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Description of a New Species with a Remarkable Cyst Structure in the Genus *Naegleria*: *Naegleria angularis* sp. n.

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Summary. A vahlkampfiid amoeba was isolated from a water sample collected in Peru. Ribosomal DNA sequence analysis showed that the isolate belongs to the genus *Naegleria*, that it represents a novel species and that its closest relative is *N. pussardi*. Although there is very little sequence difference between the new isolate and *N. pussardi*, and both species grow at 40°C, their cyst morphology is very different. The cysts of the new species are angular to star-like in appearance and so do not resemble the cysts of any described *Naegleria* species. The strain did not form flagellates in distilled water but did transform in Prescott's and James's solution. The name *N. angularis* sp. n. is proposed for this isolate with angular cysts.

Key words: 5.8S rDNA, cyst morphology, *Naegleria angularis* sp. n.

INTRODUCTION

Over the last decade, ribosomal DNA (rDNA) sequences of many strains of the family Vahlkampfiidae have been compared and the evolutionary relationships which have been inferred from these analyses are not congruent with the phenotype-based classification of vahlkampfiid species (De Jonckheere and Brown 1995, De Jonckheere 1997, De Jonckheere *et al.* 1997, Brown and De Jonckheere 1999). The least controversial consequence was the reassignment of most species of the phenotypically-diverse and poorly-defined genus *Vahlkampfia* to other genera (Brown and De Jonckheere 1999). Several *Vahlkampfia* species were reassigned to the genus *Tetramitus*, but these, and other vahlkampfiid species which cluster with the type species *T. rostratus* in rDNA phylogenetic trees, do not conform to the morphological definition of the genus (De Jonckheere and Brown 2003). Consequently the genus *Tetramitus* now includes phenotypically-diverse species which may (or may not) have a flagellate stage with (or without) a rostrum and with two (or four) flagella, and whose cysts may (or may not) have pores. Despite the observed phenotypic variation, all *Tetramitus* species have very similar internal transcribed spacer (ITS) and 5.8S rDNA sequences.

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The genus *Naegleria* is also now defined by molecular criteria (De Jonckheere 2002) but, in contrast with *Tetramitus*, somewhat less morphological variation exists within the genus, whilst ITS and 5.8S rDNA sequences of *Naegleria* species are more divergent. The morphological variation in *Naegleria* is mostly in the presence or absence of the flagellate stage (De Jonckheere *et al.* 2001), and whether or not the flagellate stage can divide. For example, *N. minor* was originally described as *Willaertia minor* on the basis that the flagellates can divide, as is the case in *W. magna* (Dobson *et al.* 1993). However, small subunit (SSU) rDNA sequence analysis demonstrated that *W. minor* is, in fact, a *Naegleria* sp. Similarly, *N. robinsoni* has a 5.8S rDNA sequence which clusters within the genus *Naegleria*, although also in this species the flagellates do divide (De Jonckheere and Brown 1999). Within the genus some slight variation is seen in the cyst morphology. Page (1975) attempted to differentiate *N. gruberi* strains on the basis of the cyst wall morphology, which he characterised as smooth, rough or angular. In fact, the use of molecular methods established later that the *N. gruberi* strains he investigated represented several distinct species. These species are *N. pringsheimi*, *N. pagei* (De Jonckheere 2002), *N. schusteri* and *N. americana* (De Jonckheere 2004). Subsequently, Pussard and Pons (1979) tried to differentiate *Naegleria* isolates on the basis of the number of pores in the cyst wall and the shape of the plugs in these pores. In addition to those species which Page investigated, Pussard and Pons included in their study the *N. gruberi* strains which were later differentiated by molecular techniques as *N. gallica*, *N. americana* (De Jonckheere 2004) and *N. gruberi sensu stricto* (De Jonckheere 2002), and two even turned out to belong to another genus, *Willaertia* (De Jonckheere *et al.* 1984). However, despite the fact that Page (1975) and Pussard and Pons (1979) observed significant differences in cyst morphology amongst *Naegleria* isolates which we now know are different species, they failed to obtain morphological criteria that could be used in species identification, because cyst morphology varies according to culture age and conditions.

Here we present a new *Naegleria* sp. which has a pronounced and consistent difference in cyst morphology from its closest relative, *N. pussardi*. These two species are very closely related: there is only a small difference in their ITS sequences (4 bp in the ITS1 and 3 bp in the ITS2) and their 5.8S rDNA sequences are identical.

MATERIALS AND METHODS

Strain T692 was isolated from a sample kindly provided by Humberto Guerra. The freshwater sample containing sediment was collected in Iquitos, Peru, on June 24, 2003, and held at ambient temperature until February, 2004, before it was processed for the isolation of amoebae. Sediment in the water sample was placed on non-nutrient (NN) agar streaked with *Escherichia coli* and the plates were incubated at 37°C. The vahlkampfiid strain which was isolated was later tested for growth at 40°C and 42°C. Light microscopical observations were made using Nikon Eclipse TS100 and Olympus BH2 microscopes.

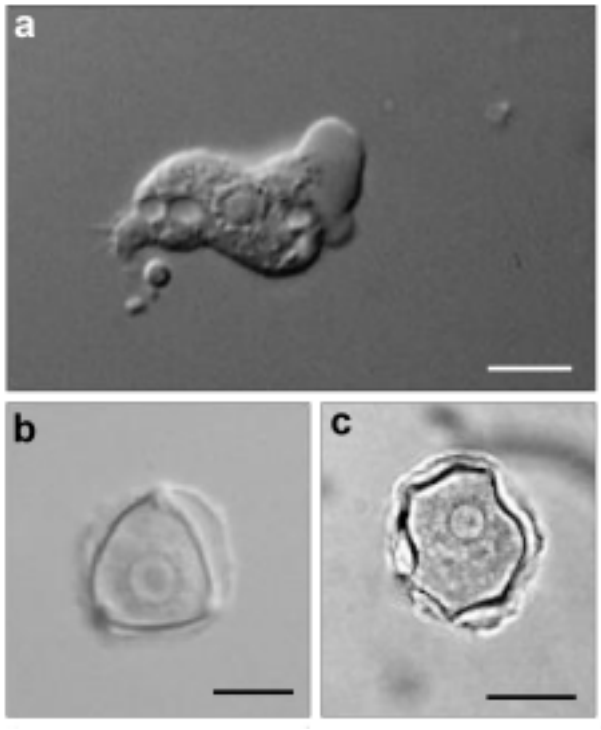
DNA was isolated from pelleted trophozoites of strain T692 using the UNSET method (Hugo *et al.* 1992). The ITS1, 5.8S and ITS2 rDNA were PCR-amplified using the ITS forward primer and the ITS reverse primer, corresponding to the 3' end of the SSU rDNA and the 5' end of the large subunit (LSU) rDNA, respectively (De Jonckheere 2004). Two pairs of ITS primers were employed, one pair designed for amplifying from *Naegleria* spp. specifically (De Jonckheere 1998) and a second less specific pair for amplifying from vahlkampfiid species (De Jonckheere and Brown 2003). Employing the latter primer pair the PCR product was sequenced (both strands) without cloning using a Beckman CEQ2000 sequencer using the CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., Fullerton, CA, USA).

The 5.8S rDNA sequences were aligned using ClustalX (Thompson *et al.* 1997) and the alignment was manually adjusted in SepPub9 (D. G. Gilbert, http://iubio.bio.indiana.edu). Because of long indels in the ITS1 and ITS2 sequences, these sequences were not included in the alignment. Phylogenetic trees were constructed from these alignments of 177 nucleotides using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (Neighbor joining and UPGMA) and Drawgram programs of the PHYLIP (version 3.572c) package (Felsenstein 1989).

The ITS1, 5.8S rDNA and ITS2 sequence of strain T692 has been deposited in EMBL with the accession number AJ785756.

RESULTS

Trophozoites of strain T692 (Fig. 1a) move very rapidly across the substratum. During steady, unidirectional, locomotion the trophozoites are limax, with length 28.8-40.0 µm (mean 33.5 µm) and breadth 4.8-8.0 µm (mean 6.9 µm); L/B ratio 3.8-5.8 (mean 4.9). Locomotory cells are not markedly eruptive, but the hyaloplasm does spill (from the front) to alternate sides, creating a slight left, then right, pattern of progression. The frontal hyaline zone is relatively-large; usually deeper than broad, often accounting for one third of the cell length. Trophozoites make frequent changes in direction and are very eruptive when changing direction and when stationary. All trophozoites observed had a perinuclear layer of small granules. The nucleus is vesicular and round, or oval if squashed. The nucleolus has a diameter approx.



Figs 1a-c. a - trophozoites of strain T692 showing the typical eruptive pseudopodes during locomotion; **b** - angular cyst of strain T692 with three pores clearly visible; **c** - more irregularly shaped cyst. Bars 10 µm.

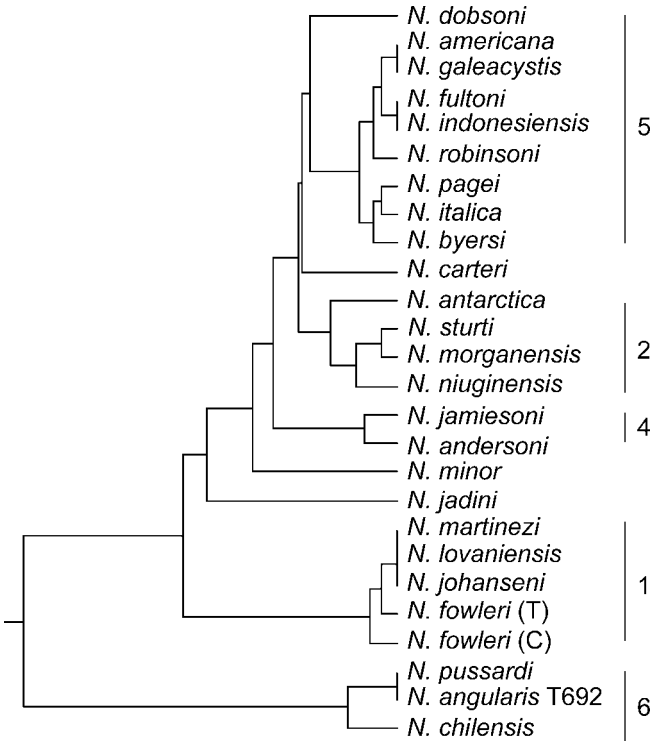


Fig. 2. Phylogenetic tree inferred from comparisons of the 5.8S rDNA sequences using the Kimura two-parameter correction and the UPGMA method. Numbers at the right indicate the clusters as defined previously (De Jonckheere 2004). Only cluster 3 (*N. carteri* and *N. minor*) does not form here.

one third to a half the diameter of the nucleus. All trophozoites observed were uninucleate, with one, two or three contractile vacuoles per cell. The posterior of the cell is rounded. Uroidal filaments were observed at the rear of a small proportion of cells.

Cysts have two walls and are unevenly-round or irregular in shape (Fig. 1c). The two walls always meet at a point at excystment pores. The cyst shape is often angular, usually with three or four points each ending at a pore. In the latter cases the cysts have a star-like appearance (Fig. 1b). The triangular and quadrangular cysts each account for approximately one third of cysts in a culture. Exact proportions cannot be provided as they vary from culture to culture. The proximity of the outer and inner cyst walls varies greatly, even around the perimeter of a single cyst. The diameter of the outer wall varies from 12.9-24.6 µm (mean 18.6 µm). All were uninucleate with a perinuclear layer of small granules.

A temporary flagellate stage was not observed when the trophozoites were suspended in distilled water. However, after suspension in Prescott's and James's solution (Page 1988) flagellates did appear. Strain T692 grows at 40°C but not at 42°C.

A strong PCR product was obtained when using the general vahlkampfiid ITS primers while only a faint band was obtained with the more specific *Naegleria* ITS primers. Therefore, the PCR product using the vahlkampfiid primers was sequenced using the same primers. The sequence lengths of the ITS1, 5.8S and ITS2 of strain T692 are 40, 176 and 91 bp, respectively. While the 5.8S rDNA sequence is identical to that of *N. pussardi* (De Jonckheere 2004), the strain differs from the latter species in 4 bp in the ITS sequence (Table 1) and 3 bp in the ITS2 sequence (Table 2). Because the 5.8S rDNA sequence of strain T692 is the same as that of *N. pussardi* both cluster in phylogenetic analyses of the aligned 5.8S rDNA with other *Naegleria* (Fig. 2), whichever method used.

DISCUSSION

Prior to the DNA sequence analysis, our initial phenotypic observations made us uncertain about which vahlkampfiid genus strain T692 belongs to. The amoe-

Table 1. ITS1 sequence.

	10 20 30 40
T692:	5′TTGGTAAAAGATTTGGGTAAAACCAAATTTT**T**TT**G**CC**TA**C3′
N.pussardi:	5′TTGGTAAAAGATTTGGGTAAAACCAAATTTT**A**TT**A**CC**CC**C3′

Table 2. ITS2 sequence.

	10 20 30 40
T692:	5′GCTGCTTTCGAGTTTGCGCCCTATTCAGAGGAAGCGTGTCAAAA**AAG**T
N.pussardi:	5′GCTGCTTTCGAGTTTGCGCCCTATTCAGAGGAAGCGTGTCAAAA-**TT**T
	50 60 70 80 90
	ATTTTTGATACGAATTTTAATCCGAAAGGATTTCAATTATTAC3′
	ATTTTTGATACGAATTTTAATCCGAAAGGATTTCAATTATTAC3′

bae have a layer of refractile granules around the nucleus which seems to be typical for the genera *Naegleria* and *Willaertia* (Page 1988), but the morphology of the cysts, together with the absence of transformation in distilled water, are not characteristic of these genera. Even so, the 5.8S rDNA sequence conclusively placed the strain within the genus *Naegleria*, although a better PCR product was obtained with the more general vahlkampfiid primers than with the more specific *Naegleria* primers. This unexpected result prompted the re-isolation, PCR-amplification and sequencing of DNA from strain T692 to check that the first result was not the consequence of mislabelling. However, the same result was obtained and was confirmed by determining the sequence three times in both directions.

Phylogenetic analyses of the 5.8S rDNA sequences with those of other *Naegleria* species revealed that strain T692 is most closely related to *N. pussardi* (Fig. 2). These two species, which have a 5.8S rDNA of 176 bp, constitute a separate branch 6 (De Jonckheere 2004) with *N. chilensis*, which has a 5.8S rDNA of 177 bp. The latter is the longest 5.8S rDNA length detected in *Naegleria*, as deduced from an alignment of *Naegleria* 5.8S rDNA sequences which is more accurate than the alignment published originally (De Jonckheere 2002). All other *Naegleria* spp. have a 5.8S rDNA length of 175 bp, except *N. andersoni*, *N. jamiesoni* (the two species in branch 4), and *N. carteri* (one of the two species in branch 3), which have a length of 174 bp.

Cysts of *N. pussardi* are typical in appearance for the *Naegleria* genus: round in shape with a double wall, and with plugged pores (Pernin and De Jonckheere 1996). The cysts of the new isolate T692 also have a double wall with pores, but the cyst shape is usually angular, often with three or four points ending in a pore. Although Page (1975) described angular cysts in *Naegleria* spp. he did not depict or describe cysts that had the star-like appearance which we observed in strain T692. When cysts have the star-like appearance they bear a superficial resemblance to those of *Acanthamoeba* strains, especially *A. triangularis*. Therefore the cyst also resembles the cyst of *Vahlkampfia angularis* (Robinson 1980). The species *V. angularis* is not a valid name, because it has never been published in a regular journal but a description of it is in preparation using strains that were isolated more recently (Robinson *et al.*, in preparation). The absence of the flagellate stage made Robinson assign his isolates to the genus *Vahlkampfia*, despite the presence of pores in its cysts. Unpublished molecular results (JFDJ) indicate that the new strains of *V. angularis* do not belong to the genus *Naegleria* nor to the genus *Vahlkampfia*, but to the genus *Tetramitus*. Hence the strains of B. Robinson will be described as *T. angularis*. In contrast to *T. angularis*, which was reported to be unable to grow above 30°C, strain T692 does grow very quickly at 37°C. It even grows at 40°C, but not at 42°C. In its temperature tolerance it resembles *N. pussardi*, its phylogenetically closest relative.

Because of the morphological similarity of the cysts with those of *V. angularis* we decided to also use the species name *angularis* for strain T692 which, however, belongs to the genus *Naegleria*. The special morphology of the cysts of this new species further increases the degree of phenotypic diversity observed within the genus *Naegleria*.

Description of *Naegleria angularis* sp. n.

The trophozoites have the typical appearance of *Naegleria* and they can be induced to transform into flagellates. The cysts have an angular shape, with the points ending in a pore. Because of the angular shape a high proportion of the cysts have a star-like appearance, not seen in any other described *Naegleria* sp. The strain grows at 40°C, but not at 42°C.

Because of the morphological similarity of the cysts with those of *T. angularis* molecular identification is required, especially since flagellates might be difficult to obtain in *N. angularis*. The species can be identified from the ITS1 and ITS2 sequences, which differ by 4 bp and 3 bp, respectively, from those of *N. pussardi*. The 5.8S rDNA sequence is identical to that of *N. pussardi*.

The type strain was isolated from a water/sediment sample collected in Peru.

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