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An Endocytobiont Harbouring *Naegleria* Strain Identified as *N. clarki* De Jonckheere, 1994

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Summary. A *Naegleria* strain harbouring two different populations of bacterial endocytobionts, together inhibiting the encystation of their host was isolated from a garden pond. Interestingly, the ability to form cysts was regained after removing one endocytobiont by antibiotic treatment. The trophozoite and the flagellate stage were investigated by light and electron microscopy and the temperature tolerance, under-agarose migration and tissue culture pathogenicity of the strain were assessed. Moreover, the entire gene of the SSU rRNA was sequenced and compared to published sequences of various *Naegleria* species and a cluster analysis was performed in order to reveal the phylogenetic position of this endocytobiont harbouring *Naegleria* strain. The amoeba was shown to grow well at 37°C, to migrate under-agarose and to lyse human HEp-2 cells, properties related to pathogenicity. With the help of SSU rDNA sequencing and subsequent cluster analysis the strain N_DMLG was identified as *N. clarki*. The *N. clarki* strains altogether formed a cluster with *N. gruberi* and the potentially pathogenic *N. italica, N. australiensis* and *N. tihangensis*.

Key words: amoebae, endocytobionts, *Naegleria clarki*, SSU rDNA.

INTRODUCTION

Naegleriae are free-living amoebae occurring predominantly in freshwater and soil. *N. fowleri* is pathogenic for man causing the almost always fatal primary amebic meningoencephalitis (Carter 1970). Besides their active pathogenicity free-living amoebae can also be of clinical relevance by acting as hosts and vehicles

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for pathogenic bacteria. Rowbotham (1980) was the first who confirmed the ability of *Legionella pneumophila* - the causative agent of legionnaires' disease - to grow within vacuoles of acanthamoebae. He also demonstrated the growth of this pathogen within *Naegleria* sp. While acanthamoebae and also hartmannellae frequently harbour intracellularly replicating bacteria (Barker and Brown 1994; Michel and Hauröder 1997; Michel *et al*. 1998, 2003; Horn *et al*. 2000, 2001; Greub and Raoult 2004) - some authors report that occasionally more than 20% of isolated *Acanthamoeba* strains are naturally infected with bacteria (Fritsche *et al*. 1993) - only very few naegleriae

naturally harbouring bacteria have been found. Harf (1993) reported on the occurrence of intracellular Gram negative rods in *Naegleria* sp., Michel *et al.* (1998) found naturally occurring intracellular cocci within a *Naegleria* strain isolated from an aquarium and Thom *et al.* (1992) have shown experimentally within a period of 24 h, that *Vibrio cholerae* grow better in the presence of acanthamoebae and naegleriae. *Naegleria* spp. are also often found in *Legionella*-positive waters, but they usually do not carry bacteria inside their cells (Grimm *et al.* 2001).

A *Naegleria* strain (strain N_DMLG) isolated from a small private garden pond attracted our attention by its inability to form cysts. An initial microscopic study revealed that this strain harboured two different populations of bacterial endocytobionts - one (pc) replicating within the cytoplasm and the other (pn) growing within the nucleus of its host amoeba (Michel *et al.* 1999). The current study is a continuation of that investigation in order to characterise and identify this *Naegleria* strain serving as a host for such a rarely found double infection.

MATERIALS AND METHODS

Organisms and culture. The strain N_DMLG was isolated from a mixed population of various species of FLA from a garden pond (Michel *et al.* 1999) and was cultured by the non nutrient (NN)-agar plate culture method according to Page (1988). Briefly, mud samples were inoculated onto non-nutrient agar plates coated with a 24 h old culture of *Enterobacter cloacae* and incubated at RT. Initial cultures were diluted in order to eliminate co-contaminants by cutting a small piece of agar with a sterile scalpel and applying it centrally onto a fresh plate. For obtaining uniform genetic populations for taxonomic investigations, the isolate was cloned by transferring a single cyst to a fresh plate with a micromanipulator.

In order to confirm the ability of the strains to transform into the characteristic flagellate stage the trophozoites of a 48-72 h culture were transferred from the agar surface into amoeba-saline or distilled water and observed for one to two hours. The strain was maintained on NN-agar plates, subcultured by monthly serial transfers and later transferred to axenic SCGYE-medium (De Jonckheere 1977) under antibiotic treatment (200 IU penicillin and 200 µg/ ml streptomycin). Morphological investigations included measurements made with a calibrated ocular micrometer.

Electron microscopy. For electron microscopical investigations amoebae and flagellates were harvested from the plates and pelleted by centrifugation (500 g) for 10 min. The resulting pellets were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, washed twice in the same buffer, postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), and embedded in Spurr resin. Finally, the sections were stained with uranyl acetate and

Reynold's lead citrate and examined using a Leo EM 910 transmission electron microscope (Leo, Oberkochen).

Physiological investigations. Temperature tolerance was assessed by incubating fresh cultures at a range of temperatures (30°C, 34°C, 37°C, 40°C and 42°C) and observing them daily using phase contrast microscopy. The strain was considered tolerant to a certain temperature, if it was able to survive and to multiply at this temperature. Altogether, the growth of the amoebae was observed at each temperature for two weeks. The ability of the amoebae to migrate into the agar was recorded after 72 h. The ability of the amoebae to lyse human cells was revealed by coincubating the amoebae with a human cell monolayer (HEp-2 cells). The HEp-2 cells were cultured in a 1:1 mixture of PC-1TM (Bio Whittaker, Walkersville, Maryland) and CO_2 independent medium (Life Technologies Ltd., Paisley, Scotland) supplemented with L-glutamine (2 mM) in 75 cm^2 tissue culture flasks (Corning, Costar, Bodenheim, Germany) at 37°C under sterile conditions. 1 ml of a 10^5 cell/ ml axenized suspension was inoculated onto a monolayer of HEp-2 cells. Cocultures of amoebae and tissue cells were incubated at 30°C, 37°C and 42°C, respectively. Pathogenicity was defined as complete lysis of the monolayer within 48 h at 37°C. All experiments were carried out in triplicate and were repeated after 6 weeks.

Isolation of DNA. For molecular biological investigations ~10^6 amoebae from actively growing cultures were harvested and washed 3x in sterile 0.9% NaCl by centrifugation 500 ×g/ 7 min. Whole-cell DNA was isolated by a modified UNSET-procedure (Hugo *et al.* 1992). Briefly, the amoebae were suspended in 500 µl of UNSET-lysis buffer, overlaid with 500 µl phenol-chloroform-isoamylalcohol (PCI) and shaken gently for 5 h. DNA was extracted by multiple PCI-extraction, precipitated in alcohol, air dried and resuspended in 30 µl of sterile double-distilled water.

PCR and sequence analysis. The SSU rRNA gene (small subunit ribosomal RNA gene) was amplified using universal eukaryotic primers complementary to the conserved ends of the eukaryotic SSU rRNA genes (Gast *et al.* 1996) and a standard amplification program (30 cycles; 95°C 1 min, 51°C 2 min., 72°C 3 min.). The amplified gene (3285 bp long) was visualized by ethidium-bromide in an agarose-gel electrophoresis and then sequenced stepwise (by constructing the following internal primers, given in 5'-3' direction:
N1-CTGCAGCGATAATACTTG,
N2-GGAATCAGTGTTCGATTCC,
IN1-GTAGTGACAAGCTATAGTG,
IN2-CTGTAGGATGGTAACAAGC,
IN3-CAAGATTGATCGCGAATC,
IN4-CGAATGCTTGATTGGTGC,
IN5-CTGAGGAGGTCTCAATC,
IN6-GAATTGATGTTCGGTAGTC,
Nrev1-CAACGAGCTGATAGGTAAG,
Nrev2-GTTATCTACACCCAAATC,
Nrev3-CTGCAGTCCCATGCTAAATC,
INrev1-CTCGTTGCCGAGCGGTAC,
INrev2-CAATGGTCTGCCGTGTAG,
INrev3-GCAGTTTGACAACCATTC,
INrev4-CACAGCCTCCATTACCAC,
INrev5-CACTCTTAGGTATACTAC)
by direct sequencing from the PCR-product using the BigDye sequencing kit and a 310 ABI PRISM® automated sequencer (PE Applied Biosystems, Langen, Germany). Sequences were obtained from both strands.
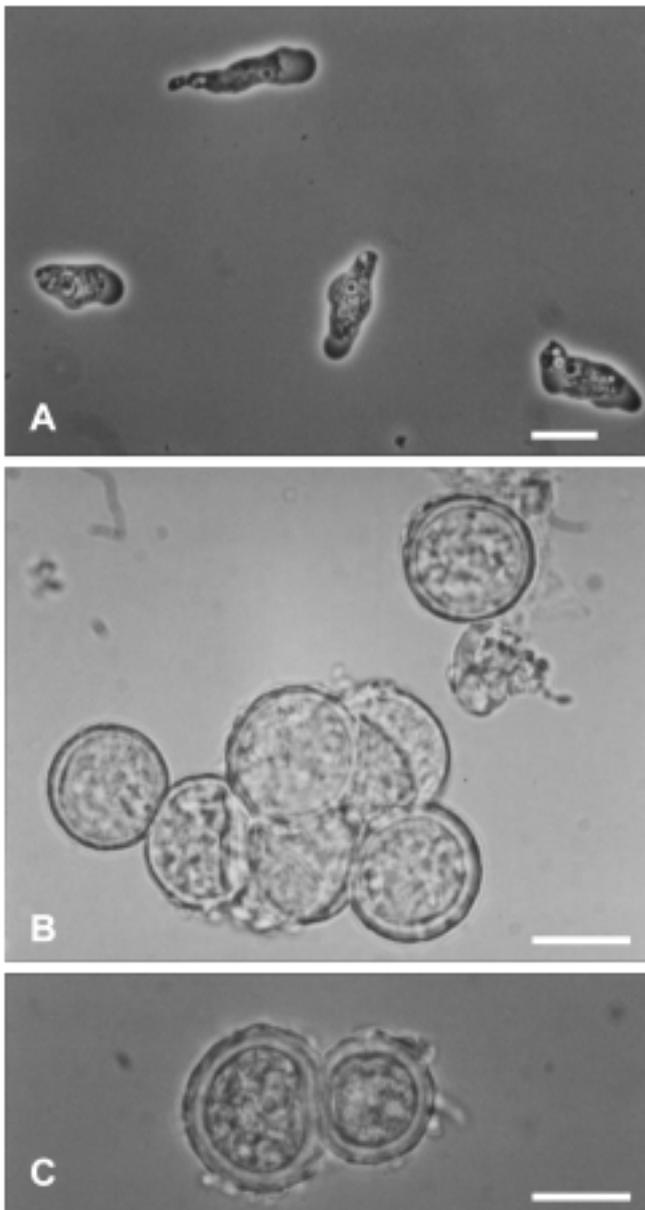
Figs 1 A-C. *Naegleria clarki* strain N_DMLG. **A** - trophozoites in phase contrast, endocytobiotic bacteria visible; **B, C** - cysts, note the extremely thick cyst wall, encystation induced after antibiotic treatment in order to kill the endocytobiotic bacteria. Scale bars 10 µm (A); 15 µm (B, C).

Sequence data were processed with the GeneDoc (Nicholas *et al.* 1997) sequence editor and compared to the ones of strains published in GenBank using BLAST Search (Altschul *et al.* 1990). Multiple alignment was performed and sequence dissimilarities to published sequences of *Naegleria* spp. were assessed.

Phylogenetic analyses. Two cluster analyses were performed. One including various vahlkampfiid amoebae in order to proof the

position of strain N_DMLG within the genus *Naegleria* using *Acrasis rosea* as outgroup and a second, more detailed cluster analysis in order to find out the exact position of strain N_DMLG within the genus *Naegleria* and in order to reveal the detailed branching order of the closely related species *N. clarki, N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. In this second analysis *N. fowleri* and *N. lovaniensis* were used as an outgroup. Multiple sequence alignments were performed by subsequent pairwise alignment using the Clustal X application (Thompson *et al.* 1997). The alignments were controlled by eye and corrected manually.

Phylogenetic analyses were made using the PHYLIP (Felsenstein 1989) package. Primer sites, unique gaps, ambiguously aligned sites and introns were excluded from the analyses. The analyses were performed with different evolutionary models including maximum likelihood, neighbour joining and maximum parsimony. The confidence of the branching order was proven by the generation of 100 bootstrap replicates. Maximum likelihood was performed with invariable sites and a gamma distribution of among-site rate variation, neighbour joining with a Kimura two parameter corrections and maximum parsimony employing heuristic search with a random sequence addition option and counting all steps. Consensus trees were made from the resulting trees using Consense and drawn with the TREEVIEW software (Page 1996).

Sequence data. The sequence reported in this paper is available at GenBank under the following accession number: AF426157.

RESULTS

Morphology and physiology. The strain N_DMLG readily transformed to the flagellate stage when incubated in amoeba saline, but the amoebae were obviously unable to encyst. In order to prove that this was due to the intracellularly "parasitising" bacteria and also in order to be able to study the cyst morphology, it was aimed to induce encystment by eliminating the bacteria. Indeed, after several rounds of antibiotic treatment (200 IU penicillin and 200 µg/ ml streptomycin) and thus after elimination of at least the cytoplasmic population of the endocytobiotic bacteria, the amoebae did form cysts. Figure 1 shows trophozoites (Fig.1A) and cysts (Figs 1B, C). The cysts are round, 8-16 µm in diameter with clearly separated cyst walls and a slightly wrinkled ectocyst.

In order to investigate the endocytobiotic bacterial populations electron microscopy was performed. At the ultrastructural level we discovered not only coccoid organisms of a very prickly appearance (pc) within the cytoplasm but in addition cells with a more smooth outline within the nucleus - especially on the surface of the prominent nucleolus looming into the karyoplasm (pn) (Fig. 2). Both endocytobiotic bacterial populations were shown to be Gram negative and both could not be
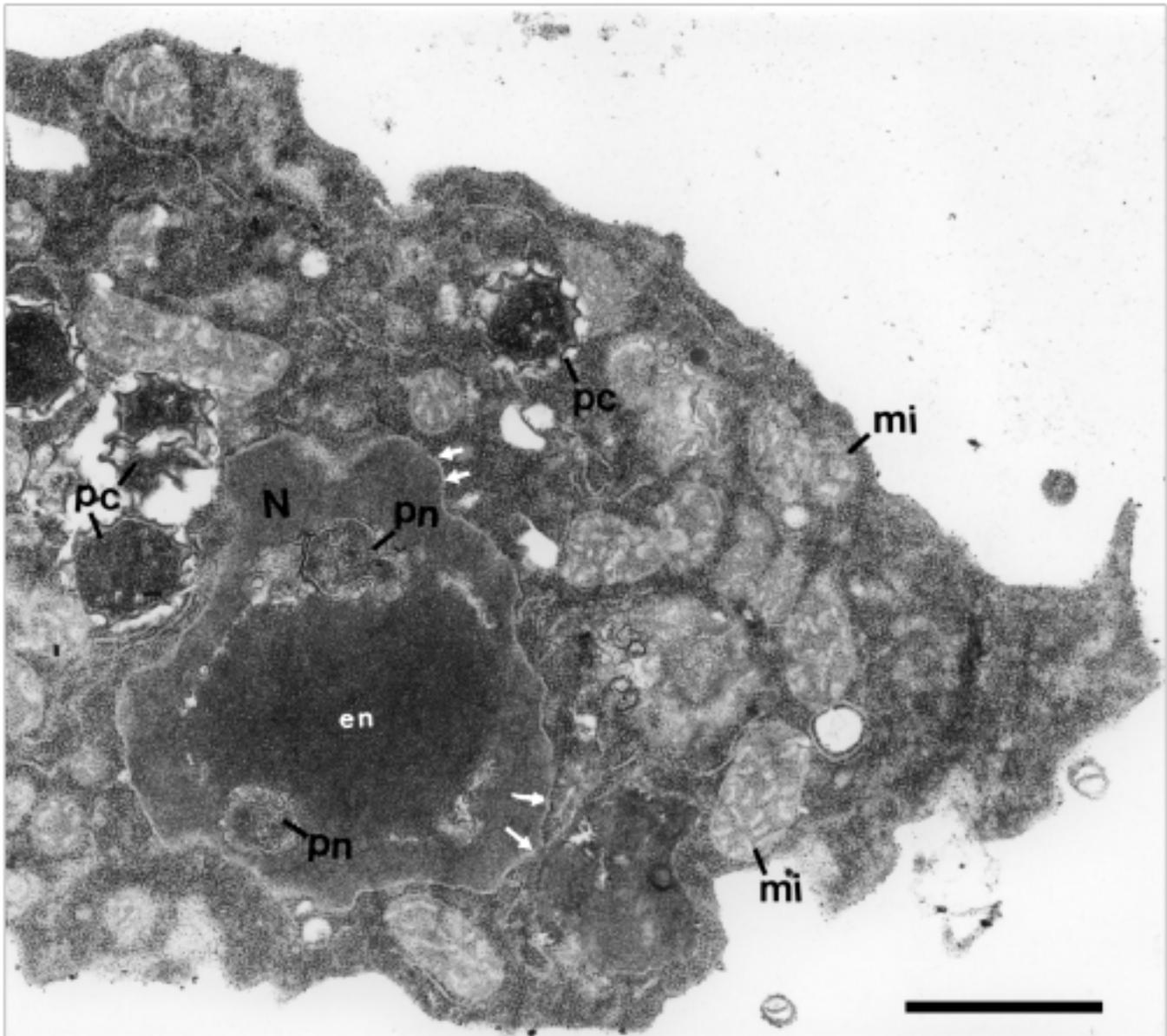
Fig. 2. *Naegleria clarki* strain N_DMLG trophozoite harbouring two different populations of endocytobiotic bacteria: one (pc) is multiplying within the cytoplasm, the other one (pn) is located within the nucleus (N) at the interphase between the prominent nucleolus (en) and the karyoplasm. mi - mitochondria. Scale bar 1 μm (30 000×).

grown on various synthetic media (Michel *et al.* 1999). However, the two bacterial populations could be grown separately as a "single infection" by variation of the culture conditions and application of antibiotics - both within substrains of the same host amoeba. Thus, it can be concluded, that indeed two different populations were present in the original isolate (Michel *et al.* in preparation). An example of a trophozoite harbouring solely the

intranuclear population is shown in Fig. 3A. The original isolate with "double infection" as well as the experimentally derived amoebae with only a single infection were still able to transform into the flagellate stage thereby maintaining their respective endocytobionts such as the intranuclear population for instance shown in Fig. 3B.

Physiological investigations revealed the amoebae to be thermophilic (growing well at 37°C), to be able to
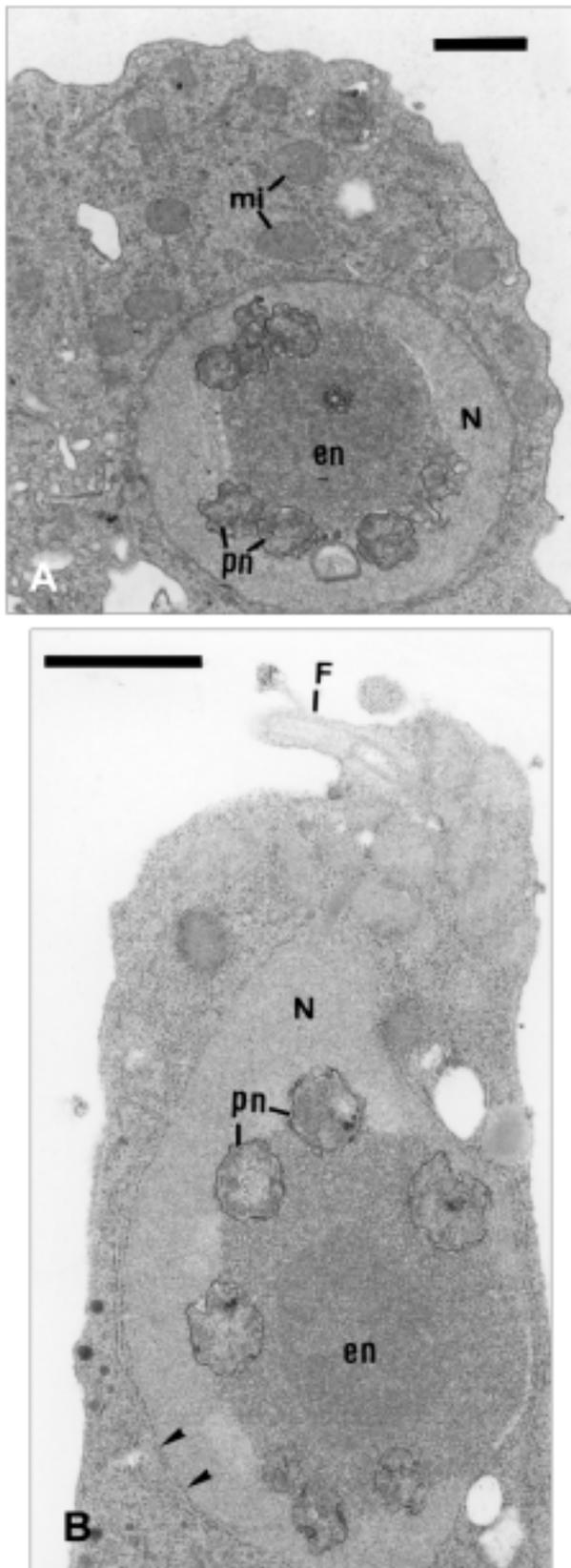
migrate under-agarose and to completely lyse a mono-layer of human HEp-2 cells within 48 h, independently of the presence of the bacterial populations.

Molecular biology. It was shown that the SSU rDNA of the *Naegleria* strain N_DMLG is 3285 bp long. It has a group I intron of 1305 bp from base 631-1936 and a G + C content of 44.18 (47.53 without insert). Comparison to published sequences revealed that N_DMLG shows highest SSU rDNA sequence identity (99.95%) to strain 4564/IV of *N. clarki* (GenBank Accession No.: AF338419) isolated from the kidney of a *Perca fluviatilis* (Dykova *et al.* 2001). The sequence dissimilarity of N_DMLG is <0.36% to all *N. clarki* strains - to the most closely related one only 0.05% (the dissimilarities between all published *N. clarki* strains are up to 0.56%), while it is 0.66 to *N. gruberi*, the closest relative to *N. clarki* (from which it, however, differs by the intron N_DMLG and all *N. clarki* strains have, and which *N. gruberi* sensu stricto does not have), and >1% to all other naegleriae - to *N. fowleri*, *N. lovaniensis, N. jamiesoni* and *N. andersoni* even >5%.

Cluster analysis. In the first phylogenetic analysis (Fig. 4) it was shown that the strain N_DMLG grouped within the species *N. clarki* and the *N. clarki* strains altogether formed an imperfectly resolved cluster with *N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. The whole genus *Naegleria* appeared as a monophyletic group with *Willaertia* being most closely related.

In the second analysis (Fig. 5) it was shown that the strain N_DMLG indeed groups with the 5 *N. clarki* strains isolated from organs of freshwater fish. More-over, it was shown, that *N. gruberi* is the nearest relative and this grouping was supported by high boot-strap values. The subsequent closest relative was *N. italica* and these altogether formed a cluster with *N. australiensis* and *N. tihangensis*.

Figs 3A, B. *Naegleria clarki* strain N_DMLG. **A** - trophozoite harbouring only the **intranuclear** endocytobiotic population (pn) located at the interphase between nucleolus (en) and karyoplasm. N - nucleus, mi - mitochondria. Scale bar 1 μm (13 200×); **B** - flagellate stage harbouring the intranuclear population (pn) at the margin of the nucleolus (en). F - flagellum, N - nucleus. Arrow-heads - nuclear membrane. Scale bar 1 μm (21 000×).
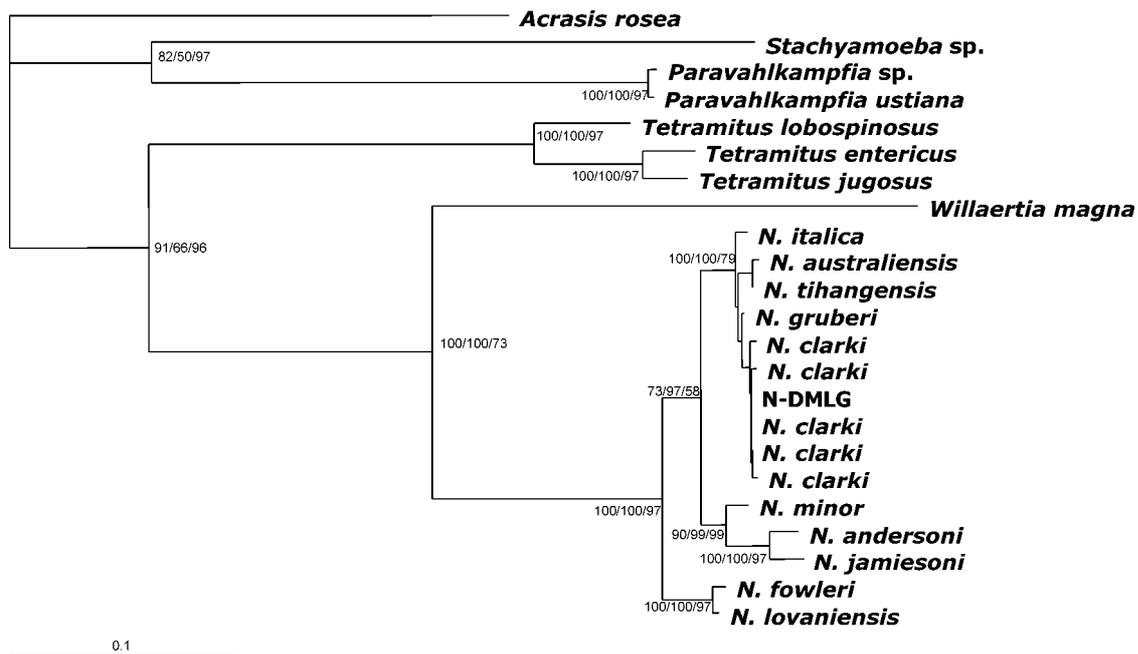
Fig. 4. 18S rDNA maximum likelihood analysis of the relationship of different vahlkampfiid amoebae using *Acrasis rosea* as outgroup. Bootstrap values are based on 100 replicates and are given at the nodes (ML/NJ/MP). The scale bar indicates the number of substitutions per site. Observe the clustering of *N. clarki* with *N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. Strains and GenBank accession numbers: *Acrasis rosea* (strain T-235, AF011458), *N. andersoni* (strain PPMFB-6, U80057), *N. australiensis* (strain CB2B/I, AF338421), *N. clarki* (strain Pd72Z/I, AF338417; strain 4177/I, AF338418; strain 4564/IV, AF338419; strain 4709/I, AF338420; strain Pd56Z/I, AF338422), *N. fowleri* (strain MCM, U80059), *N. gruberi* (strain ATCC 30224, M18732), *N. italica* (strain AB-T-F3, U80060), *N. jamiesoni* (strain T56E, U80061), *N. lovaniensis* (strain C-0490, U80062), *N. minor* (strain WTO43, X93224), *N. tihangensis* (isolate TT, AY321362), *Paravahlkampfia* sp. (isolate li3_03, AJ550994), *P. ustiana* (strain CCAP 1588/6, AJ224890), *Stachyamoeba* sp. (strain ATCC 50324, AF011461), *Tetramitus entericus* (strain CCAP 1588/5, AJ224889), *T. jugosus* (strain ATCC 30703, M98050), *T. lobospinosus* (strain ATCC 30298, M98052), *Willaertia magna* (strain A1,2 PWCl1, AY266315)

DISCUSSION

A *Naegleria* strain isolated from a garden pond and harbouring two different populations of bacterial endocytobionts, one replicating within the cytoplasm, the other growing within the nucleus of the amoeba - together inhibiting the encystation of their host, was identified as *Naegleria clarki* De Jonckheere, 1994, by SSU rDNA sequencing.

Identification

Morphology alone is unfortunately not discriminative in the genus *Naegleria* (De Jonckheere 2002), and in this case the identification was especially intricate as these amoebae initially did not form cysts. All amoeboflagellates with plugged pores and with not dividing flagellate stages with two flagellae and without a cytostome have been assigned to the genus *Naegleria*. However, meanwhile it has become clear, that there are

naegleriae that have no flagellates and there are also naegleriae that have dividing flagellates. Up to date more than 20 different species of *Naegleria* have been described (De Jonckheere 2002, 2004), whereby ribosomal DNA sequencing has contributed the main part of recent classification and identification of *Naegleria* isolates.

The SSU rDNA of strain N_DMLG was shown to be highly identical to 5 *N. clarki* strains isolated from organs of freshwater fish in the Czech Republic (Dykova *et al.* 2001) and as N_DMLG also clustered with these strains in phylogenetic analyses we identify our strain as *N. clarki*. The strains isolated from fish have not been described morphologically, so no light- and electron microscopical comparison to these strains was possible. The initially described *N. clarki* strains, strain RU30 and RU42, had been isolated from a spring in Rotorua (New Zealand). Also these two strains have a group I intron in their SSU rDNA (De Jonckheere 1993, 1994a), however, no entire SSU rDNA sequences are available at
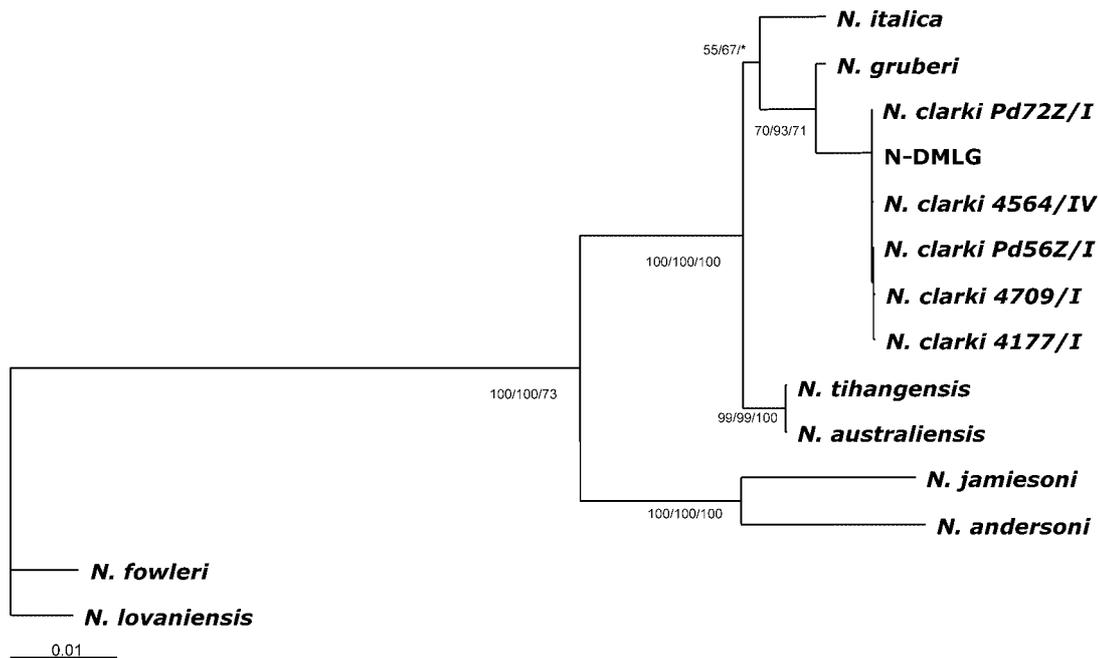
Fig. 5. 18S rDNA maximum likelihood cluster analysis of strain N_DMLG and different *Naegleria* spp. using *N. lovaniensis* and *N. fowleri* as outgroup. Strain N_DMLG clearly clusters with the *N. clarki* strains isolated from freshwater fish in the Czech Republic and *N. gruberi* is the closest relative of this group. Strains and GenBank accession numbers see legend of Figure 4.

GenBank, so no thorough molecular comparison was possible. Recently, De Jonckheere (2004) identified another *N. clarki* strain (strain BG6), but also for this strain no entire SSU rDNA sequence is available at GenBank.

The SSU rDNA of strain N_DMLG was shown to contain a 1305 bp group I intron and this intron was identical to the one Dykova *et al*. (2001) found in their 5 *N. clarki* strains and also to the group I intron of strain RU30 (De Jonckheere 1994a). Several species of *Naegleria* have group I introns in their SSU rDNA (De Jonckheere 1994a, De Jonckheere and Brown 1998). These group I introns of *Naegleria* are twintrons with two catalytic RNAs, which is rather unusual and only also found in *Didymium*, a myxogastrid mycetozoan (Einvik *et al*. 1998). De Jonckheere demonstrated that the *Naegleria* SSU rDNA group I intron was acquired in an ancestral state and lost in most *Naegleria* species (De Jonckheere and Brown 1994, De Jonckheere 2002).

Interesting is also the obviously broad temperature tolerance spectrum of the species *N. clarki*. The two New Zealandian *N. clarki* isolates came from a spring, the 5 Czech isolates from freshwater fish and our strain from a garden pond. The *N. clarki* strains isolated from fish all had a temperature limit below 37°C, while our isolate, although coming from a rather cold habitat exhibited temperature tolerance (growth at 37°C). Also the type strain of *N. clarki* RU30, as the majority of *N. clarki* strains, has an upper temperature limit of 37°C, however, the species *N. clarki* De Jonckheere, 1994 also includes a strain growing up to 40°C (De Jonckheere 2002). Griffin (1972) established temperature tolerance (at least 37°C) as a criterion for pathogenicity. All pathogenic *Naegleria* isolates are thermophilic, however, also several non-pathogenic species have been shown to be thermophilic, e.g. *N. lovaniensis*. Altogether, there are thermophilic isolates in 12 different *Naegleria* species (De Jonckheere 2002). Three species of *Naegleria* have been shown to kill mice after intranasal instillation, *N. fowleri*, *N. australiensis* and *N. italica*, although, until now, neither *N. australiensis* nor *N. italica* have been involved in human disease. The first two *N. clarki* strains, the ones from New Zealand, had initially been shown to be pathogenic for mice, but in later pathogenicity tests they did not kill mice after intranasal instillation (Willaert 1976) and the whole species was thus considered as

non-pathogenic. However, these strains had been isolated long before and only later assigned to this genus and it is known that at least *N. fowleri* strains tend to loose their pathogenicity after long term culture (Wong *et al*. 1977).

Cluster analysis

Strain N_DMLG clearly clustered with the *N. clarki* strains isolated from freshwater fish in the Czech Republic and the *N. clarki* strains altogether formed a cluster with *N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. *N. tihangensis* was newly erected out of several *N. gruberi* strains (see below). *N. australiensis* was described in 1981 (De Jonckheere 1981). It grows up to 42°C and has no intron. The type strain had been isolated from water in Australia, but the species is meanwhile considered to occur all over the world. Recently, *N. australiensis* has also been isolated from the brain of fishes in the Czech Republic (Dykova *et al*. 2001). *N. italica* De Jonckheere, Pernin, Scaglia *et* Michel, 1984, was initially described as a subspecies of *N. australiensis*. Later it was found that the differences justify an own species name for these amoebae (Adams *et al*. 1989, De Jonckheere 1994b) and also in our analysis it rather grouped with *N. gruberi* and *N. clarki* than with *N. australiensis*, although this clustering was not supported by really high bootstrap values. *N. italica* has in contrast to *N. australiensis* a group I intron in its SSU rDNA (De Jonckheere 1993) and is also more virulent than *N. australiensis* (De Jonckheere 2002). *N. italica* has long been thought to occur only in Italy, however, recently two strains of *N. italica* have been isolated in Australia with an sequence identity of 100% in the 5.8S rRNA gene and the flanking ITS (Henderson *et al*. 2001, Robinson *et al*. 2004). Interestingly, the Australian isolate seems to be non-pathogenic to mice after intranasal instillation. While *N. lovaniensis* and *N. australiensis* are considered to be ubiquitously distributed (De Jonckheere 2004), it has not yet been clarified, if this is true for all *Naegleria* strains. A major problem is that from many strains only so few isolates have been investigated that no final decision can be drawn on this.

N. gruberi has long been a most heterogenous species, which was to a large extent also due to the fact, that before 1970, when the species *N. fowleri* was established (Carter 1970) for the pathogenic isolates, all *Naegleria* isolates were assigned to this in those days only described species. *N. gruberi* was first described

in 1899 (Schardinger 1899) and is therefore the first described *Naegleria* species, although it was initially described as *Amoeba gruberi* and only later assigned to the newly established genus *Naegleria* Alexeieff, 1912. Today *N. gruberi* is considered to represent a complex of at least 10 species (Clark *et al*. 1989, De Jonckheere 2002), it has been divided into four clusters on the basis of rDNA sequence analyses (Clark *et al*. 1989) and these clusters were later given the species status (*N. gruberi* sensu stricto*, N. pringsheimi, N. pagei* and *N. tihangensis*, whereby *N. tihangensis* was then considered a subgroup of *N. australiensis* and is now considered the sister species to *N. australiensis*) (De Jonckheere 2002). Also the genus Vahlkampfia has been reevaluated and the species have now been divided between the four genera *Tetramitus, Vahlkampfia, Neovahlkampfia* and *Paravahlkampfia* (Brown and De Jonckheere 1999).

Endosymbionts in *Naegleria*

Our isolate was the first *N. clarki* shown to harbour bacteria and, moreover, the first *Naegleria* strain harbouring two different bacterial populations at the same time, one in the cytoplasm and one in the nucleus. At another occasion a similar coccoid Gram negative bacterium ("Knic") had been shown to replicate within the cytoplasm of a *Naegleria* sp. isolated from an aquarium (Michel *et al*. 1998). This host-amoeba was lost unfortunately whereas the endoparasites could be transferred and maintained on other *Naegleria* strains (Michel *et al*. 2000). They have a more smooth outline compared to "Pc" and look like little golf balls seen with SEM. The taxonomic position of both the endocytobionts of the present paper as well as of "Knic" could not yet be identified unequivocally. Endoparasites in the nucleus are altogether very rare. Sabaneeva *et al*. (2002) reported on intranuclear bacteria in *Paramecium*.

In summary, it was shown that *N. clarki* can harbour living bacteria, and thus can act as vehicle for bacteria. Moreover, the results of our study corroborate the close relationship between *N. clarki* and the potentially pathogenic *N. italica, N. australiensis* and *N. tihangensis* and demonstrate that also *N. clarki* shows physiological properties related to pathogenicity.

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