Walochnik and Mulec 2009).

The length of the ITS 1 is 133 bp, the 5.8S rDNA 154 bp, and the ITS2 125 bp (EBI accession N° FR719837) for both isolated strains, FUM1 and FUM4, and the sequences are identical. Therefore, all experiments were only performed with strain FUM1. There is very low % identity in the ITS sequence of strain FUM1, including the 5.8S rDNA, with the *Paravahlkampfia* spp. and *A. spelaea* (Table 2).

DISCUSSION

Phylogenetic analysis based on SSU rDNA sequences shows that strain FUM1, together with clone RT5in38, form a clade which lies outside the clade formed by an environmental heterolobosean isolate LA and the *Paravahlkampfia* spp. Both clades are totally unrelated to the *Neovahlkampfia* and *Allovahlkampfia* branches (Fig. 3). While the SSU rDNA sequence of *P. ustiana* is 100% identical to that of *P. francinae*, strain FUM1 shares 81% residues with the former two and 76% with clone RT5in38 (Table 1). A percentage of 81is too low for strain FUM1 to belong to the genus *Paravahlkampfia*, while the sequence of the LA isolate is 98% identical to the *Paravahlkampfia* spp. Thus the LA strain probably belongs to the genus *Paravahlkampfia*. The SSU rDNA sequence of *P. lenta* is not available, only the ITS, including the 5.8S rDNA, sequence has

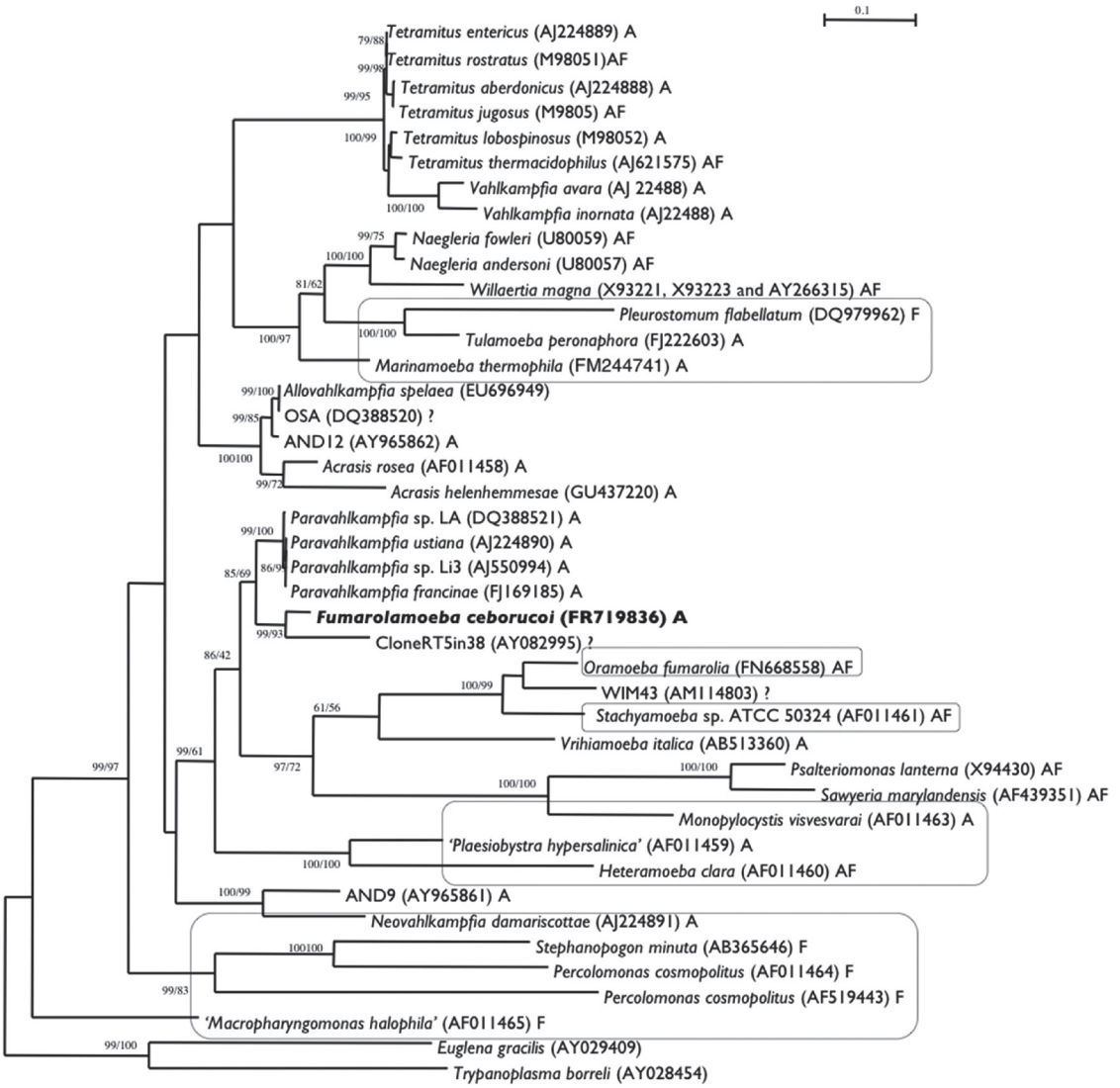

Fig. 3. SSU rRNA genetree (of the PhyML analysis) showing the phylogenetic position of *Fumarolamoeba ceborucoi,* gen. nov. sp. nov. relative to 39 heterolobosean and two outgroup taxa. Support values for the nodes from Bayesian (500,000 generations) and PhyML (100 bootstrap samplings) analyses are indicated. Support values below 50% are not shown. Accession numbers of each taxon are presented in parentheses. The horizontal bar represents 10 substitutions per 100 nucleotides. "A" denotes species which have only an amoebic stage, "AF" denotes amoeboflagellate species, "F" denotes species which have only a flagellate stage, "?" denotes unknown (uncultured environmental sequence). Marine and halophilic species are boxed, the others are species or sequences from freshwater or soil.

been determined (Brown and De Jonckheere 2004). The ITS, including the 5.8S rDNA, sequence differences (Table 2) of the *Paravahlkampfia* spp. are large enough (25%, 28% and 32%, respectively) to consider them as separate species within the genus. The differences of strain FUM1 with the *Paravahlkampfia* spp. is almost 60%, which is very similar to that of *A. cavaea* with the *Paravahlkampfia* spp. However, this sequence of strain FUM1 differs also with that of *A. cavaea* by 42%. Together with the SSU rDNA sequence analysis this is further evidence that strain FUM1 belongs to another genus.

The SSU rDNA of strain OSA (DQ388520; Shutt and Gray, unpublished) is 97% identical with that of *A. spelaea*, and only 90% identical to the SSU rDNA sequence of strain AND12 (Lara *et al.* 2007). Therefore,

Table 1. Percentage identity matrix obtained with ClustalX alignments of SSU rDNA sequences.

strain	1	2	3	4	5	6	7	8	9
1. *A.spelaea*	100								
2. Heterolobosea OSA	97	100							
3. Heterolobosea AND12	90	90	100						
4. *Paravahlkampfia* sp. Li3	74	74	74	100					
5. *Paravahlkampfia* sp. LA	74	75	74	98	100				
6. *P. ustiana*	74	75	74	99	98	100			
7. *P. francinae*	74	75	74	100	99	100	100		
8. FUM1	75	75	75	81	81	81	82	100	
9. Clone RT5in38	71	71	70	74	74	74	74	76	100

Table 2. Percentage identity matrix obtained with ClustalX alignments of ITS, including 5.8S rDNA, sequences.

Strain	1	2	3	4	5
1. *P. lenta*	100				
2. *P. francinae*	72	100			
3. *P. ustiana*	68	75	100		
4. *A. spelaea*	37	40	35	100	
5. FUM1	42	41	39	58	100

the former sequence probably belongs to the genus *Allovahlkampfia*, while strain AND12 should be considered a separate genus or species.

It is interesting to note that the only strain (AND9) mentioned in the literature to be a close relative to *N. damariscottae* (Lara *et al.* 2007) also has only 72% identity in the SSU rDNA (Table 1) with the latter and, therefore, does not belong to the *Neovahlkampfia* genus. This is supported by the fact that *N. damariscottae* is a marine organism (Table 3) while strain AND9 was isolated from soil. In the Heterolobosea all strains of a genus are either freshwater or marine isolates (Fig. 3).

The morphology of the FUM1 amoebae seems to be similar to that of *Paravahlkampfia* spp. by the fact that it forms hemispherical bulges in all directions (Brown and De Jonckheere 2004), while the limax form is infrequently observed, although in other *Paravahlkampfia* reports the monopodal-type of locomotion seems to be more the norm (Schuster *et al.* 2003; Visvesvara *et al.* 2009). Uroidal filaments are common in *Paravahlkampfia* spp., but we did not observe them in strain FUM1. The biggest difference might seem to be the presence of multinucleated trophozoites, but it has been reported that species of several vahlkampfiid genera have a strong tendency to supernumerary nuclei (Page 1988). The cysts appear to be similar to those of *Paravahlkampfia* spp. by the fact that the outer cyst wall often is detached from the inner wall. None of the *Paravahlkampfia* spp. grows at a temperature higher than 42°C (Table 3), while strain FUM1 was isolated at 44°C and grows at a temperature up to 50°C.

In *Allovahlkampfia* the outer wall is closely attached to the endocyst, and the trophozoites are mostly monopodial and show prominent, very long uroid filaments (Walochnik and Mulec 2009). Also in *N. damariscottae* uroidal filaments seem to be common, but this marine species does not form cysts (Page 1983). None of the mentioned genera, and strain FUM1, have pores in the cysts. Nothing is known on the morphology of clone RT5in38 as the sequence was obtained from an environmental sample, without culturing the amoeba (Zettler *et al.* 2002). But the low % identity (Table 1) of the SSU rDNA sequences of clone RT5in38 with strain FUM1 precludes that they could belong to the same genus.

The morphology supports the conclusion from the molecular data that strain FUM1 belongs to a new species and new genus, *Fumarolamoeba ceborucoi,* gen. nov., sp. nov. It is also obvious that some of the sequences of Heterolobosea reported unnamed in the databases do belong to novel genera, and the organisms, if isolated (e. g. AND9 and AND12), should be investigated in detail to give them a proper scientific name.

Table 3. Comparison *of Fumarolamoeba ceborucoi*, gen. nov., sp. nov., to the *Paravahlkampfia* spp., *Neovahlkampfia damariscottae* and *Allovahlkampfia cavaea.*

Species	Strain	Length in bp				Cyst size in µm (range)	Max. temp. (°C)	Growth requirements
		ITS1	5.8S	ITS2	Total			
F. ceborucoi	FUM1	133	154	125	412	6.2 (5.5–11.0)	50	Bacteria
P. ustiana	CCAP1588/6	129	158	316	603	(30–65)	≥ 37	Bacteria
P. lenta	6/3Ab/1B	120	159	397	676	18.1 (12.8–22.4)	34	Bacteria
P. francinae	CDC:V595	129*	158	270*	557*	17.5 (15–21)	42	Mammalian cells
Paravahlkampfia sp.	Li3	ND**	ND	ND	ND	(10–15)	42	Mammalian cells
Paravahlkampfia sp.	SO/1P	ND	ND	ND	ND	(5–15)	35	Bacteria
*N. damariscottae****	CCAP1588/7	140	153	121	414	No cysts	< 37	Bacteria
A. spelaea	SK1	166	161	108	435	(16–25)	30	Bacteria

* Length corrected as the ITS1 and ITS2 sequences at GenBank (FJ169186) contain SSU and LSU rDNA bp, respectively.
** Not Done.
*** from seawater, all others are freshwater strains.

DIAGNOSIS

Fumarolamoeba ceborucoi, gen. nov., sp. nov.

Most trophozoites are rounded displaying markedly eruptive pseudopodes in all directions. In young cultures most trophozoites have two nuclei, but up to six can be observed. This number diminishes to one nucleus per cell as the culture ages. The cysts lack pores and in most cysts the outer cyst wall is detached from the inner wall. No flagellates are observed. The maximum growth temperature is 50°C. The organism does grow in liquid medium but not on agar, except if the latter is covered with liquid.

The species has a unique ITS, including 5.8S rDNA, sequence which allows it to be identified from other Vahlkampfiidae, especially from *Paravahlkampfia* spp. which seems to be the closest related genus.

Observed habitat: soil near a fumarole at the volcano Ceboruco in Nayarit, Mexico.

Etymology: *Fumarolamoeba ceborucoi, gen. nov.,* sp. nov. is named to denote the origin of the type strain, the fumaroles at the Ceboruco volcano in Nayarit, Mexico.

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