

A Century of Research on the Amoeboflagellate Genus *Naegleria*

Johan F. De JONCKHEERE

Protozoology Laboratory, Scientific Institute of Public Health, Brussels, Belgium

Summary. The amoeboflagellate genus *Naegleria* contains pathogenic and nonpathogenic species. As most species are morphologically indistinguishable, species are defined and identified by molecular methods. For routine identification, isoenzyme analyses are performed. For the description of a new species, sequences of ribosomal DNA are increasingly used and the analyses of these sequences also allow us to define the phylogenetic relationships between species and strains. In the present monograph 27 *Naegleria* lineages are discussed and identified as separate species. Using molecular methods, *Naegleria* spp. have been identified which either form dividing flagellates or which do not form flagellates at all, thus contradicting the accepted definition of the genus. *Willaertia*, which forms dividing flagellates, is the genus that is the closest relative of the genus *Naegleria*. The genus *Naegleria* has some particularities in its molecular biology, such as circular ribosomal DNA plasmids, group I introns in the small and large subunit ribosomal DNA, and an unusual pyrophosphate-dependant phosphofructokinase. The phylogeny of the *Naegleria* spp. is compared to the situation concerning the other genera of the family Vahlkampfiidae. Also discussed is the state of affairs concerning species designation based on phylogeny in the genus *Acanthamoeba*, another free-living amoeba with species pathogenic to man.

Key words: *Acanthamoeba*, *Balamuthia*, dividing flagellates, group I introns, *Hartmannella*, *Naegleria pagei* sp. n., *N. pringsheimi* sp. n., *N. tihangensis* sp. n., non-flagellating, phylogeny, ribosomal DNA, *Vahlkampfiia*.

Abbreviations used: AP - acid phosphatase, ATCC - American Type Culture Collection, bp - basepairs, CCAP - Culture Collection of Algae and Protozoa, CSF - cerebrospinal fluid, EMBL - European Molecular Biology Laboratory, IC - intracerebral, IN - intranasal, ITS - internal transcribed spacer, LSU - large subunit, mt - mitochondrial, NACM - *Naegleria* amoebae cytopathogenic material, NRS - non-ribosomal sequence, ORF - open reading frame, PAM - primary amoebic meningoencephalitis, PE - propionyl esterase, PCR - polymerase chain reaction, PPI-PFK - pyrophosphate-dependant phosphofructokinase, RAPD - random amplified polymorphic DNA, rDNA - ribosomal DNA, RFLP - restriction fragment length polymorphism, SSU - small subunit.

INTRODUCTION

A century ago Schardinger (1899) discovered an *Amoeba. lobosa* that could transform into a flagellate stage, and called it *Amoeba gruberi*. The genus

name *Naegleria* was suggested much later by Alexeieff (1912).

Before 1970 *Naegleria* was studied mainly as a model for transformation because the amoebae easily transform into flagellates (Willmer 1956, Chang 1958, Fulton and Dingle 1967). However, the genus attracted much more attention, especially from the biomedical world, when it was found that some *Naegleria* isolates cause a fatal brain infection, primary amoebic meningoencephalitis (PAM) in humans. The infection almost invariably results in death. The *Naegleria* isolates that

Address for correspondence: Johan F. De Jonckheere, Research Unit for Tropical Diseases (TROP), Christian de Duve Institute of Cellular Pathology (ICP), Avenue Hippocrate 74-75, B-1200 Brussels, Belgium; Fax: 32-2-762.68.53; E-mail: djonckh@ben.vub.ac.be

cause PAM were given species status and named *N. fowleri*, after Malcolm Fowler who first recognized the disease in Australia (Carter 1970). Cases of PAM were soon afterwards detected all over the world. The most recent review on the diseases produced by *N. fowleri* and other opportunistic free-living amoebae, belonging to the genera *Acanthamoeba* and *Balamuthia*, can be found in Martinez and Visvesvara (1997).

Between October 1970 and October 1972 four PAM cases were diagnosed in Belgium in children aged between 11 and 14 years old (Hermanne *et al.* 1973). All cases were diagnosed around the city of Antwerp. Although *N. fowleri* was never isolated from the swimming pools where the four children had been swimming before becoming ill, it was presumed that these swimming pools were implicated. Because the swimming pools are filled with water from the drinking water suppliers, the latter were accused of introducing the pathogenic amoeba into the swimming pools. Therefore, the Belgian water distributors decided to have the water of their network investigated for the presence of *N. fowleri*. This is where my involvement with *Naegleria* started. As a young biologist, with no experience in protozoa at all, I was hired to work on the grant that was assigned to a university laboratory, that also had no experience in protozoology whatsoever.

It was quite a coincidence that a new case of PAM was diagnosed in Belgium (Van Den Driessche *et al.* 1973) only six months after I started to investigate methods for the identification and isolation of *N. fowleri*. Not only was the timing useful, the place where the 14 year-old boy probably became infected gave a clue to the ecology of the pathogenic *N. fowleri*. The deceased boy had been swimming in a brook that received cooling water from a metallurgical factory, and we were able to demonstrate the presence of *N. fowleri* in that water (De Jonckheere *et al.* 1975). Therefore, an investigation was started on the presence of *N. fowleri* in warm water discharges of different industries in Belgium. This investigation demonstrated that cooling waters were indeed the places where this pathogen could proliferate (De Jonckheere and van de Voorde 1977a). In addition, the absence of *N. fowleri* in drinking water and swimming pools in Belgium was also subsequently proven (De Jonckheere 1979a). This is in contrast to the situation in other parts of the world where higher annual water temperatures are prevalent and the presence of *N. fowleri* in drinking water is not uncommon (South

Australia is the most notorious example Dorsch *et al.* 1983).

In the attempts to isolate *N. fowleri*, many different *Naegleria* strains were isolated that have properties that did not fit the descriptions of either *N. gruberi* (non-pathogenic) or *N. fowleri* (pathogenic), the only two species described at that time. At the time of writing this monograph, 20 *Naegleria* spp. have been fully described. Three strains are given species status here, and a few more descriptions are in preparation (Table 1).

It is mostly due to the use of molecular biology techniques that species descriptions are possible in a genus where morphology is not discriminative. However, this latter statement may now have to be reconsidered. Until recently all vahlkampfiids with dividing flagellates had been classified in genera other than *Naegleria*. It has been found that some amoebflagellates whose flagellates can divide (Dobson *et al.* 1993, B. Robinson personal communication) are in fact *Naegleria* spp. (De Jonckheere and Brown 1995, 1999b). In addition there are two *Naegleria* strains that do not form flagellates under laboratory conditions (De Jonckheere *et al.* 2001), and a few *N. fowleri* strains from one location in France have never formed flagellates. A *Naegleria* strain also exists that fails to form cysts, but this seems to be due to the presence of a bacterial parasite (Michel *et al.* 2000). Infection of other *Naegleria* strains with the parasite impaired their capacity to form cysts. This bacteria did not interfere with the transformation to the flagellate stage. As cyst morphology is informative for identifying amoebflagellate genera, and because the bacteria also infects other genera of amoebae, the investigation of whether the originally-infected amoebflagellate does indeed belong to the genus *Naegleria* is recommended.

In 1988 the definition of the *Naegleria* genus was: these are vahlkampfiids whose flagellate stage normally has two flagella, lacks a cytostome, and does not divide. The cysts have plugged pores through which the amoeba excysts (Page 1988). Although the statement about the plugs in the cysts remains valid the rest of the definition of *Naegleria* should be emended as follows: these are vahlkampfiid amoebae with a temporary flagellate stage in most species, but lacking or difficult to induce in some species and in individual strains of others. Where present, the flagellate stage lacks a cytostome, is usually biflagellate and incapable of division. In at least two lineages, flagellates initially have four flagella and divide once to

Table 1. Species of the genus *Naegleria*

Species	author, year	Max. °C	Flagellates	EMBL*
<i>N. gruberi</i>	Schardinger, 1899, emend. De Jonckheere, this paper	39	+	M18732
<i>N. fowleri</i>	Carter, 1970	45	+	U80059
<i>N. jadini</i>	Willaert and Le Ray, 1973	35	+	-
<i>N. lovaniensis</i>	Stevens, De Jonckheere and Willaert, 1980	45	+	U80062
<i>N. australiensis</i>	De Jonckheere, 1981	42	+	U80058
<i>N. italica</i>	De Jonckheere, Permin, Scaglia and Michel, 1984	42	+	U80060
<i>N. andersoni</i>	De Jonckheere, 1988	40	+	U80057
<i>N. jamiesoni</i>	De Jonckheere, 1988	42	+	U80062
<i>N. clarki</i>	De Jonckheere, 1994	37	+	-
<i>N. galeacystis</i>	De Jonckheere, 1994	35	+	-
<i>N. minor</i>	De Jonckheere and Brown, 1995	38	divide	X93224
<i>N. pussardi</i>	Permin and De Jonckheere, 1996	41	+	-
<i>N. carteri</i>	Dobson, Robinson and Rowan-Kelly, 1997	45	+	Y10189
<i>N. morganensis</i>	Dobson, Robinson and Rowan-Kelly, 1997	44	+	Y10188
<i>N. niuginensis</i>	Dobson, Robinson and Rowan-Kelly, 1997	45	+	Y10186
<i>N. sturti</i>	Dobson, Robinson and Rowan-Kelly, 1997	44	+	Y10185
<i>N. robinsoni</i>	De Jonckheere and Brown, 1999	38	divide	AJ237786
<i>N. fultoni</i>	De Jonckheere, Brown, Dobson, Robinson and Permin, 2001	35	+	AJ243440
<i>N. chilensis</i>	De Jonckheere, Brown, Dobson, Robinson and Permin, 2001	30	-	AJ243442
<i>N. indonesiensis</i>	De Jonckheere, Brown, Dobson, Robinson and Permin, 2001	38	-	AJ243441
<i>N. tihangensis</i>	De Jonckheere, this paper	42	+	-
<i>N. pringsheimi</i>	De Jonckheere, this paper	37	+	-
<i>N. pagei</i>	De Jonckheere, this paper	37	+	-
<i>N. philippinensis</i>	In preparation	40	+	NA
WA variant <i>N. lovaniensis</i>	In preparation	45	+	Y10187
NG597	In preparation	42	+	Y10184
antarctic <i>Naegleria</i> sp.	In preparation	<30	+	ND

* - EMBL accession N° of SSUrDNA

- - not at EMBL, but partial sequences have been published (De Jonckheere 1994a, Permin and De Jonckheere 1996)

NA - not available yet

ND - not done (DNA could not be isolated because of poor growth)

form typical biflagellate cells. *Naegleria* can only be identified to species level by biochemical and molecular techniques.

(De Jonckheere 1982a) or cellulose acetate electrophoresis (Robinson *et al.* 1992), and the bands of enzyme activity were visualized according to procedures published by these authors.

DNA sequence analysis

DNA is extracted from cell pellets using either a phenol-chloroform-isoamyl alcohol method or a guanidium thiocyanate-sarkosyl method (Pitcher *et al.* 1989). The small subunit ribosomal DNA (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and the internal transcribed spacer (ITS) regions, including the 5.8S rDNA, are amplified using primers and polymerase chain reaction (PCR) conditions described by De Jonckheere (1994a, 1998). In preparation for sequencing PCR products were treated with exonuclease I and shrimp alkaline phosphatase for 15 min. at 37°C. After inactivating these enzymes by heating at 80°C for 15 min., the PCR products were sequenced using the Sequenase PCR product sequencing kit (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) using either [³²P]-dATP or [³³P]-dATP. SSU rDNA and ITS amplification and conserved internal primers were used (De Jonckheere 1994a, 1998). Approximately 800 basepairs (bp)

MATERIALS AND METHODS

Culture

Established cultures of *Naegleria* strains are grown either monoxenically on non-nutrient agar plates with *Escherichia coli* (Page 1988) or axenically in a liquid medium (De Jonckheere 1977).

Isoenzyme analysis

Protein extracts are prepared by adding 0.25% Triton X-100 to amoebae concentrated by centrifugation. The suspensions are frozen and thawed several times to make the amoebae burst. For isoenzyme analyses the proteins are separated by agarose gel isoelectric focusing

between two conserved Pst I sites within the *Naegleria* SSU rDNA were sequenced and used for phylogenetic analysis (De Jonckheere 1994a). Sequences of group I introns in the SSU and LSU rDNA are determined using internal rDNA and group I intron primers (De Jonckheere 1993). The nucleotide sequence data reported in this paper are available in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database and the accession numbers are indicated at each species description.

Phylogenetic analysis

The DNA sequences are aligned by eye using the Eyeball Sequence Editor (ESEE) (Cabot and Beckenbach 1989). Phylogenetic trees are constructed from the aligned sequences using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (Neighbor joining and UPGMA), FITCH (Fitch-Margoliash), KITCH (Fitch-Margoliash with evolutionary clock) and SEQBOOT (bootstrapping) programs of the PHYLIP (version 3.572c) package (Felsenstein 1989). For phylogenetic analyses of proteins the PROTPARS and PROTDIST programs of the same package are used.

MOLECULAR BIOLOGY OF THE GENUS *NAEGLERIA*

Chromosomes and ploidy

Naegleria has an intranuclear mitosis, called promitosis, following the classical pattern of chromosome separation, but the chromosomes are too small to be counted by conventional histological techniques (Fulton 1970). However, it has been possible to enumerate the chromosomes with the use of pulsed field gel electrophoresis. The number of chromosomes and their size differ between species and even between strains of the same species. Two strains of *N. gruberi sensu lato* have 23 chromosomes, but the size of some chromosomes differ (Clark *et al.* 1990). These two strains are considered now to belong to two different species, *N. gruberi sensu stricto* and *N. pringsheimi* (see below). Within the species *N. fowleri* differences in number and size of chromosomes are observed with different isolates (De Jonckheere 1989).

The ploidy of the *Naegleria* genome is still not known. The sum of the chromosome sizes (approximately 19 Mb) does not equal the expected genome size (approximately 104 Mb), which indicates that *Naegleria* might be polyploid (Clark 1990). It has been demonstrated that differences in ploidy exist between strains (Fulton 1993), and isoenzyme studies of *Naegleria* spp. usually imply diploidy (Cariou and Pernin 1987, Adams *et al.* 1989). Isoenzyme studies also reveal that genetic exchange occurs in *N. lovaniensis* but not in other

species (Pernin *et al.* 1992). Of course, genetic recombination does not mean sexuality, which involves meiosis to form monoploid cells, and karyogamy. It has been argued that the flagellates of *Naegleria* are gametes (Fulton 1993) and the fact that in some *Naegleria* spp. the flagellates divide once (De Jonckheere and Brown 1995) could be in support of monoploidy formation. However, meiosis in *Naegleria* has not been proven experimentally.

rDNA plasmid

In *N. gruberi*, the rRNA genes are carried exclusively on a 14-kp circular plasmid, and each plasmid contains only one rDNA repeat unit (Clark and Cross 1987). The number of rDNA circles per cell was estimated to be 4,000. This circular plasmid is a general feature of the rDNA genes in all the vahlkampfiids (Clark and Cross 1988a). The length of the rDNA plasmid varies according to the species and strain investigated. It is not known whether different numbers of rDNA repeats per plasmid, as was found in the anaerobic *Entamoeba histolytica* (Bhattacharya *et al.* 1998), contribute to the plasmid length differences. Length differences in the ribosomal genes themselves are mainly due to the presence of group I introns. The SSU rDNA of several species carry these introns (De Jonckheere 1994b). Length differences in the ITS1 and/or ITS2 also contribute to repeat unit size variability (De Jonckheere 1998). In a few *Naegleria* strains, group I introns are also present in the LSU rDNA (De Jonckheere and Brown 1998a, 2001).

The rDNA plasmid of *N. gruberi* strain EG_B has been completely sequenced. The molecule is 13,996 bp in length with an overall G+C content of 40.7% (Mulligan, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA:1-163,1995). A putative open reading frame (ORF) for a heat shock protein is detected in the non-ribosomal sequence (NRS) of the *N. gruberi* plasmid (Mulligan and Tracy 1993). No similarities were observed in the NRS between *N. fowleri* (strain LEE) and *N. gruberi* (strain NG_B).

Another plasmid has been detected in *N. minor* (De Jonckheere and Brown 1995). The function of this 6.0 kb plasmid is unknown but it seems not to be involved in flagellate division as it is not found in *N. robinsoni*, currently the only other *Naegleria* species with dividing flagellates.

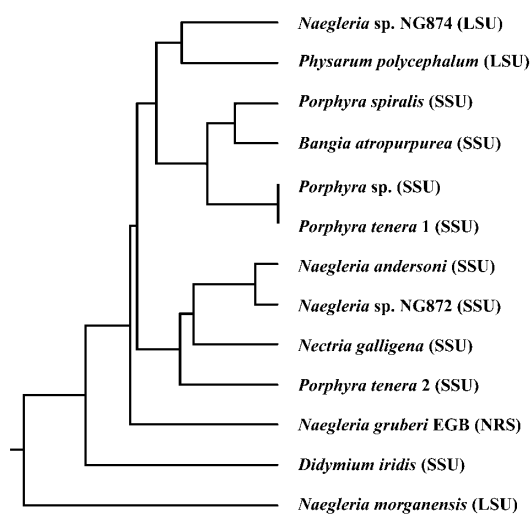


Fig. 1. Phylogenetic tree inferred from the amino acid alignments of the His-Cys box in the ORF of the group I introns

Group I introns

Group I introns are catalytic RNA molecules that occur within transcribed sequences and are able to self-excise. The group I intron in the SSU rDNA of *Naegleria* spp. is a twintron (Einvik *et al.* 1998), consisting of two distinct ribozymes (catalytic RNAs) and an ORF encoding a homing endonuclease with a His-Cys box (Johansen *et al.* 1993). Endonucleases with His-Cys boxes are uncommon (Johansen *et al.* 1997). A similar twintron has only been found in the myxomycete *Didymium* (Einvik *et al.* 1998). In one *Naegleria* lineage the twintron has lost the ribozyme that carries the endonuclease (De Jonckheere and Brown 1994). The group I introns in the LSU rDNA of *Naegleria* either carry an endonuclease or do not (De Jonckheere and Brown 1998a, 2001).

In the genus *Naegleria* the group I intron seems to be transferred vertically in the SSU rDNA (De Jonckheere 1994b) and horizontally in the LSU rDNA (De Jonckheere and Brown 1998a, 2001). From this it is inferred that the SSU rDNA group I intron was acquired in an ancestral state and lost in most of the *Naegleria* spp. In all described *Naegleria* spp. with a group I intron in the SSU rDNA, the presence of this intron is a property of the species. Only the WA variants of *N. lovaniensis*

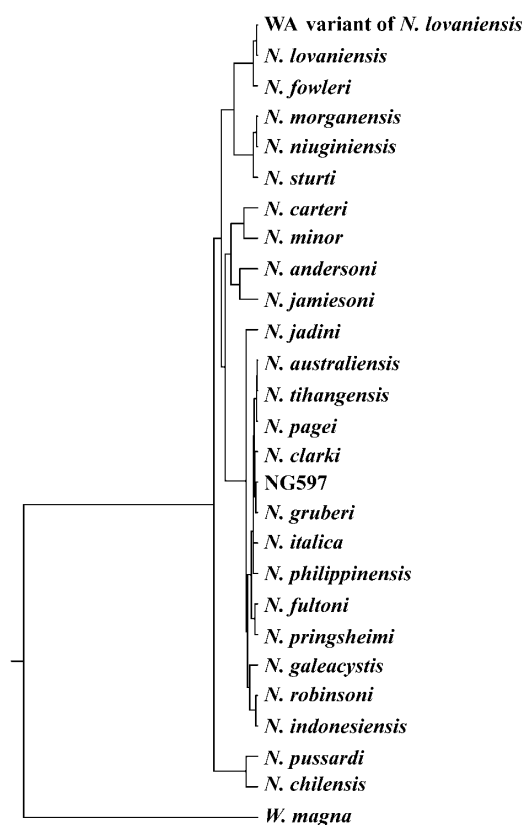


Fig. 2. Phylogenetic tree inferred from partial SSU rDNA of species in the genus *Naegleria* and its closest relative *Willaertia magna*

could be exception to this rule. An ORF with approximately 30% identity to the ORF in the SSU rDNA group I intron of *N. pringsheimi* has been found in the NRS of the rDNA plasmid of strain EGB_b of *N. gruberi* (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA: 1-163, 1995). It is transcriptionally silent and may be a remnant of the group I intron that was lost from the SSU rDNA of *N. gruberi*. The His-Cys box is still present in the ORF in strain EGB_b, but comparison with His-Cys boxes in the SSU rDNA introns of other *Naegleria* spp. shows it is phylogenetic distinct (Fig. 1).

It may have evolved faster because it is silent and thus under different constraint, or it was introduced horizontally from another organism.

Standard introns

Standard introns are not prevalent in any of the structural genes sequenced to date in *Naegleria*, except for two introns present in the calcineurin B gene (EMBL accession N° U04380). The introns are 258 and 55 bp long, respectively, and flanked by characteristic splice junction sequences (Remillard *et al.* 1995). *Naegleria* might be the earliest branching eukaryote known to contain canonical introns in only one gene. Together with the fact that this is the first gene encoding calcineurin B found in an organism other than metazoa and yeast, one wonders whether this gene might have been acquired by horizontal transmission.

mtDNA

Recently, the circular 49,843 bp mitochondrial (mt) DNA of strain NEG-M of *N. gruberi sensu stricto* has been sequenced and the sequence is available at EMBL under accession N° AF288092. Non-coding sequences amount to only 8%. Two genes have been identified that have previously only been detected in jakobid mtDNA (Lang *et al.* 1999). The *Naegleria* mtDNA seems to share an evolutionary history with that of the jakobids, even though the mtDNA in the latter is linear. It will be interesting to see whether the *Naegleria* mtDNA show other peculiarities, such as RNA editing as found in transfer RNAs in mtDNA of *Acanthamoeba* (Lonergan and Gray 1993).

Pyrophosphate-dependant phosphofructokinase

From isoenzyme studies it is known that several glycolytic enzymes are present in *Naegleria*. Few have been studied in detail probably because it has been assumed that these enzymes are rather similar in all eukaryotes. However, *Naegleria* seems to be one of the eukaryotes that have a pyrophosphate-dependant phosphofructokinase (PPi-PFK), and shares this property with the amoeba *Entamoeba*, the flagellates *Trichomonas*, *Tritrichomonas* and *Giardia*, the ciliate *Isotricha* and the apicomplexa *Toxoplasma*, *Eimeria* and *Cryptosporidium*. The *Naegleria* PPi-PFK is regulated by AMP, a property that distinguishes it from all of its counterparts, which are either unregulated or stimulated by fructose 2,6-biphosphate (Mertens *et al.* 1993). The enzyme was investigated both in *N. fowleri* and in

a strain of *N. gruberi sensu lato*. The cloned PPi-PFK gene of *N. fowleri* has been sequenced (EMBL accession N° U11733) and the expressed protein had the same properties (Wessberg *et al.* 1995) as the native protein.

OVERVIEW OF SPECIES

Fulton (1993) has argued strongly against naming too many *Naegleria* spp. He reasoned that we might end up with “more than two-dozen species of *Naegleria*, many identifiable only by isoenzyme patterns, a level of obscurity so wonderful that everyone would wisely ignore those of us who worked on these impossibly classified organisms”. Fulton used *N. gruberi* as an example of a species that is an easily recognizable species by conventional criteria. Unfortunately, he did not say what these criteria were, but at the same time he admitted that *N. gruberi* is a polyglot assembly of diverse isoenzyme types. Page (1988) called *N. gruberi* “intolerably heterogeneous on detailed study”. It is this heterogeneity of a single species that will scare people away from studying the organism, unless everybody works on the same strain, i.e. EG.

The heterogeneity in *N. gruberi* is really not so surprising. Until recently, most *Naegleria* isolates that didn't fit into the few described *Naegleria* spp. were called *N. gruberi*. Fortunately, new isolates which are not further typed are now called *Naegleria* sp. instead of *N. gruberi*.

Several investigations have indicated that *N. gruberi* is in fact a complex of at least 10 species (De Jonckheere 1982a, Adams *et al.* 1989, Clark *et al.* 1989, Pernin and Cariou 1989, Robinson *et al.* 1992). *N. gruberi* strains fall into distinct clusters, separated by genetic distances similar to those separating the better-characterised taxa *N. fowleri*, *N. lovaniensis*, *N. jadini*, *N. australiensis*, *N. italica*, *N. andersoni* and *N. jamiesoni* (Robinson *et al.* 1992). Allozymes and rDNA sequences give the same conclusions for delineating *Naegleria* spp. (Dobson *et al.* 1997, De Jonckheere and Brown 1997). In the present monograph, the four clusters that have been recognized previously in *N. gruberi* (Clark *et al.* 1989), are given separate species status. Clearly new species names are needed rather than lumping everything under *N. gruberi*.

There is a good reason for naming new species. When the species name is given one knows already some of the characteristics of the strain under study, for

example its pathogenicity, thermotolerance, capacity to transform into flagellates and whether the latter are able to divide. I strongly support the notion that, apart from the species name, the strain identification number should always be given (Fulton 1993) as interstrain differences exist. It is obvious that different clonotypes can evolve (Fulton 1993), but these clonotypes never form different clusters in allozymes-derived trees. Slight allozyme differences between strains of the same species have been demonstrated in *N. fowleri* (Pernin *et al.* 1985, De Jonckheere 1987, Adams *et al.* 1989), *N. lovaniensis* (De Jonckheere 1982a, Pernin *et al.* 1985, Adams *et al.* 1989, Robinson *et al.* 1992), *N. australiensis* (De Jonckheere *et al.* 1984, Pernin *et al.* 1985, Adams *et al.* 1989, Robinson 1992), *N. andersoni* (De Jonckheere 1988a, Robinson 1992), *N. jamielsoni* (De Jonckheere 1988a, Robinson 1992), *N. sturti* and *N. carteri* (Dobson *et al.* 1997), but these are much smaller than those between different species and therefore, creating a subspecies would be invalid (see Rivera *et al.* 1990, De Jonckheere 1994a).

Since 1994 I have based species delineation on SSU rDNA sequences (De Jonckheere 1994a) because not only are the differences easier to quantify, but they also allow more distantly-related organisms to be compared than when using isoenzyme differences. SSU rDNA sequences have been used for delineating species of other protists such as *Giardia* and *Cryptosporidium* (Thompson *et al.* 2000, Xiao *et al.* 2000). rDNA sequencing provides unambiguous data which is reproducible between laboratories. However, sequencing is also prone to errors. Generally, small sequencing errors will not influence the phylogenetic identification of a strain under study to a high degree, but if errors become numerous they could influence species identifications, especially in cluster 5 (Fig. 2).

Restriction fragment length polymorphism (RFLP) analysis of PCR amplified ribosomal DNA, or riboprinting, produces similar phylogenetic trees as SSU rDNA sequencing (De Jonckheere 1994c) and constitutes less work. However, the latter is less accurate because in riboprinting less information is obtained. Also the treebuilding can be biased by the presence of group I introns (De Jonckheere 1994b). Riboprinting the LSU rDNA is preferred (De Jonckheere 1994c) as in the genus *Naegleria* group I introns are found infrequently in the LSU rDNA (De Jonckheere and Brown 1998a, 2001) compared to the SSU rDNA. Sequence analysis

of ITS1, 5.8S rDNA and ITS2 has confirmed the species delineation and also aids finetuning the descriptions (De Jonckheere 1998).

Eight species descriptions in the *Naegleria* genus are based on only one isolate, while different strains of three species came from the same place. The "one strain" species are *N. jadini*, *N. pussardi*, *N. niuginensis*, *N. galeacystis*, *N. minor*, *N. robinsoni*, *N. chilensis* and *N. indonesiensis*. The three species with strains from only one place are *N. italica*, *N. morganensis* and *N. fultoni*. Therefore, the variability within these species is unknown. For all these species, data on the rDNA sequences, especially ITS sequences, from different strains are needed to assess the variability within the species. A recent isolate of *N. italica* from Australia shows indeed ITS2 variation in this species (Henderson *et al.* 2001). Until now, only *N. fowleri* has been thoroughly studied, and a great deal of variability in ITS1 sequence was detected (De Jonckheere 1998).

Thermophilic species

The genus *Naegleria* comprises pathogenic and non-pathogenic species. All pathogenic species are thermophilic but not all thermophilic species are pathogens. Thermophilic is defined here as the ability to grow at 40°C or higher. It is important to know one is working with a pathogenic species as special precautions have to be taken.

It had been observed that *N. fowleri* can grow at temperatures up to 45°C while nonpathogenic *Naegleria* spp. did not tolerate such a high temperature (Griffin 1972). This approach was used in the routine testing of water samples for *N. fowleri* where concentrated water samples were incubated at temperatures between 42°C and 45°C in an attempt to suppress the growth of other amoebae. When using this isolation method it was soon realized that more *Naegleria* strains than those belonging to *N. fowleri* can grow at these high temperatures. There are currently 12 named thermophilic *Naegleria* spp. (Table 1). The term thermophilic is arbitrarily chosen with some using the borderline of 42°C (Robinson *et al.* 1992) instead of 40°C (De Jonckheere 1988a). In addition, differences in thermophilic growth temperatures have been noted for the same strain (see below).

Pathogenic thermophilic species

Besides the human pathogen *N. fowleri*, also *N. australiensis* and *N. italica* kill experimental ani-

mals. However, no human infection due to these two other pathogenic *Naegleria* spp. has been diagnosed to date. Although the amoeba was found not to be responsible for the disease, *N. lovaniensis* has been found as a contaminant in the cerebrospinal fluid (CSF) of a patient in Mexico (Rivera *et al.* 1989).

About 15 years ago a *Naegleria* was isolated from human CSF. It will be described as a new species. (see *N. philippinensis*). I have tested the pathogenicity of the latter isolate recently in experimental animals and no mice were killed. Therefore, it is important to discuss virulence and pathogenicity tests. In some of the species that are described as nonpathogenic, positive mouse tests have been reported.

In some of the pathogenicity tests with nonpathogenic variants of *N. fowleri*, later described as *N. lovaniensis*, one out of five mice died after intranasal (IN) inoculation but amoebae could not be recovered from the brain (De Jonckheere and van de Voorde 1977b). Virulence could not be induced in these strains, in contrast to strains of *N. fowleri* with attenuated virulence (De Jonckheere 1979b), and it was concluded *N. lovaniensis* is a nonpathogenic species. In the USA a *Naegleria* isolated at an incubation temperature of 42°C killed one mouse out of three and was identified on this basis as being *N. australiensis* (John and Howard 1995). However, this isolate (EPA-741) was later identified as *N. lovaniensis* by immunofluorescence (John *et al.* 1998) and this identity was confirmed by isoenzyme analysis (De Jonckheere unpublished). This isolate should not be considered a pathogenic strain of *N. lovaniensis*, not only because only one mouse died, but because it also died after one day (John and Howard 1995), which is too short a time after IN instillation for the amoeba to be the cause of death. In addition, subsequent inoculations with isolate EPA-741 did not kill mice (John *et al.* 1998). As a result, *N. lovaniensis* should still be considered a nonpathogenic species.

Also one mouse died after the first intracerebral (IC) inoculation with the type strain of *N. jadini*, but subsequent attempts to prove pathogenicity of the strain remained unsuccessful. It was concluded that the presumed pathogenicity was probably due to contaminating bacteria and fungi, which is highly possible after IC inoculation (Willaert and Le Ray 1973).

Two mice each out of six were killed after IN inoculation with strains RU30 and RU42 (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of

Science thesis, Adelaide, 1-48, 1975). These *Naegleria* isolates were later described as *N. clarki*. Subsequent attempts with amoebae isolated from the mouse brain continued to kill some mice with strain RU30, but the results didn't show an increased virulence as found for strain PP397, which was later described as pathogenic *N. australiensis*. Pathogenicity tests with strains RU30 and RU42 appeared to remain negative on later attempts (Willaert 1976) and it was concluded *N. clarki* is a nonpathogenic species (De Jonckheere 1994a), although *N. clarki* is closely related to the pathogenic *N. italica*.

Some *Naegleria* isolates from a swimming pool in Czechoslovakia did kill a few mice soon after isolation, but lost that capacity upon subculture (Kadlec 1981). From the serological tests it can be deduced that some might have been *N. lovaniensis* but that some of the isolates belonged to other species.

Because of the difficulty in interpreting some of the pathogenicity results the Culture Collection of Algae and Protozoa (CCAP) decided to put a warning of "possible pathogen" on all *Naegleria* spp. At the American Type Culture Collection (ATCC) a public health permit is necessary only for *N. fowleri*, *N. australiensis* and *N. italica*.

Naegleria fowleri Carter, 1970

Both in the USA and in Australia reports were published in 1968 on the isolation of a *Naegleria* sp. from human CSF which was different from all the known *N. gruberi* strains (Butt *et al.* 1968, Callicott *et al.* 1968, Culbertson *et al.* 1968, Carter 1968). At that time, pathogenicity had only been proven experimentally in free-living amoebae belonging to the genera *Hartmannella* - *Acanthamoeba*, so similar cases had been previously attributed to *Hartmannella* - *Acanthamoeba*. At that time confusion existed over the genera *Hartmannella* and *Acanthamoeba*. In all older literature where *Hartmannella* is indicated as a human pathogen it probably refers to *Acanthamoeba*. Because the amoebae could not be isolated from CSF of these first human cases they could not be studied in detail.

In 1970 Carter distinguished the human pathogenic *Naegleria* from the common *N. gruberi* and named it after M. Fowler who first described the disease in Australia (Carter 1970). This species distinction was based exclusively on morphological differences and the pathogenicity of the isolates. The separate species status of the pathogen was later confirmed using serological,

biochemical and molecular techniques. The human pathogenic *Naegleria* sp. has also been called *N. aerobia* (Singh and Das 1970) but the use of the name, mainly in India, has been abandoned. The junior synonym *N. invadens* (Chang 1971) has also disappeared rapidly from the literature.

The disease PAM occurs worldwide and very few people survive an infection with *N. fowleri*. Only Amphotericin B seems to be really effective in curing the disease if given at a very early stage of the infection. The best documented cases of successful treatment occurred in California (Seidel *et al.* 1982) and in Australia (Anderson and Jamieson 1972). There are some additional reports from Hong Kong, Thailand, Italy, England and Mexico, but there is doubt whether these patients really suffered from PAM.

Mice are the preferred animals for testing the pathogenicity of *Naegleria* isolates. Although some differences in susceptibility to *N. fowleri* infections are observed between different mice breeds, the age of mice is more important (De Jonckheere 1979b). The amoebae preferentially migrate to the brain where they multiply to high numbers while eating the tissue. However, in experimental infections of mice pneumonitis and hepatitis are also observed with masses of amoebae present in the tissues. Splenitis occurs frequently, while amoebae are often found in glomular capillaries as well (Carter 1970). However, kidney and liver transplants from a 11 year old donor who had died of a *N. fowleri* infection did not induce infection in three different organ recipients (Kramer *et al.* 1997). Also, guinea pigs (Philips 1974), old world monkeys (Wong *et al.* 1975), sheep (Simpson *et al.* 1982), rabbits (Smego and Durack 1984), squirrels, cotton rats and muskrats (John and Hoppe 1990) are susceptible to experimental infection with *N. fowleri*. On the other hand, cottontail rabbits, opossums and raccoons seem to be not susceptible (John and Hoppe 1990). A natural *N. fowleri* infection has been detected in a South American tapir (Lozano-Alarcon *et al.* 1997).

Upon isolation pathogenic strains of *N. fowleri* are highly virulent, but tend to lose virulence after longterm axenic culturing (Wong *et al.* 1977, De Jonckheere 1979b). In reports of *N. fowleri* strains with low virulence immediately after isolation (Kadlec 1981) the isolates were probably strains of *N. australiensis* because, contrary to *N. fowleri* strains, they were difficult to axenise and their temperature optimum decreased

rapidly after isolation. On the other hand, *N. italica* was first reported as being most probably an isolate of *N. fowleri* (Scaglia *et al.* 1983). Contrary to other *Naegleria* spp., strains of *N. fowleri* adapt easily to an axenic medium, which makes this a quick method for separating this pathogen from other *Naegleria* spp. (De Jonckheere 1977). Other *Naegleria* spp. can be adapted only slowly to axenic growth. A chemically-defined medium has been described in which *N. fowleri* can be cultured (Nerad *et al.* 1983).

Most strains of *N. fowleri* transform into flagellates, but several strains isolated in France could never be induced to transform (De Jonckheere *et al.* 2001). There has been a report of dividing *N. fowleri* flagellates in a brain infection (Clavel *et al.* 1996), however, the isolated strain turned out not to be a *Naegleria* sp. but some kind of *Platyamoeba* and is considered to be a contaminant (De Jonckheere and Brown unpublished). The photographs of the organism that was purportedly present in the CSF of this case, showed the morphology and the typical axostyle of a *Trichomonas* sp. (De Jonckheere unpublished), which explains why the flagellates were seen to divide. In the meantime, another case of *Trichomonas* meningoencephalitis has been reported (Okamoto *et al.* 1998).

Restriction endonuclease digestion of whole-cell DNA (De Jonckheere 1988a) reveals differences between *N. fowleri* strains from different places, differences that are confirmed by isoenzymes, random amplified polymorphic DNA (RAPD) and ITS sequences analyses. The most clearcut divide is found between the Australian-Asian strains and those from other regions. A point mutation is found in the conserved 5.8S rDNA of these two different *N. fowleri* lineages (De Jonckheere 1998). Further differentiations of *N. fowleri* strains can be made by using the different lengths found in the ITS1 (Table 2) (De Jonckheere 1998, Pélandakis *et al.* 2000).

Other variations in *N. fowleri* strains have been observed. While DNA RFLP made similar distinctions between strains of different continents, a *N. fowleri* isolate from a surviving patient showed the most different DNA RFLP (De Jonckheere 1987), and the strain showed swellings of the flagella that looked like paddles, not found in any other *Naegleria* strain (John *et al.* 1991).

The ITS1, 5.8S rDNA and ITS2 sequences of different *N. fowleri* strains are available at EMBL under accession numbers X96561 till X96567, while the SSU

Table 2. Length in bp of ITS1, 5.8S rDNA and ITS2 in different *Naegleria* spp.

Cluster	Species	Strain	ITS1	5.8S rDNA	ITS2	Total	EMBL	
1	<i>N. fowleri</i>	KUL	86	175	106	367	X96561	
		LEE	86	175	106	367	X96562	
		M4E	142	175	106	423	X96563	
		AR12	42	175	106	323	X96564	
		Northcott	84	175	106	365	X96565	
		Mst1	84	175	106	365	X96566	
		J/16/1/42E	84	175	106	365	X96567	
		<i>N. lovaniensis</i>	Aq/9/1/45D	41	175	103	319	X96568
	WA variant	F9	41	175	103	319	X96569	
		NG872	36	175	103	314	Y10191	
	2	<i>N. morganensis</i>	NG236	34	175	226	435	Y10192
<i>N. sturti</i>		NG334	35	175	118	328	Y10195	
<i>N. niuginiensis</i>		NG427	46	175	117	338	Y10193	
3	<i>N. carteri</i>	NG055	34	174	100	308	Y10197	
	<i>N. minor</i>	WTO43	30	175	528	738	X96577	
4	<i>N. andersoni</i>	A2	35	174	100	309	X96572	
	<i>N. jamiesoni</i>	T56E	34	174	100	308	X96570	
5	<i>N. australiensis</i>	PP397	33	175	100	308	X96573	
		LSR34	33	175	100	308	AJ132034 ^b	
	<i>N. philippinensis</i>	RJTM	33	175	105	313	-	
	<i>N. italica</i>	AB-T-F3	33	175	162	370	X96574	
	<i>N. clarki</i>	RU30	33	175	201	409	X96575	
	<i>N. fultoni</i>	NG885	33	175	106	314	AJ243445	
	<i>Naegleria</i> sp.	NG597	33	175	115	323	Y10194	
	<i>N. gruberi</i>	EG _B	33	175	114	322	^a	
		AUD1	33	175	114	322	AJ132031 ^b	
		DRI	33	175	114	322	-	
	<i>N. tihangensis</i>	T2A	33	175	102	310	-	
		NG202	33	175	102	310	-	
	<i>N. pringsheimi</i>	CCAP1518/1D	33	175	134	342	-	
	<i>N. pagei</i>	CCAP1518/1E	33	175	165	373	-	
		CCAP1518/1F	33	175	153	361	AJ132022 ^b	
	subcluster	<i>N. jadini</i>	O400	35	175	>519	>729	X96576
<i>N. galeacystis</i>		AV500	33	175	181	389	X96578	
<i>N. indonesiensis</i>		NG945	33	175	182	390	AJ243444	
<i>N. robinsoni</i>		NG944	33	175	176	384	AJ237787	
6		<i>N. pussardi</i>	EDF258	38	174	92	304	X96571
		<i>N. chilensis</i>	NG946	152	175	129	456	AJ243443

Results are from my laboratory except for ^a (Mulligan 1995) and ^b (Pélandakis *et al.* 2000)

rDNA sequence of strain MCM is under number U80059. The partial SSU rDNA sequence of strain KUL has been published (De Jonckheere 1994a).

The type strain Nf66 of *N. fowleri* is available from ATCC (N° 30214) together with other *N. fowleri* isolates from all over the world.

Naegleria australiensis De Jonckheere, 1981

The type strain of *N. australiensis* (PP397) was originally isolated from water in Australia (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975). It is virulent for

Table 3. Summary on the presence and length (in nt) of group I introns in the nuclear rDNA of the genus *Naegleria*

Cluster	Species	SSU rDNA	EMBL	LSU rDNA	EMBL
1	<i>N. fowleri</i>	-	-	-	-
	<i>N. lovaniensis</i>	-	-	-	-
	WA variant NG874	+ (+ ORF) ^a	-	869 (+ ORF)*	AJ271406
	WA variant NG872	1318 (+ ORF)	AJ001399	474 (- ORF)	AJ001316
	WA variant NG881	-	-	+	-
2	WA variant NG876	-	-	-	-
	<i>N. morganensis</i>	-	-	389 (- ORF) \$	AJ001314
3	<i>N. sturti</i>	-	-	919 (+ ORF)	AJ001315
	<i>N. niuginensis</i>	-	-	-	-
	<i>N. carteri</i>	+1324(+ ORF)	Y10190	-	-
4	<i>N. minor</i>	-	-	-	-
	<i>N. andersoni</i>	1309 (+ ORF)	X78280	-	-
5	<i>N. jamiesoni</i>	1307 (+ ORF)	X78279	-	-
	<i>N. australiensis</i>	-	-	-	-
	<i>N. philippinensis</i>	1297 (+ ORF)	-	-	-
	<i>N. italica</i>	1319 (+ ORF)	X78277	-	-
	<i>N. clarki</i>	1305 (+ ORF)	X78281	-	-
	<i>N. fultoni</i>	-	-	-	-
	<i>N. gruberi</i>	-	-	-	-
	<i>N. tihangensis</i>	-	-	-	-
	<i>N. pringsheimi</i>	1316 (+ ORF)	X78278	-	-
	<i>N. pagei</i>	-	-	-	-
subcluster	<i>N. jadini</i>	-	-	-	-
	<i>Naegleria</i> sp. NG597	375 (- ORF)	X79070	-	-
	<i>N. galeacystis</i>	-	-	-	-
	<i>N. indonesiensis</i>	-	-	-	-
	<i>N. robinsoni</i>	-	-	-	-
6	<i>N. pussardi</i>	-	-	-	-
	<i>N. chilensis</i>	-	-	-	-

* - at slightly different location from in NG872, \$ - different group I introns at different locations in the same strain, ^a - present but sequence not determined

mice but the virulence is lower than that of *N. fowleri*. Generally strains of *N. australiensis* kill fewer animals and require a longer incubation time (De Jonckheere *et al.* 1983a). Moreover, *N. australiensis* loses virulence more quickly than *N. fowleri* in axenic culture (De Jonckheere 1981), probably due to this lower virulence. Therefore, a negative virulence test does not indicate the strain under investigation does not belong to *N. australiensis*. The species differs antigenically (De Jonckheere 1981) from *N. fowleri* and can be separated by allozyme (De Jonckheere 1982a) and DNA (De Jonckheere 1994a) analyses. Intraspecies differences in allozyme and whole rDNA plasmid restriction patterns are observed and the latter indicated that this species might have a European origin (Clark *et al.* 1989). However, the ITS1, 5.8S rDNA and ITS2 sequences of strain PP397 from Australia (De Jonckheere 1998) are

exactly the same as those of strain LSR34 from France (Pélandakis *et al.* 2000). Therefore, the difference in whole rDNA plasmid restriction patterns observed between those two strains must be due to sequence differences in the NRS of the plasmid.

The maximum temperature tolerated for growth by this species is 42°C, which is 3°C lower than for *N. fowleri*. Strains of *N. australiensis* occur worldwide in warm waters and a strain of *N. australiensis* has been isolated from the brain of a fish (Dykova *et al.* 2001) but they have never been isolated from a human being. This species is found very frequently in cooling waters in Belgium (De Jonckheere unpublished) because the incubation of concentrated water samples at 44°C allows the isolation of species that only tolerate 42°C. The exact reason for this is unknown, but it could be that the strains are adapted to higher temperatures in

the environment or because the interior of the agar plates does not attain 44°C during the short incubation time for isolation. It has been reported that the upper temperature tolerance limits of described *Naegleria* spp. vary narrowly (<2°C) between many isolates (Robinson *et al.* 1996a). Some strains of *N. fowleri* reported to have low virulence upon isolation, with a temperature optimum that rapidly decreases after isolation (see above) and which also do not adapt readily to axenic growth (Kadlec 1981) might actually belong to the species *N. australiensis*.

The ITS1, 5.8S rDNA and ITS2 sequences of strain PP397 and LSR34 are available at EMBL under accession number X06573 and AJ132034 respectively, while the SSU rDNA sequence of strain PP397 is under U80058. The partial SSU rDNA sequence of strain PP397 has also been published (De Jonckheere 1994a).

Strains of *N. australiensis* were adapted to axenic growth. A chemically-defined medium has been described in which *N. australiensis* can be cultured. This is a modification of the one in which *N. fowleri* and *N. lovaniensis* are grown (Nerad *et al.* 1983). The type strain PP397 is available from ATCC (N° 30958), as are three additional strains from France, Australia and the USA, respectively. The strain from the USA was isolated from kidney tissue of a goldfish and submitted to my laboratory for typing (De Jonckheere unpublished).

Naegleria italica De Jonckheere, Pernin, Scaglia and Michel, 1984

When *N. italica* was isolated for the first time it was reported as probably being a strain of *N. fowleri* (Scaglia *et al.* 1983). Because of high crossreaction with antiserum against *N. australiensis* it was subsequently described as a subspecies of the latter (De Jonckheere *et al.* 1984). However, allozyme studies suggested that the subspecies deserved species level rank (Adams *et al.* 1989), and the genetic distance between the SSU rDNA sequences of the subspecies are comparable to those between other *Naegleria* spp. (De Jonckheere 1994a). Therefore the subspecies of *N. australiensis* were given species status. In contrast with *N. australiensis* (Table 3), *N. italica* has a group I intron in the SSU rDNA (De Jonckheere 1993). Although the pathological findings in mice are the same as for *N. australiensis*, *N. italica* is much more virulent.

The maximum temperature tolerated for growth by *N. italica* is 42°C and strains of *N. italica* have been adapted to axenic growth. Until recently, this species has

never been isolated from anywhere other than the spa in Italy where it was originally found and continued to be detected on subsequent occasions (Scaglia *et al.* 1987). In February 2000, two strains isolated from an artificial water body in Western Australia have been identified as *N. italica* on the basis of nearly identical allozyme profiles (Henderson *et al.* 2001). The 5.8S rDNA with flanking ITS sequences gave 98% identity with the published sequence for *N. italica*. The Australian strains have the same insert in the ITS2 that is typical for *N. italica*, but it is in this stretch that the sequence divergence with the type strain is observed. Based upon the extensive sampling for *Naegleria* spp. in Australia, *N. italica* seems to be a rare organism on that continent as well.

The ITS1, 5.8S rDNA and ITS2 sequences of strain AB-T-F3 are available at EMBL under accession number X96574, while the partial SSU rDNA sequence is under U80060. The sequence of the SSU rDNA group I intron of strain AB-T-F3 is available at EMBL under accession number X78277. The partial SSU rDNA sequence of strain AB-T-F3 has also been published (De Jonckheere 1994a).

The type strain AB-T-F3, relabeled SWL NG-073, is available from ATCC (N° 50347).

Nonpathogenic thermophilic species

Most *Naegleria* strains isolated from the environment are nonpathogenic. It is important to identify them and find out whether they could be indicators for the presence of the pathogenic species.

Naegleria lovaniensis Stevens, De Jonckheere and Willaert, 1980

This species was described initially as a nonpathogenic variant of *N. fowleri* (De Jonckheere and van de Voorde 1977b) because the amoebae reacted positively with antiserum against the pathogenic *N. fowleri*, yet these isolates were not pathogenic. There have been some reports on positive pathogenicity tests with *N. lovaniensis* strains, but the amoebae were probably not the cause of death (see above). More detailed studies showed that, although antigenically being the closest relative, *N. lovaniensis* differs from *N. fowleri* in many more aspects than by virulence alone. Therefore, it was given separate species status (Stevens *et al.* 1980). Also, in phylogenetic analysis based on SSU rDNA sequences, *N. lovaniensis* is the closest relative of *N. fowleri* (De Jonckheere 1994a). In contrast to

N. fowleri, no length variation was found in the ITS1 of five *N. lovaniensis* strains investigated (De Jonckheere 1998).

Strains of *N. lovaniensis* have been found worldwide leading to the hypothesis of a common origin and recent dispersion throughout the world (Pernin *et al.* 1992). The *N. lovaniensis tarasca* subspecies and purepecha variant of *N. lovaniensis* from Mexico (Rivera *et al.* 1990) fall within the variability of zymograms observed in the species and, therefore, are invalid names (De Jonckheere 1994a). The particular nucleolar morphology in these Mexican strains had been observed repeatedly in one natural population of *N. lovaniensis* in Australia. Evidence has been presented for genetic recombination in *N. lovaniensis* (Cariou and Pernin 1987, Pernin *et al.* 1992) but genetic exchange could not be proven in other *Naegleria* spp. investigated, such as *N. fowleri*, *N. australiensis* and *N. gruberi sensu lato* (Pernin and Cariou 1997).

A fast method to separate *N. lovaniensis* from *N. fowleri* isolates is the use of a liquid axenic medium, in which only *N. fowleri* grows immediately to high numbers, while *N. lovaniensis* needs a lot of time to adapt (De Jonckheere 1977). Also, other *Naegleria* spp. can be grown in this medium but only after long adaptation, by each week decanting the medium and adding fresh medium to the tube. A chemically-defined medium has been described in which *N. lovaniensis* can be cultured; a property it shares with *N. fowleri* (Nerad *et al.* 1983).

The ITS1, 5.8S rDNA and ITS2 sequences of strains Aq/9/1/45D and F9 are available at EMBL under accession numbers X96568 and X96569, while the SSU rDNA sequence of strain C-0490 is under U80062. The partial SSU rDNA sequence of strain Aq/9/1/45D has also been published (De Jonckheere 1994a).

Strains LvH1 (30811), TS (30569) and K-1 (30467) of *N. lovaniensis* are present in ATCC although they had originally been submitted under different species names. The three strains were isolated in Belgium, the USA and Australia, respectively.

Naegleria andersoni De Jonckheere, 1988

Naegleria andersoni is a thermophilic species defined on the basis of isoenzyme patterns and rDNA sequences (De Jonckheere 1988a). It is closely-related to *N. jamiesoni*, which was originally described as a subspecies of *N. andersoni*. A group I intron is found in the SSU rDNA of six strains investigated of both species (De Jonckheere 1993). The combined ITS1, 5.8S rDNA

and ITS2 PCR product of both species have a similar length in all six strains (De Jonckheere 1998) but the sequence of only one strain of each species was determined. The ITS1 seems to be 1 bp longer in *N. andersoni* than in *N. jamiesoni* (Table 2), but in the combined ITS1, 5.8S rDNA and ITS2, 17 bp differences exist between the two species. The 5.8S rDNA in both *N. andersoni* and *N. jamiesoni* is 1 bp shorter than in the majority of *Naegleria* spp., a character they share with *N. carteri* and *N. pussardi*.

Strains of *N. andersoni* were first isolated in Australia (strains A2 and PPMFB6 in Willaert 1976) and later in Belgium from water associated with imported fish from Malawi, Singapore, Nigeria and Brazil (De Jonckheere 1988a). Strains of *N. andersoni* have been isolated again in Australia from an aquarium and from the public water supply (Robinson *et al.* 1992) and in Japan from industrial cooling water (De Jonckheere *et al.* 1991). This species has not been detected in cooling waters in Belgium, probably because the upper temperature limit for *N. andersoni* is 40°C to 41°C, while the samples are incubated at 44°C. Strains of *N. andersoni* are sometimes isolated from surface water in Australia where different incubation temperatures are used for isolating *Naegleria* spp. (Robinson personal communication). Strains A2 and PPMFB6 were shown to be nonpathogenic in experimental animals (Willaert 1976).

The ITS1, 5.8S rDNA and ITS2 sequences of strain A2 are available at EMBL under accession number X96572, while the SSU rDNA sequence of strain PPMFB6 is under U80057. The partial SSU rDNA sequence of strain A2 has also been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain A2 is available at EMBL under accession number X78280.

Strains of *N. andersoni* have been adapted to axenic growth. Type strain Aq/4/1H is available from CCAP under accession N° 1518/16.

Naegleria jamiesoni De Jonckheere, 1988

This species was originally described as a subspecies of *N. andersoni* on the basis of similarities in isoenzyme and DNA restriction patterns (De Jonckheere 1988a). However, allozyme studies suggested that the subspecies could be regarded as separate species (Robinson *et al.* 1992). Also, the difference in the SSU rDNA sequence between the two subspecies far exceeded those found between some described *Naegleria* spp. Therefore, the subspecies were given species status, but they do form a cluster in phylogenetic trees (De

Jonckheere 1994a). As with its closest relative, *N. andersoni*, *N. jamielsoni* has a group I intron in the SSU rDNA (Table 3). Strains of this species were originally isolated in Belgium from water associated with imported fish from Malawi and Singapore. This species is found sporadically in cooling waters in Belgium (De Jonckheere unpublished) and in the environment in Australia (Robinson personal communication). Incubating the samples at 44°C apparently allows species to be isolated which have a maximum temperature tolerance of 42°C (see also *N. australiensis*).

The ITS1, 5.8S rDNA and ITS2 sequences of strain T56E are available at EMBL under accession number X96570, while the SSU rDNA sequence is under U80061. The partial SSU rDNA sequence of strain T56E has also been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain T56E is available at EMBL under accession number X78279.

Although strains of *N. jamielsoni* have been adapted to axenic growth there is currently no strain available at either ATCC or CCAP.

Naegleria pussardi Pernin and De Jonckheere, 1996

Based on allozyme and SSU rDNA sequences, the species *N. pussardi* appeared to be the most distantly-related *Naegleria* sp. (Pernin and De Jonckheere 1996). It is one of the few *Naegleria* spp. that shows a morphological particularity; during promitosis the nucleolus tends to fragment in an unequal way in prophase. The description of this species is based on one single isolate (EDF258) from river water in France. The maximum temperature tolerated for growth is 41°C. Therefore, as for *N. jamielsoni*, there is little chance that it will be isolated while incubating water samples at 44°C for *N. fowleri* detection. In a tree based on 5.8S rDNA and ITS sequences strain NG260 (allozyme cluster B in Robinson *et al.* 1992) clusters with *N. pussardi* (Pélandakis *et al.* 2000). The 5.8S rDNA is 174 bp long, which is the same as in the typestrain EDF258. The ITS1 is, however, two bp shorter in the typestrain of *N. pussardi*. Also the maximum growth temperature is only 37°C for strain NG260.

The ITS1, 5.8S rDNA and ITS2 sequences of strain EDF258 are available at EMBL under accession number X96571. The partial SSU rDNA sequence of strain EDF258 has been published (Pernin and De Jonckheere 1996).

The typestrain EDF258 is available from ATCC (N° 50564) and another strain (VA-1) that was reclassified from *Mastocystis marylandensis* to *N. pussardi*

is also available from ATCC (N° 50652). It is not certain whether this strain was reclassified on the basis of its morphological particularity or on the basis of isoenzyme analysis.

Naegleria carteri Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. carteri* was based on allozyme studies of nine strains isolated from different parts in Australia. Strains of this species were also isolated from Sri Lanka (Dobson *et al.* 1997). Slight differences in allozymes are detected between different strains of *N. carteri*, and it shares with *N. fowleri* and *N. lovaniensis* the capacity to grow at 45°C (Dobson *et al.* 1997). The validity of the separate species status was confirmed by rDNA sequence analysis of reference strain NG055 (De Jonckheere and Brown 1997). In phylogenetic trees based on SSU rDNA sequences *N. carteri* forms a cluster with *N. minor*, which is known for the capacity of its flagellates to divide. The strain of *N. carteri* investigated (NG055) has a group I intron in the SSU rDNA. *N. carteri* is one of the four *Naegleria* spp. in which the 5.8S rDNA is one bp shorter (Table 2).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG055 are available at EMBL under accession number Y10197, the partial SSU rDNA sequence under Y10189, and the SSU rDNA group I intron under Y10190.

Strains of *N. carteri* have never been grown axenically and there is currently no strain of *N. carteri* available at either ATCC or CCAP.

Naegleria morganensis Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. morganensis* was based on allozyme studies of four strains isolated from the River Murray in South Australia. It grows at 44°C (Dobson *et al.* 1997), and the validity of the separate species status is confirmed by rDNA sequence analysis of the typestrain NG236 (De Jonckheere and Brown 1997). In phylogenetic trees based on SSU rDNA sequences *N. morganensis* forms a cluster with two other *Naegleria* spp. that grow at 44–45°C, *N. niuginensis* and *N. sturti*. In this cluster, *N. morganensis* is the only described species with group I introns in the LSU rDNA (De Jonckheere and Brown 1998a). Actually, it has two group I introns in the LSU rDNA, one with and one without an ORF (Table 3). The description of the only other species known to have group I introns in the LSU rDNA, is in preparation

(see WA variant of *N. lovaniensis* in preparation). The only other strains known to belong to the *N. morganensis* lineage were isolated from the same location as the type strain. One of these other strains (NG258) was found to generate the same length LSU rDNA PCR product as NG236, indicating the presence of the same two introns as in the type strain. When isolating other strains of *N. morganensis* in the future, they should be investigated for the presence of group I introns in the LSU rDNA as these introns are quite unusual. The type strain of *N. morganensis* has no group I intron in the SSU rDNA (Table 3).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG236 are available at EMBL under accession number Y10192, while the partial SSU rDNA sequence is under Y10188. The sequence of the two group I introns in the LSU rDNA of strain NG236 are available at EMBL under accession numbers AJ001314 and AJ001315 respectively.

The type strain NG236 of *N. morganensis* is available from ATCC (N° 50351) and strain NG237 from CCAP (N° 1518/22).

Naegleria niuginensis Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. niuginensis* was based on allozyme studies of only one strain (NG427) isolated from lake sediment in New Guinea. It shares with *N. fowleri* and *N. lovaniensis* the capacity to grow at 45°C (Dobson *et al.* 1997). The validity of the separate species status is confirmed by rDNA sequence analysis of the type strain (De Jonckheere and Brown 1997).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG427 are available at EMBL under accession number Y10193, while the partial SSU rDNA sequence is under Y10186.

The only known strain (Dobson *et al.* 1997) of *N. niuginensis* is currently unavailable at either ATCC or CCAP.

Naegleria sturti Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. sturti* was based on allozyme studies of four strains isolated from water in Australia and Asia. Strains of *N. sturti* grow at 44°C (Dobson *et al.* 1997). The validity of the separate species status is confirmed by rDNA sequences of reference strain NG334 (De Jonckheere and Brown 1997). Differences in allozymes detected between dif-

ferent strains divides the species into two subgroups (Dobson *et al.* 1997). The allozyme differences between strains NG334/NG390 and NG221/277, respectively, are intermediate and in the same order as those between *N. australiensis* and *N. tihangensis*. The latter was previously called "sister species" of *N. australiensis* (Adams *et al.* 1989), later the spa variant of *N. australiensis* (Robinson, B. Protozoology, State Water Laboratory, Engineering and Water Supply Department, South Australia, Protozoology, Report No. 39, 1992), more recently a subgroup of *N. australiensis* (Dobson *et al.* 1997) and is given species status in the present monograph (see *N. tihangensis*). Therefore, it is possible that *N. sturti* comprises actually two different species. Because the SSU rDNA sequence of strain NG334 has been determined to substantiate the establishment of *N. sturti*, sequencing the SSU rDNA of strain NG277, which is available from ATCC, will resolve this issue.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG334 are available at EMBL under accession number Y10195, while the partial SSU rDNA sequence is under Y10185.

Only strain NG277 of *N. sturti* is available from ATCC (N° 50356).

Naegleria tihangensis sp. n.

The *N. gruberi* complex (Clark *et al.* 1989) is divided in this monograph into four different species. The second cluster of the *N. gruberi* complex contains strains that were isolated from Belgium (De Jonckheere *et al.* 1983b) and Mexico (De Jonckheere unpublished) in 1980 and 1983, respectively, while trying to isolate *N. fowleri* at 44°C. Therefore, the isolates are thermophilic but the maximum temperature tolerated is only 42°C. The Belgian strains came from a fish farm that used the cooling water from a nuclear power station, while the Mexican strains were isolated from geothermal water. The cluster is given species status based on SSU rDNA (De Jonckheere 1994a) and ITS DNA (Table 2) sequence analyses. The SSU rDNA of strain T2A of *N. tihangensis* differs only in one bp (0.125%) from that of strain NG202 within the 800 bp sequenced (De Jonckheere unpublished). This base substitution is in the highly variable loop 17 of the secondary structure. The ITS1, ITS2 and 5.8S rDNA of the two strains are identical (De Jonckheere unpublished). Also the LSU riboprints had been proven to be identical (Brown and De Jonckheere 1997). Strain NG202 is a representative of what was first called a "sister species" of

N. australiensis (Adams *et al.* 1989), later the spa variant of *N. australiensis* (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992), and more recently a subgroup of *N. australiensis* (Dobson *et al.* 1997). Therefore, the “sister species”, spa variant, or subgroup of *N. australiensis* belongs to *N. tihangensis*. The latter species name refers to the location of the power plant in Tihange, Belgium, from which cooling water strains of this species were isolated. Strains of *N. tihangensis* are isolated frequently during attempts to isolate *N. fowleri* in Australia (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992) and Belgium (De Jonckheere unpublished). Strain EDF145, isolated in France, (Pernin and De Jonckheere 1996) has the same acid phosphatase (AP) and propionyl esterase (PE) isoenzymes, as well as an identical partial SSU rDNA sequence, and belongs, therefore, to this new species, *N. tihangensis*.

Although the original Belgian and Mexican strains are no longer available the species can still be identified based on the partial SSU rDNA sequences of strain T2A that has been published (De Jonckheere 1994a).

Strain NG202 from Australia is available from CCAP (N° 1518/21).

Nonthermophilic species

Naegleria gruberi, the first *Naegleria* sp. described turned out to be a large species complex (De Jonckheere 1982a, Adams *et al.* 1989, Clark *et al.* 1989, Pernin and Cariou 1989, Robinson *et al.* 1992).

The flagellates of two non-thermophilic species, *N. minor* and *N. robinsoni*, have the capacity to divide. The amoebae of two species, *N. chilensis* and *N. indonesiensis* could not be induced to transform into flagellates (De Jonckheere *et al.* 2001).

A note on N. gruberi sensu lato. The four clusters distinguished previously by rDNA RFLP plasmid typing (Clark *et al.* 1989) in *N. gruberi sensu lato* are given the status of species in the present paper (Table 4). One of the clusters retains the species name *gruberi*. Strains belonging to cluster 2 are considered thermophilic as they were isolated at 44°C (De Jonckheere *et al.* 1983b). They are named *N. tihangensis* (see above) and correspond to what was first called a “sister species” of *N. australiensis* (Adams *et al.* 1989), and later the spa variant of *N. australiensis* (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water

Supply Department. South Australia. Protozoology. Report No. 39, 1992). More recently they have been considered a subgroup of *N. australiensis* (Dobson *et al.* 1997). It is not unexpectedly that a thermophilic cluster is found within the *N. gruberi sensu lato* as other thermophilic species are found to branch in the midst of the complex (Clark *et al.* 1989, Robinson *et al.* 1992, De Jonckheere 1994a). Two other clusters in *N. gruberi sensu lato* are named *N. pringsheimi* and *N. pagei*, respectively.

With allozymes it was demonstrated that strains assigned to *N. gruberi sensu lato* consist of at least 10 species (Adams *et al.* 1989). Although some of these clusters correspond to presently described species, more new species descriptions might be expected (Table 5). Strains of some of these unnamed allozyme clusters are available from ATCC and can thus be studied by anyone interested. I stated previously (De Jonckheere 1994a) that it would be preferable to study all the other allozyme groups before giving species rank to the riboprint clusters in *N. gruberi*, but it seems the latter will not be achieved soon. Therefore, I decide to upgrade those that have been studied most extensively.

Over the years some doubts have developed over the authenticity of certain CCAP strains assigned to *N. gruberi sensu lato*. Page suspected that strain CCAP 1518/1E might have been transposed and allozymes of the strain were indeed found to be identical to the ones of the Pringsheim strains (De Jonckheere 1987, Adams *et al.* 1989). This is the reason why CCAP decided not to supply strains CCAP 1518/1A, 1B, 1C, 1D, 1E and 1S anymore, but a strain called CCAP 1518/1X (Table 4), which would correspond to *N. pringsheimi* (Brown personal communication). I have done an in depth analysis of this problem by looking at my old lab notes and correspondence with CCAP. I have used strains CCAP 1518/1E and CCAP 1518/1F as references in comparisons of isoenzyme patterns (De Jonckheere 1982a, De Jonckheere *et al.* 1984) and unpublished identifications of Belgian and Mexican *Naegleria* isolates in 1984 and 1985. In these studies CCAP 1518/1E was different from CCAP 1518/1F, but also from CCAP 1518/1D, one the Pringsheim strains. In 1986 (De Jonckheere 1987), the isoenzyme pattern of CCAP 1518/1E had suddenly changed and was identical to those of the Pringsheim strains CCAP 1518/1A, 1C, 1D and 1S. However, in the same publication the DNA RFLP of the latter strains were identical to each other, but that of CCAP 1518/1E did not correspond to it. In my correspondence I noticed that all the CCAP 1518 strains

Table 4. Strains of *N. gruberi sensu lato*

Strain	Max. °C	Analysis	Species
EG and descendants	39	allozyme, SSUrDNA, ITS, riboprints	<i>N. gruberi sensu stricto</i>
AUD1	(?)	riboprints, ITS, allozyme	<i>N. gruberi sensu stricto</i>
DRI	38	RFLP, SSU rDNA, riboprints, ITS, allozyme	<i>N. gruberi sensu stricto</i>
CCAP1518/1A(<1986)	37	riboprints	<i>N. pringsheimi</i>
CCAP1518/1C(<1986)	37	riboprints	<i>N. pringsheimi</i>
CCAP1518/1D(<1986)	37	riboprints, SSU rDNA	<i>N. pringsheimi</i>
CCAP1518/1S(<1986)	37	riboprints	<i>N. pringsheimi</i>
CCAP1518/1X	37	riboprints	<i>N. pringsheimi</i>
BL (contaminant)	(?)	riboprints	<i>N. pringsheimi</i>
CCAP1518/1E(<1986)	37	riboprints, SSUrDNA, allozyme	<i>N. pagei</i>
CCAP1518/1F	39	riboprints, ITS, allozyme	<i>N. pagei</i> (?)
CCAP1518/1G	(?)	allozyme	(?)
CCAP1518/7	37	riboprints, allozyme	<i>N. pagei</i>
Philar	(?)	riboprints, allozyme	<i>N. pagei</i>
B14	(?)	riboprints, allozyme	<i>N. pagei</i>
B26	(?)	riboprints, allozyme	<i>N. pagei</i>
B27	(?)	riboprints	<i>N. pagei</i>
R6a	(?)	allozyme	<i>N. pagei</i>
113/1	(?)	riboprints	<i>N. galeacystis</i>
T2A	42	riboprints, SSU rDNA, ITS	<i>N. tihangensis</i>
T2S	42	riboprints	<i>N. tihangensis</i>
Mx6J	42	riboprints	<i>N. tihangensis</i>
Mx6G	42	riboprints	<i>N. tihangensis</i>
NG202	42	SSU rDNA, ITS	<i>N. tihangensis</i>

used in this last isoenzyme analysis had been sent to me from CCAP on September 1, 1986, while the DNA of CCAP 1518/1E and CCAP 1518/1F had been prepared from the strains CCAP 1518/1E and CCAP 1518/1F that I had already in my laboratory before that. So it turned out that a transposition of strains had occurred during the difficult process that preceded the transition of the CCAP collection from Cambridge to Windermere. Strains of CCAP 1518/1E used in different laboratories might therefore not be the original strain if obtained after 1985. It is comforting to know that all DNA studies I have done with this strain were, and still are, with the DNA from the original CCAP 1518/1E strain. The mislabeled strain was sent to me on September 1986, while the first publication in which I used the DNA of the original CCAP 1518/1E was submitted on August 2, 1986 (De Jonckheere 1987). Also the isoenzyme studies I published before 1987 were performed with the original CCAP 1518/1E strain.

Because of change in cyst diameter over a 10 year period Page mentioned that a possible transposition of

labels for CCAP 1518/1E and CCAP 1518/1F might have happened already between 1964/1965 and 1974 (Page 1975). However, a transposition at that time can be excluded, as both strains still appeared to be different (De Jonckheere 1982a, De Jonckheere *et al.* 1984). Thus the change in cyst diameter must have been caused by changes over the years in culture, the second possible explanation (Page 1975).

In the older studies most emphasis was given to the identification of pathogenic and thermophilic strains, while it was only stated that there was an extreme heterogeneity in strains of *N. gruberi sensu lato*. However, serious consideration was given to the mesophilic *Naegleria* in Australia (Adams *et al.* 1989, Robinson *et al.* 1992). In examining my published studies and unpublished isoenzyme gels I can conclude a few things on the strains of *N. gruberi sensu lato* (Table 4). According to allozyme results strain R6a belongs to *N. pagei*, while CCAP 1518/1F might not belong to it. Strain CCAP 1518/1G would still belong to an un-named species other than the four into which *N. gruberi sensu*

lato is here divided Robinson *et al.* 1992, De Jonckheere unpublished).

Naegleria gruberi Schardinger, 1899

This is the first described species of *Naegleria* (Schardinger 1899), and was originally described as *Amoeba gruberi*, but later redefined as a separate genus (Alexeieff 1912, Calkins 1913). The whole history of how the genus *Naegleria* was born has been described in detail (Fulton 1970). Until the description of *N. fowleri* in 1970, all amoeboflagellates that conformed to the genus *Naegleria* were assigned to the species *gruberi*. Due to newer techniques that were introduced for more rapid identification of the pathogen *N. fowleri*, it was realised that there is an enormous diversity within the strains that were catalogued as *N. gruberi*. By using rDNA analyses it was possible to group *N. gruberi* strains into four clusters (Clark *et al.* 1989). The first cluster contains strain EG, and its descendants, that are used in different laboratories as the model for differentiation from amoebae to flagellates (Fulton 1993). In the absence of any type material, it was decided to retain the species name *gruberi sensu stricto* for strains that cluster with this strain EG and its descendants. Strain EG was originally isolated in the early sixties by F. Schuster in California from an eucalyptus grove, hence its designation EG (Fulton 1993). The first SSU rDNA sequence from the genus *Naegleria* (EMBL accession N° M18732) was obtained (Clark and Cross 1988b) from strain NEG-M, a descendant of strain EG, which has a ploidy double that of the original strain (Fulton 1993). The same descendant was used to determine the full sequence of the mtDNA (Lang *et al.* 1999). For obtaining the full sequence of the plasmid containing the rDNA (Mullican and Tracy 1993) strain NG_B was used, the EG strain kept by Balamuth. The EG strain that contains virus-like particles is called EG_S (Fulton 1993).

It was mentioned that strain NEG, a clonal strain of EG, will grow at up to 40–41°C with a maximum rate at about 33°C (Fulton 1970). Since the temperature optimum for each species is approximately 4°C lower than the upper temperature limit (Robinson *et al.* 1996a), the latter temperature is probably around 37°C for *N. gruberi*, otherwise it would be a thermophilic species. Strain ATCC 30544, corresponding to Fulton's strain NEG was reported to tolerate a maximum temperature of 39°C (Robinson *et al.* 1992). It has also been reported that strains can decrease their maximum temperature tolerance after maintenance in the laboratory for a certain amount of time (Dobson *et al.* 1997).

Different culture conditions could also explain the difference in maximum temperature noted between strain CCAP 1518/1E (see *N. pagei*) from CCAP and that from ATCC (Robinson *et al.* 1992). A maximum temperature tolerance of 38°C is observed (Brown personal communication) with strain DRI, which belongs to *N. gruberi sensu stricto* (see below).

Naegleria amoebae cytopathogenic material (NACM) kills various cell cultures (Dunnebacke and Walen 1999) and was isolated from strain EG_S, the descendant of strain EG with virus-like particles. NACM is a small acidic protein, that resists inactivation by irradiation, nucleases, and a number of proteases, while it is inactivated by proteinase K and at elevated temperatures.

The reduced species *N. gruberi sensu stricto* still has a worldwide distribution. Other strains belonging to the emended *N. gruberi* species are strains AUD1 isolated from a swimming pool filter in France and strain DRI isolated from a water station drain on a golf course in Australia (Table 4). I have sequenced part of the SSU rDNA of strain DRI (De Jonckheere 1994a) and there is only one bp difference with that published for strain NEG-M. This indel is thought to be probably a sequencing error in strain NEG-M, as the deletion is a nucleotide that is conserved not only in all other *Naegleria* spp., but in all eukaryotes. In the sequence published for strain EG_B (Mullican 1995), another descendant of strain EG, there is, indeed, no such deletion (I made the same sequencing error in the SSU rDNA of *N. minor* strain WT043). The combined ITS1, 5.8S rDNA and ITS2 PCR product has the same length in strains AUD1, DRI and EG_B (De Jonckheere 1998). The sequences of these areas in strain EG_B (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA: 1-163, 1995) in strain AUD1 (Pélandakis *et al.* 2000) and DRI are identical (Table 2). Strains that cluster in allozyme analysis with strain NEG of *N. gruberi sensu stricto* have been isolated from Australia, Japan, Germany and Ireland (Robinson *et al.* 1992). These isolates have all an upper temperature tolerance for growth of 38–39°C.

The ITS1, 5.8S rDNA and ITS2 sequences of strain EG_B have been determined (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA: 1-163, 1995) while those of strain AUD1 are available at

EMBL under accession number AJ132031. These sequences of strain DRI are in Table 2. The partial SSU rDNA sequence of strain NEG-M is available at EMBL under accession number M18732 while that of strain DRI has been published (De Jonckheere 1994a).

Strain DRI is currently available from CCAP under accession number 1518/17. Strain EG and descendants of this strain, NEG, NEG-M and EG_s are available from ATCC under accession numbers 30311, 30233, 30224 and 30540, respectively.

Naegleria pringsheimi sp. n.

The third cluster observed by Clark *et al.* (1989) in the *N. gruberi* complex contains strains isolated before 1950 by Pringsheim, of which CCAP 1518/1D is a representative. The clustering of these strains by DNA analysis was confirmed by allozyme analysis (Adams *et al.* 1989). This cluster is given species status based on SSU rDNA sequences (De Jonckheere 1994a). All five strains of *N. pringsheimi* studied have a group I intron in the SSU rDNA (De Jonckheere 1993) and the combined ITS1, 5.8S rDNA and ITS2 PCR product has the same length in those five strains (De Jonckheere 1989). Strains CCAP 1518/1D and CCAP 1518/1S were reported to grow at a maximum temperature of 37°C (Griffin 1972) as do the other CCAP strains belonging to *N. pringsheimi* (Robinson *et al.* 1992). Strain CCAP 1518/1S came from Singh and could be derived from CCAP 1518/1C, while CCAP 1518/1D came from Fulton with the information that it came to him from Balamuth as strain NB-1, which was derived from a subculture of CCAP 1518/1A (Page 1975). The latter is supported by the fact that CCAP 1518/1A and CCAP 1518/1D both have cysts of the rough type, but CCAP 1518/1S has the angular-smooth cyst type (Page 1975). However, Pussard and Pons (1979) believe that the morphology of the cysts depends too much on the condition of the medium on which they are formed and they found extreme differences in cyst morphology of clonal cultures. According to Page (1975) strain CCAP 1518/1A is the one used by Willmer in his experimental study of amoeba to flagellate transformation published in 1956. According to Pussard and Pons (1979), strain CCAP 1518 that Willaert used corresponds to CCAP 1518/1A or CCAP 1518/1D, which are identical (Page 1975). It is important to know the exact origin and history of a strain as conclusions drawn from using different strains may actually apply to several descendants of the same strain. For example, in a study of cytopathic effect of *N. gruberi sensu lato*, eight strains were used

(John and John 1990) but there were probably only three strains with different origin and the others being descendants of strain EG. Riboprinting (Clark *et al.* 1989) demonstrated a slightly higher variation in *N. pringsheimi* strains (cluster 3 of *N. gruberi sensu lato*) than in *N. gruberi sensu stricto* strains (cluster 1 of *N. gruberi sensu lato*).

Strain BL also clusters with the Pringsheim strains, and although strain BL was claimed to have been isolated from a New Zealand case in 1968, the strain turned out to be nonpathogenic and reacts with *N. gruberi* antibodies and not *N. fowleri* antibodies. It is clear that the strain must have been mislabeled at some point and the BL strain that I used (De Jonckheere 1993, 1998) certainly clusters with the Pringsheim strains. Also, allozyme analysis groups strain BL with CCAP 1518/1D (Pernin and Cariou, 1989). On the other hand, strain BL available at ATCC (N° 22758) might be the original *N. fowleri* strain. It was submitted as a *Echinostelium* sp. by Mandal, one of the original authors of the PAM case in New Zealand (Mandal *et al.* 1970). The reason for this assumption is that in a recent study (Pélandakis *et al.* 2000) the ITS and 5.8S rDNA of strain BL correspond to what is found in *N. fowleri* (De Jonckheere 1998). As strain BL in different labs might be a mislabeled strain, except the one at ATCC, it appears that all strains of *N. pringsheimi* known at present are restricted to Europe.

The species *N. pringsheimi* can be identified based on the DNA sequences of the partial SSU rDNA sequence of strain CCAP 1518/1D that has been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain CCAP 1518/1D is available at EMBL under accession number X78278. The ITS1, 5.8S rDNA and ITS2 sequences of strain CCAP 1518/1D are in Table 2.

Unfortunately, CCAP 1518/1D is no longer available, but CCAP 1518/1A and CCAP 1518/1C are at ATCC under N° 30874 and 30875, respectively.

Naegleria pagei sp. n.

Several strains isolated by F. Page in the USA (of which CCAP 1518/1E is a representative), strain Philar isolated in 1981 from the river Nile in Egypt, and strains isolated in 1980 from different lakes in Belgium, form a fourth cluster in the *N. gruberi* species complex (Clark *et al.* 1989). This cluster is now given species status on the basis of SSU rDNA sequences (De Jonckheere 1994a). The name is given in recognition of the contribution of F. C. Page to the knowledge

Table 5. Identification of clusters defined on the basis of allozymes by Robinson *et al.* (1992)

Cluster	N° of strains	Type strain	Max. °C	Culture collection N°	Group I intron*	Species
A	10	NG257	34	ATCC 50354	-	TD
B	2	NG260	37	ATCC 50355	-	<i>N. pussardi</i> (?)
C	2	NG158 (T56E)	42	-	+	<i>N. jamiesoni</i>
D	6	NG160 (Aq/4/1H)	40	CCAP 1518/16	+	<i>N. andersoni</i>
E	11	NG152 (CCAP 1518/1E)	35	ATCC 30876	-	<i>N. pagei</i>
F	2	NG006 NG608	36 39	CCAP 1518/1G -	- ND	TD TD
G	6	NG282 NG010 (PD)	37 39	ATCC 50357 ATCC 30544	+ ND	<i>N. clarki</i> idem
H	9	NG206 NG250	40 40	ATCC 50349 ATCC 50352	- ND	TD TD
I	5	NG106 NG230	37 37	CCAP 1518/7 ATCC 50350	- -	<i>N. pagei</i> idem
J	2	NG395	40	-	ND	TD
K	2	NG005	40 (39)	CCAP 1518/1F (ATCC 50348)	-	<i>N. pagei</i> (?)
L	8	NG008 (NEG)	39	ATCC 30223	-	<i>N. gruberi</i>
M	5	NG001	37	CCAP 1518/1A (ATCC 30874)	+	<i>N. pringsheimi</i>
N	2	NG408	<30	-	ND	Antarctic <i>Naegleria</i> sp.
Gr-3#	2	NG256	37	ATCC 50353	-	TD

* - De Jonckheere and Brown unpublished, # - in Adams *et al.* 1989, ND - not done, TD - to determine

of amoebae and who isolated several of the strains belonging to this species. The species has a worldwide distribution. With riboprinting (Clark *et al.* 1989) *N. pagei* strains (cluster 4 of *N. gruberi sensu lato*) were found to be more variable than *N. pringsheimi* strains (cluster 3 of *N. gruberi sensu lato*). The difference between CCAP 1518/1E and CCAP 1518/1F with the other *N. pagei* strains is greater than that between *N. italica* and *N. clarki* (Clark *et al.* 1989). Of the five strains of *N. pagei* investigated only strain CCAP 1518/1F has a combined ITS1, 5.8S rDNA and ITS2 PCR product that is a little shorter than the other strains (De Jonckheere 1998). Also, the isoenzyme patterns of CCAP 1518/1F do not correspond to that of CCAP 1518/7 (De Jonckheere 1987). Strain CCAP 1518/1F grows at a maximum temperature of 40°C (only slightly according to Pernin and Cariou 1989), while the other strains of *N. pagei* only tolerate 37°C (Robinson *et al.* 1992). The maximum temperature tolerance of strain

CCAP 1518/1F has been recently confirmed to be 39°C (Brown personal communication). Severe differences in cyst morphology between strains CCAP 1518/1E and CCAP 1518/1F have been reported (Pussard and Pons 1979) and no cross-reaction was obtained with strain CCAP 1518/1F when antiserum prepared against strain CCAP 1518/1E was used (De Jonckheere *et al.* 1974). Furthermore, in allozyme studies strain CCAP 1518/1F does not cluster with CCAP 1518/7 of *N. pagei* (Robinson *et al.* 1992). Because of difference in ITS2 length, differences in isoenzymes and different maximum temperature tolerance, cyst morphology and antigens, strain CCAP 1518/1F does not really belong to *N. pagei* and needs further investigation. According to Pernin and Cariou (1989) strain CCAP 1518/1E would not grow at 37°C, but I found in my labnotes of 1974 the strain does grow at this temperature. A discrepancy between the maximum temperature tolerated (Robinson *et al.* 1992) was found between the CCAP 1518/1E strain received

from CCAP and ATCC. See above (*A note on N. gruberi sensu lato*) for information on the CCAP strains. Page suspected that strain CCAP 1518/1E might have been transposed and allozymes for the strain were found to be indeed identical to the ones of the Pringsheim strains (Adams *et al.* 1989). However, the DNA analyses (Clark *et al.* 1989, De Jonckheere 1994a) indicate that they have been performed on the DNA from the original strains before the transposition (see *A note on N. gruberi sensu lato*). These strains have indeed been acquired by me from CCAP in the early seventies. I even found in my labnotes of 1974 the results of the temperature tolerance tests on these strains, so that I was able to compare these to those published (Adams *et al.* 1989, Robinson *et al.* 1992).

The species *N. pagei* can be identified based on the partial SSU rDNA sequence of strain CCAP 1518/1E that has been published (De Jonckheere 1994a). The ITS1, 5.8S rDNA and ITS2 sequences of strain CCAP 1518/1F are available at EMBL under accession number AJ132022, while that of CCAP 1518/1E are in Table 2.

Strains of *N. pagei* are still available at CCAP (N° 1518/1F and N° 1518/7) and ATCC (N° 30876), but it should be investigated whether the latter corresponds indeed to the original CCAP 1518/1E (see above). Because strain CCAP 1518/1F seems to be the most different strain I propose to use CCAP 1518/7 as the type strain of *N. pagei*.

Naegleria jadini Willaert and Le Ray, 1973

The type strain of *N. jadini* was isolated from a swimming pool in Antwerp, Belgium. Only one strain of this species is known and it was identified by immunoelectrophoretic analysis, a technique not currently employed in species identification (Willaert and Le Ray 1973). The species can be differentiated from other species by isoenzyme analysis, a technique used more frequently in the identification of *Naegleria* isolates (De Jonckheere 1982a). However, *N. jadini* is unlikely to be encountered in routine *Naegleria* analyses as during these isolation procedures high incubation temperatures are used, and *N. jadini* does not grow above 35°C (Robinson *et al.* 1992). However, even the Australian Water Quality Centre which uses different incubation temperatures on samples from all over the world never seems to have encountered this species (Robinson *et al.* 1992).

The type strain of *N. jadini* appears to have one of the longest ITS2 region in the genus *Naegleria*

(Table 2). The larger size is due to a number of repeats (De Jonckheere 1998). On the other hand, *N. jadini* has one of the smallest rDNA plasmids reported (Clark *et al.* 1989).

The ITS1, 5.8S rDNA and ITS2 sequences of strain 0.400 are available at EMBL under accession number X96576, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a).

The type strain of *N. jadini* has been adapted to axenic growth. The type strain 0400 is available from ATCC (N° 30900) and from CCAP (N°1518/2).

Naegleria clarki De Jonckheere, 1984

Two *Naegleria* strains from New Zealand were found to form a separate cluster related to *N. italica* in a tree based on rDNA restriction patterns (Clark *et al.* 1989). They have special isoenzyme patterns and total DNA patterns not found in described species (De Jonckheere 1988b). The SSU rDNA of strain RU30 was sequenced and on this basis this cluster was given species status (De Jonckheere 1994a). The two strains of *N. clarki* have a group I intron in the SSU rDNA (De Jonckheere 1993). The two type strains of *N. clarki*, RU30 and RU42, were isolated from water at the Golden Springs in Rotorua, New Zealand (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975). When tested for upper temperature limit strain RU30 was found to grow at 37°C (Brown personal communication). Based on allozyme, strains of this species have been identified in water samples from the USA, Australia, Korea, China and Guyana, of which the majority grows at an upper temperature limit of 37°C, a few at 38°C and only one at 40°C (Dobson personal communication). Strains closely related to *N. clarki*, based on SSUrDNA sequences, have been isolated from organs of fish as well (Dykova *et al.* 2001). It is of interest to note that soon after isolation the two strains of *N. clarki* killed some mice after IN instillation (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975), but later attempts could not confirm the pathogenicity of this species (Willaert 1976).

The ITS1, 5.8S rDNA and ITS2 sequences of strain RU30 are available at EMBL under accession number X96575, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain RU30 is available at EMBL under accession number X78281.

Strains of *N. clarki* can be grown axenically. Strains RU30 and RU42 are available from CCAP (N° 1518/14 and 1518/15, respectively).

Naegleria galeacystis De Jonckheere, 1994

The strain that was used in different laboratories as the type strain of *Adelphamoeba galeacystis* (ATCC 30294) appears now to be a strain that was mislabelled. Adams *et al.* (1989) already considered ATCC strain 30294 to be a *Naegleria* species. Since this mislabelled strain showed SSU rDNA (De Jonckheere 1994a) and ITS rDNA (De Jonckheere 1998) sequences compatible with the genus *Naegleria*, but different from described *Naegleria* spp., it was given separate species status within the genus and the species name was retained. A strain isolated in South Australia, that conformed to the original morphological description for *A. galeacystis*, turned out to have identical SSU rDNA sequences as *Didascalus thorntoni* (De Jonckheere *et al.* 1997). Thus *Adelphamoeba* is a nonvalid junior name of *Didascalus*. Therefore, it is acceptable to use the species name *galeacystis* for this *Naegleria* sp.

The mislabelling of this isolate probably occurred in Napolitano's laboratory before it was distributed, because the typestrain available from ATCC and submitted by Napolitano, is also a *Naegleria* strain. The close similarity of strain *A. galeacystis* from ATCC with strains of *N. gruberi* had already been observed by studying rDNA restriction patterns (Clark and Cross 1988a). Strain 113/1 from France probably belongs to *N. galeacystis* (Clark *et al.* 1989). I have done a phylogenetic analysis of the partial SSU rDNA published for a *Naegleria* isolated from a necrotic lesion in a reptile (Walochnick *et al.* 1999). The sequence invariably clusters with that from *N. galeacystis*, although the sequence is not identical. The reptile isolate should be further studied for proper identification to species level.

The ITS1, 5.8S rDNA and ITS2 sequences of strain A.V.500 are available at EMBL under accession number X96578, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a).

Strain A.V.500 of *N. galeacystis* has been adapted to axenic growth. Typestrain A.V. 500 is available from ATCC (N° 30294) and from CCAP (1506/1).

Naegleria fultoni De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

Strains described as *N. fultoni* are different from other *Naegleria* spp. in allozyme pattern (Robinson

personal communication), while the SSU rDNA and the ITS1, 5.8S rDNA and ITS2 sequences of the typestrain NG885 are also novel (De Jonckheere *et al.* 2001). Strains of this species grow at a maximum temperature of 35°C and only form flagellates under certain conditions (De Jonckheere *et al.* 2001). Several strains of this new species were isolated from a reservoir in South Australia but, as far as I know, not from other places. Strains of the WA variant of *N. lovaniensis* with a group I intron in the LSU rDNA were also found in this reservoir (see WA variant of *N. lovaniensis* in preparation).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG885 are available at EMBL under accession number AJ243445, while the partial SSU rDNA sequence is under AJ243440.

Typestrain NG885 of *N. fultoni* is not yet available from either ATCC or CCAP.

Naegleria spp. with dividing flagellates

Although flagellates with different numbers of flagella are seen in different *Naegleria* spp. (Fulton 1970, John *et al.* 1991), the flagellates were never observed to divide although some scanning electron micrographs (John *et al.* 1991) might suggest that they were in the process of division (De Jonckheere and Brown 1995). Recently two *Naegleria* spp. with dividing flagellates have been described, and they belong to different phylogenetic clusters within the genus *Naegleria*.

Naegleria minor De Jonckheere and Brown, 1995

As in *Willaertia magna*, the flagellates of *N. minor* (strain WTO43) have four flagella, but the flagellates of *N. minor* can divide only once and the daughter cells have only two flagella. Strain WTO43 was originally described as a new *Willaertia* sp., i.e. *W. minor* (Dobson *et al.* 1993) but SSU rDNA sequence comparisons showed that the type strain of *W. minor* clusters within the genus *Naegleria* and not with *W. magna*. Therefore, it was renamed *N. minor* (De Jonckheere and Brown 1995). Because of the fact that the flagellates divide only once, it is speculated that the division might be a meiosis, and that the monoploid daughter flagellates might act as gametes. Although this hypothesis has not been proven experimentally, the division of the flagellates would support the idea of the flagellates being gametes which has been proposed earlier (Fulton 1993). There is only one strain known of *N. minor* which was isolated from the Northern Territory in Australia. It is the only

Table 6. Keratitis cases implicating amoebae other than *Acanthamoeba* spp.

Reference	Country	Strains isolated	Year	Contact lens	Age	Sex
Haase and De Jonckheere, unpublished (mentioned in: De Jonckheere and Brown 1998b)	Germany	<i>Hartmannella</i> sp.	1989	?	?	F
Kennedy <i>et al.</i> 1995	Ireland	<i>H. vermiformis</i>	?	soft	17	F
Aitken <i>et al.</i> 1996	UK	<i>Hartmannella</i> sp. (+ <i>Vahlkampfia</i> sp.)	1992	+	24	M
Inoue <i>et al.</i> 1998	Japan	<i>Hartmannella</i> sp. (+ <i>Acanthamoeba</i> sp.)	1994	hard	54	F
Aimard <i>et al.</i> 1998.	France	<i>Hartmannella</i> sp. (+ <i>Acanthamoeba</i> sp.)	?	+	40	F
Dua <i>et al.</i> 1998	UK	<i>Vahlkampfia jugosa</i> * <i>Naegleria</i> sp.	1995 1997	soft soft	38 18	M F
Alexandrakis <i>et al.</i> 1998	USA	<i>Vahlkampfia</i> sp.	?	-	30	M
Belle and De Jonckheere, unpublished	Belgium	<i>Hartmannella</i> sp.	1999	-	?	M
Belle and De Jonckheere, unpublished	Belgium	<i>Vahlkampfia inornata</i>	2000	+	18	F

* - *Vahlkampfia jugosa* was renamed *Paratetramitus jugosus* (Darbyshire *et al.* 1976) and *Tetramitus jugosus* (Brown and De Jonckheere 1999)

Naegleria strain in which a plasmid has been detected other than the rDNA plasmid (De Jonckheere and Brown 1995). It is not known what this other DNA plasmid is coding for, but it might be a good candidate for DNA-mediated transformation instead of the rDNA plasmid. The strain of *N. minor* has one of the longest ITS2 in the genus *Naegleria* (Table 2) but, contrary to *N. jadini*, this is not due to repeats (De Jonckheere 1998).

The ITS1, 5.8S rDNA and ITS2 sequences of strain WT043 are available at EMBL under accession number X96577, while the SSU rDNA sequence is under X93224.

The strain of *N. minor* grows at a maximum temperature of 38°C and has been adapted to axenic growth. The typestrain WT043 is available from ATCC (N° 50320) and CCAP (N° 1518/18).

Naegleria robinsoni De Jonckheere and Brown, 1999

This species description is based on only one strain. As is the case with *N. minor*, *N. robinsoni* transforms into dividing flagellates with four flagella, and the daughter cells have only two flagella. After this division the

flagellates can no longer divide (Robinson unpublished). In phylogenetic trees based on SSU rDNA sequences *N. robinsoni* does not cluster with *N. minor* (De Jonckheere and Brown 1999b), the other *Naegleria* sp. with dividing flagellates. The typestrain, NG944, was isolated from freshwater on Kangaroo Island at the South coast of Australia and grows at a maximum temperature of 38°C, which is the same temperature as for *N. minor*.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG944 are available at EMBL under accession number AJ237787, while the partial SSU rDNA sequence is under AJ237786.

Type strain NG944 of *N. robinsoni* is available from CCAP (N° 1518/19).

***Naegleria* spp. in which no transformation to flagellates could be induced**

It has been reported that four strains of *N. fowleri* (Cable and John 1986) and four strains of *N. lovaniensis* (John *et al.* 1991) could no longer be induced to transform to flagellates in the laboratory. These strains had

formed flagellates before and this loss of ability to transform is probably due to prolonged axenic cultivation. The capacity to transform was not restored by subculturing on bacteria (John *et al.* 1991). In addition, there are a few *N. fowleri* strains from one location in France that could never be induced to form flagellates since their isolation (Pernin personal communication). In addition there are two *Naegleria* spp., *N. chilensis* and *N. indonesiensis*, that have never formed flagellates under laboratory conditions, even immediately after isolation (Robinson personal communication).

While *N. fultoni* (see above) only forms flagellates under certain conditions most *Naegleria* spp. easily form flagellates soon after isolation (Fulton 1993). In most cases I even donot need to perform a separate transformation test on new *Naegleria* isolates. They tend to form flagellates in the liquid that accumulates at the border between the agar surface and the agar block on which they are transferred for subculturing.

Naegleria chilensis De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

The type strain of *N. chilensis*, NG946, was isolated from alpine Chile and barely grows at 30°C. The strain fails to form flagellates (Robinson personal communication) but the SSU rDNA shows that it belongs to the genus *Naegleria*. Because of unique allozyme patterns and SSU rDNA sequence differences it is described as a new species (De Jonckheere *et al.* 2001). Only one strain of this species has been isolated. In phylogenetic trees based on SSU rDNA sequences *N. chilensis* clusters with *N. pussardi*, which is the most distantly related species in the genus *Naegleria*.

The type strain of *N. chilensis* has the longest ITS1 ever found in the genus *Naegleria* (Table 2).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG946 are available at EMBL under accession number AJ243443, while the partial SSU rDNA sequence is under AJ243442.

Type strain NG946 of *N. chilensis* is not yet available from either ATCC or CCAP.

Naegleria indonesiensis De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

The type strain of *N. indonesiensis*, NG945, was isolated from Bali, Indonesia, and grows at an upper temperature limit of 38°C. This strain also fails to form flagellates (Robinson personal communication) but the

SSU rDNA shows that it belongs to the genus *Naegleria*. Because of unique allozyme patterns and SSU rDNA sequence differences it is described as a new species (De Jonckheere *et al.* 2001). Only one strain of this species has been isolated. In phylogenetic trees based on SSU rDNA sequences *N. indonesiensis* clusters with *N. robinsoni*, a species with dividing flagellates.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG945 are available at EMBL under accession number AJ243444, while the partial SSU rDNA sequence is under AJ243441.

Type strain NG945 of *N. indonesiensis* is not yet available from either ATCC or CCAP.

New *Naegleria* spp. of which the descriptions are in preparation

There are three *Naegleria* groups for which most of the allozyme and rDNA typing has been done and which will be described as new species in the near future. These three are all thermophilic species. Another species description has been long in preparation for strains that were isolated from places at low temperatures, cluster N in Robinson *et al.* (1992). I have been unable to perform DNA typing on it because of difficulties in culturing these strains in the laboratory.

Naegleria philippinensis in preparation

In 1984 a *Naegleria* strain (RJTM) was isolated from the CSF of a young patient diagnosed with encephalitis in the Philippines (Matias, R. R. *Naegleria philippinensis*: characterization based on morphology, surface membrane antigens, isoenzyme patterns and hydrolases. Doctoral Dissertation, University of the Philippines, 1991). Similar strains were isolated from a thermally polluted river and from a heated swimming pool in the Philippines. A *Naegleria* isolate with similar isoenzyme patterns as the Philippine isolate was recovered from a swimming pool in Hungary (Szénasi *et al.* 1998). The Philippine strain from thermally polluted water was reported to be of low virulence as high numbers of amoebae were needed to kill mice (Simeon *et al.* 1990). I have tested the CSF strain by IN inoculation of mice with similar amounts of amoebae and found it did not cause mortality. On NNE it grows only at a temperature up to approx. 40°C. Since pathogenic *Naegleria* spp. either grow at 42°C or 45°C, the upper temperature tolerance of strain RJTM would support that it is not a pathogenic species. The positive virulence

tests early on are comparable to those obtained with nonpathogenic species, such as *N. lovaniensis*, *N. jadini* and *N. clarki* (see above).

I was able to analyse the isoenzymes of the CSF isolate only. The AP pattern is the same as for *N. tihangensis* and *N. clarki* but the PE pattern is different. However, differences in PE patterns have been observed in several species while the AP pattern seems to be more specific to the species. The AP isoenzyme pattern of the different *N. philippinensis* strains appear to be the same (Matias, R. R. *Naegleria philippinensis*: characterization based on morphology, surface membrane antigens, isoenzyme patterns and hydrolases. Doctoral Dissertation, University of the Philippines, 1991), but some of these strains may belong to *N. tihangensis*, especially since the latter seem to be very common in the environment. The isolate from Hungary was reported to have similar isoenzyme profiles as the Philippine isolate, but the former is thermophilic (Szénasi *et al.* 1998). Therefore, the Hungarian isolate might be a strain of *N. tihangensis*.

In phylogenetic trees based on SSU rDNA sequences, the CSF isolate of *N. philippinensis* (strain RJTM) clusters within the *N. australiensis* - *N. italica* group (Fig. 2). Between the two PstI sites strain RJTM differs in respectively 14 bp (1.8%) and 16 bp (2.0%) from these two pathogenic species. Strain RJTM has the same 5.8S rDNA sequence as the two pathogenic strains and *N. clarki*. I found the ITS2 to be 105 bp long, but Fontanilla *et al.* (2001) reported that it is 115 bp long in the same isolate. The ITS2 of *N. philippinensis* is 5 nt longer than in *N. australiensis* and 3 nt longer than in *N. tihangensis* (Table 2). In *N. italica* the ITS2 has a long insert. Contrary to *N. australiensis* and *N. tihangensis*, *N. philippinensis* has a group I intron in the SSU rDNA, as is the case in *N. italica* and *N. clarki*. Since group I introns in the SSU rDNA is a property of the species, *N. philippinensis* cannot belong to *N. australiensis* or *N. tihangensis*. However, it can also not belong to *N. italica* and *N. clarki* as the group I introns are quite different. In *N. philippinensis* the group I intron is 1297 bp long while the length in *N. italica* and *N. clarki* is 1319 bp and 1305 bp, respectively (Table 3).

Type strain RJTM of *N. philippinensis* is available from CCAP (N° 1518/20).

The WA variant of *Naegleria lovaniensis* in preparation

At the State Water Laboratory of South Australia several thermophilic *Naegleria* strains with an upper temperature limit of 44°C have been isolated that show allozyme divergence at nearly 30% of loci with *N. lovaniensis* (Robinson and Dobson 2001). These strains are provisionally named WA variant as they appear to be restricted to western and northern Australia. Also, in phylogenetic trees based on SSU rDNA, strain NG872 of WA variant clusters with *N. lovaniensis* (De Jonckheere and Brown 1997). The two differ by only 3 bp (0.5%) in the 800 bp sequenced. Investigation of the SSU rDNA of strain NG872 demonstrated the presence of a group I intron. Moreover, a group I intron is also present in the LSU rDNA of this strain (De Jonckheere and Brown 1998a). This is very uncommon in *Naegleria* spp. as group I introns in the LSU rDNA have only been found in *N. morganensis* and the WA variant of *N. lovaniensis*. A recent study of different isolates belonging to the WA variant of *N. lovaniensis* showed that, in fact, two different group I introns are present in this lineage depending on the strain investigated, and group I introns might be absent from either the LSU rDNA and/or the SSU rDNA of different isolates (De Jonckheere and Brown 2001). While the first detected group I intron in the LSU rDNA has no ORF (De Jonckheere and Brown 1998a), the second one is much longer and encodes an endonuclease (De Jonckheere and Brown 2001). In contrast with the group I intron in the SSU rDNA, which is transmitted vertically (De Jonckheere 1994b), the group I introns in the LSU rDNA are transferred horizontally (De Jonckheere and Brown 1998a, 2001).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG872 are available at EMBL under accession number Y10191, while the partial SSU rDNA sequence is under Y10187. The sequences of the LSU rDNA group I intron of strain NG872 and NG874 are available at EMBL under accession number AJ001316 and AJ271406, respectively, while the sequence of the SSU rDNA group I intron of strain NG872 is under AJ001399.

No strains of the WA variant of *N. lovaniensis* are available yet from either ATCC or CCAP.

Naegleria sp. NG597 in preparation

The three strains known of this lineage were isolated from Australia, Papua New Guinea and Indonesia, respectively (Dobson personal communication). The clustering of these three strains is based on allozyme studies (Robinson personal communication), and analysis of the SSU rDNA sequence of one strain (NG597) places the lineage close to *N. clarki* and *N. gruberi sensu stricto* (De Jonckheere and Brown 1997). This *Naegleria* sp. is special as it is the only known *Naegleria* lineage that has lost the group I intron ORF (De Jonckheere and Brown 1994), which is part of the twintron present in the SSU rDNA of other intron-bearing *Naegleria* spp. (Einvik *et al.* 1998). The loss of part of the twintron is found in the three different strains investigated of this lineage and the group I introns differ by only three and four bp, respectively, from each other. The NG597 *Naegleria* lineage is another thermophilic *Naegleria* sp. (upper temperature limit of 42°C), but is awaiting proper species description.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG597 are available at EMBL under accession number Y10194, while the partial SSU rDNA sequence is under Y10184. The sequences of the SSU rDNA group I introns of strains NG434, NG597 and NG650 are available at EMBL under accession numbers X79069 till X79071.

No strains of this lineage are available yet from either ATCC or CCAP.

Antarctic *Naegleria* sp. in preparation

The research group of the State Water Laboratory of South Australia has isolated *Naegleria* strains from Antarctica that only grow at temperatures lower than 30°C. Identical strains have also been reported from the northern hemisphere (Robinson personal communication). By allozyme analysis they form cluster N in Robinson *et al.* (1992), and are the closest relative of *N. jadini*, yet are certainly a different species. I have not been able to determine DNA sequences as the strains are difficult to culture. Therefore, details about their SSU rDNA phylogeny are lacking.

PHYLOGENETIC RELATIONSHIPS

The genus *Naegleria*

Although based on a limited dataset of SSU rDNA sequences, it was already known from 1989 that the

evolutionary distance between *N. gruberi sensu lato* and *N. fowleri* was as great as the distance between amphibians and mammals (Baverstock *et al.* 1989). However, the mutational distance of the SSU rDNA might not represent the true evolutionary distance since the rDNA might have evolved at different rates in different organisms. In spite of this, the mutational differences in *Naegleria* between the SSU rDNA seem to be related to other molecular distances, such as allozyme and antigenic differences. Therefore, the use of SSU rDNA sequence differences is a good quantitative method for delineating species. Although I had used RFLP of total DNA to characterise *Naegleria* spp. before (De Jonckheere 1987), I decided in 1994 to use SSU rDNA sequences for differentiating between different *Naegleria* spp. (De Jonckheere 1994a).

The genus that is most closely related to *Naegleria* is *Willaertia*, an amoeboid flagellate genus with dividing flagellates (De Jonckheere 1997). As shown above, based on SSU rDNA analysis, the other *Willaertia* sp., *W. minor*, has turned out to be a *Naegleria* sp.

The phylogenetic tree shown in Fig. 2. is based on partial SSU rDNA sequences and different clusters within the genus *Naegleria* can be discerned. It is obvious that the pathogenic species do not cluster, neither do the thermophilic species, nor the species with dividing flagellates, nor the species which do not transform into flagellates. Therefore, it is impossible to group these isolates into different genera based upon a particular behaviour of the flagellates. These new genera would have to include species with typical *Naegleria* characteristics.

The human pathogen *N. fowleri* clusters with *N. lovaniensis* and the WA variant of *N. lovaniensis*. All strains of these three *Naegleria* grow at a maximum temperature of 44-45°C. In this cluster, group I introns are found only in the WA variant of *N. lovaniensis*, in the LSU rDNA as well as in the SSU rDNA (De Jonckheere and Brown 1998a, 2001).

Three other species that grow at up to 44-45°C, *N. morganensis*, *N. niuginensis* and *N. sturti*, form a closely-related cluster. Also in this second cluster, one species (*N. morganensis*) contains group I introns in the LSU rDNA. However, group I introns are not found in the SSU rDNA of this species.

The third cluster contains *N. minor*, one of the two *Naegleria* spp. in which the flagellate stage divides. It is not thermophilic (upper growth temperature limit of 38°C), although the second species in this cluster, *N. carteri*, is thermophilic (upper growth tem-

perature limit of 45°C) and has a group I intron in the SSU rDNA.

In the fourth cluster, both species are less thermophilic (upper growth temperature limits of 40 and 42°C, respectively). They had been described originally as two subspecies, but the phylogenetic analysis of SSU rDNA sequences confirms their independent relationship. In addition, both *N. andersoni* and *N. jamielsoni* contain a group I intron in the SSU rDNA (Table 3).

Except for *N. minor*, the species in the two clusters above have a 5.8S rDNA of 174 bp length (Table 2). All other *Naegleria* spp. have a 5.8S rDNA of 175 bp, except *N. pussardi* in cluster 6 where it is also 174 bp.

Cluster 6 is the most distant cluster in the *Naegleria* tree. The two species in this cluster are also quite different from each other in many respects. While *N. pussardi* grows at a maximum temperature limit of 41°C, *N. chilensis* only grows at a maximum of 30°C. In addition, *N. chilensis* could not be induced to transform into a flagellate stage under laboratory conditions, and in *N. pussardi* the nucleolus behaves differently during prophase than those in other *Naegleria* spp.

The 14 other *Naegleria* spp. form one big cluster 5. In this cluster, *N. galeacystis*, *N. robinsoni* and *N. indonesiensis* could be considered a subcluster although the latter three species have very little in common. *Naegleria robinsoni* is one of the two *Naegleria* spp. in which the flagellate stage can divide, while *N. indonesiensis* could not be induced to transform into flagellates under laboratory conditions. However, the single strains of these three species have a similar upper temperature limit for growth between 35°C and 38°C.

The 11 other species in cluster 5 vary in pathogenicity, temperature tolerance (Table 1) and in the presence of a group I intron in the SSU rDNA (Table 3). The length of the ITS1 of all strains in this big cluster is 33 bp, except for *N. jadini*, in which it is 35 bp (Table 2). Also, in the SSU rDNA phylogeny *N. jadini* seems to be the most distant species in cluster 5 (Fig. 2). Big differences in length of the ITS2 (from 100 to over 500 bp) are observed in species belonging to this cluster. The four different clusters of the *N. gruberi sensu lato* branch within cluster 5 as well (De Jonckheere 1994a). In the present monograph these four lineages of the *N. gruberi* species complex are given species status: *N. gruberi sensu stricto*, *N. tihangensis*, *N. pringsheimi* and *N. pagei*.

It has not been the intention to split the genus into as many different species as possible. As a matter of fact,

strains of *N. lovaniensis*, a species that is closely-related to the pathogenic *N. fowleri* in many respects (except that it is not pathogenic) were called at first nonpathogenic *N. fowleri* variants (De Jonckheere and van de Voorde 1977b). The use of molecular biology techniques allowed a much more precise delineation and separation of species. Proper identification of thermophilic *Naegleria* strains is important as it was first thought that all *Naegleria* strains isolated at 45°C were pathogenic *N. fowleri*.

Our strategy of defining species on the basis of SSU rDNA can lead to other proposals than describing more species. This technique can also lead to assembling several genera under one genus name. The situation presently concerning the other vahlkampfiids is an example of this.

Other genera in the Vahlkampfiidae

The genus *Vahlkampfia* has gained notoriety recently because some cases of keratitis have been attributed as being caused by strains of this genus (Aitken *et al.* 1996, Alexandrakis *et al.* 1998, Dua *et al.* 1998) as well as by strains of *Hartmannella* (Kennedy *et al.* 1995, Aitken *et al.* 1996, Aimard *et al.* 1998, Inoue *et al.* 1998) and even by a strain of *Naegleria* (Dua *et al.* 1998) (Table 6). Yet, in none of these keratitis cases is there enough evidence for the isolated strains to be confirmed as the cause of the keratitis infection (De Jonckheere and Brown 1998b, c, d, 1999a). In a recently published study of amoeba strains from keratitis patients in Austria, no proof was found either that the three *Vahlkampfia* and two *Hartmannella* isolates were of clinical relevance (Walochnik *et al.* 2000).

The genus *Vahlkampfia*, as previously defined, consists of species in which the amoebae are unable to transform into flagellates as the common character (Page 1988). When it was found that *V. jugosa* did form flagellates after all, it was described as another genus, *Paratetramitus jugosus* (Darbyshire *et al.* 1976).

Recently we concluded that the original assignment of all vahlkampfiid species which lack a flagellate phase to a single genus was inappropriate as they form different clusters on the basis of SSU rDNA analyses. It was proposed that the seven *Vahlkampfia* spp. should be partitioned between four genera (Brown and De Jonckheere 1999): *V. avara* and *V. inornata* retain the genus name *Vahlkampfia*, while *V. aberdonica*, *V. lobospinosa* and *V. enterica* are transferred to the genus *Tetramitus*, together with *Didascalus* and *Paratetramitus*. The difference in SSU rDNA between

Paratetramitus and *Didascalus* is 1 %, while the difference between these and *Tetramitus* amount to 10 %, exactly within the range we found for *Naegleria* spp. The two other *Vahlkampfia* spp. became two new, distantly related genera: *Neovahlkampfia* and *Paravahlkampfia*. Thus, while the genus *Vahlkampfia* was split into different genera, some species of it and other genera were actually assembled in the genus *Tetramitus*. As a result the genus *Tetramitus* contains species that form flagellates, and species that do not form flagellates, as is also now the case in the genus *Naegleria*. In *Vahlkampfia*, the phylogenetic trees based on the whole SSU rDNA molecules were the same as those based on approx. 800 bp of the molecule. We are therefore confident that in *Naegleria* the phylogeny can be based on the approx. 800 bp spanned between the two Pst I sites, typical for the genus, and that it is not necessary to sequence the whole molecule.

The genus *Acanthamoeba*

The genera *Acanthamoeba* and *Balamuthia* do not belong to the Vahlkampfiidae; they are even phylogenetically unrelated. However, it is appropriate to mention the situation in these genera here because in the free-living genera *Acanthamoeba*, *Balamuthia* and *Naegleria* pathogenic species occur and they are, therefore, studied in the same laboratories. In the genus *Acanthamoeba* a SSU rDNA analysis has also been performed using the whole SSU rDNA sequence (Stothard *et al.* 1998). The situation in *Acanthamoeba* was more confused by the fact that many different species had been described already purely on the basis of morphology before biochemical and molecular typing started, which is in contrast to *Naegleria*. It turned out that different morphological species of *Acanthamoeba* cluster in sequence type 4. Although Stothard *et al.* (1998) are in favor of the proposal that each cluster should be equated with a single species, they prefer to call them sequence types until strains have been included of all the other *Acanthamoeba* spp. that were not sequenced yet. As is the case in several *Naegleria* spp., some *Acanthamoeba* sequence types are represented by only one isolate. Only in some cases *Acanthamoeba* sequence types did correspond to one described species, such as sequence type 5, which was shown to be a homogeneous group of strains of *A. lenticulata*. In sequence type four, 24 out of 25 *Acanthamoeba* keratitis isolates cluster. The other keratitis strain is a sequence type 3 (*A. griffini*). Recently, a keratitis isolate turned out to belong to sequence type 11 (Walochnik *et al.* 2000). Isolates that cause

encephalitis appear to cluster in sequence types 1 and 12 (Stothard *et al.* 1998). As is the case in the genus *Naegleria*, pathogenic strains belong to different species, or sequence types, in the genus *Acanthamoeba*.

Group I introns are also present in the SSU rDNA of *Acanthamoeba* albeit in only two species out of 16 investigated (Gast *et al.* 1994, Schroeder-Diedrich *et al.* 1998) compared to nine out of 26 *Naegleria* spp. investigated (Table 3).

It was suggested that the sequence types of cyst morphology group I in *Acanthamoeba* should probably be reclassified as one or more different genera. The situation resembles, therefore, what we observed in the genus *Vahlkampfia*, where two new genera were erected on the basis of the SSU rDNA sequences. But, as for the species reclassification, no formal proposal for genera names was made for *Acanthamoeba*. There is around 10 to 11% difference between types that could be reclassified as distinct genera in *Acanthamoeba*. Therefore, it could be argued that cluster 6 in *Naegleria*, in which *N. pussardi* and *N. chilensis* differ by about 12% from the other *Naegleria* spp. in their SSU rDNA, could also be reclassified as another genus. This would mean we would be even more like splitters than like lumpers. However, the decision to rename two *Vahlkampfia* spp. into new genera (Brown and De Jonckheere 1999) was based on a SSU rDNA difference of around 40%, and not 10%. Indeed, one has to realise that the genus *Naegleria* branched off much earlier than *Acanthamoeba* and, therefore, species in the genus *Naegleria* have had more time to diverge than species in the genus *Acanthamoeba*. Therefore, 10% evolutionary distance means more in the younger *Acanthamoeba* genus than it does in the ancient *Naegleria* genus. In species that evolved during different timescales the molecular distances can not be compared in the same way for the definition of a species.

The genus *Balamuthia*

Like *Naegleria* and *Acanthamoeba*, *Balamuthia mandrillaris* invades the brain and lungs of experimentally infected mice after IN instillation (Visvesvara *et al.* 1993). Several human brain infections, previously reported to be caused by *Acanthamoeba*, have later been attributed to *B. mandrillaris* (Visvesvara *et al.* 1990). While it was first believed *B. mandrillaris* was a leptomyxid amoeba, a recent phylogenetic analysis of the SSU rDNA of the Leptomyxidae, revealed that *B. mandrillaris* does not belong to the Leptomyxidae, but to the Acanthamoebidae (Zettler *et al.* 2000). Until

now, only one species has been recognized in the genus *Balamuthia*, and no group I introns are reported to be present.

ECOLOGY

Naegleria spp. are found in water and soil but they cannot live in seawater. The pathogenic *N. fowleri* prefers water with high temperatures and, therefore, grows abundantly in thermal waters, be it geothermal or industrial cooling water. Because of special circumstances, South Australia is about the only place where *N. fowleri* is present in public water supplies. In these water supplies a positive association between *N. fowleri* was found with temperature, bacteriological counts and other *Naegleria* spp., while a negative association was evident with free and total chlorine residuals (Esterman *et al.* 1984). In a swimming pool that had been identified as the source of an outbreak of 16 PAM cases, *N. fowleri* and other thermophilic *Naegleria* spp. were found to proliferate in a cavity behind a damaged wall of the pool (Kadlec *et al.* 1980).

Although a lot has been published on the presence of *N. fowleri* in thermal waters, very little research has been done on the accompanying *Naegleria* spp. in these waters. The first other thermophilic *Naegleria* spp. to be recognised was *N. lovaniensis*, which seems to be quite prevalent. The presence of *N. lovaniensis* has been taken as a signal that conditions are right for the growth of *N. fowleri*. It is of significance, therefore, that high numbers of *N. lovaniensis* were found in two hospital hydrotherapy pools (De Jonckheere 1982b). In aquaria *Naegleria* is the predominant amoeba genus and a high percentage of these *Naegleria* isolates is thermophilic (De Jonckheere 1979c).

It was only when allozyme and DNA techniques were used that some information on the ecology of other *Naegleria* spp. became available. To my knowledge, there are presently only three laboratories that use allozyme analyses routinely for identification of *Naegleria* spp. isolated over longer periods of time: my Protozoology Laboratory in Brussels, Belgium, Le Laboratoire de Biologie Cellulaire of Pierre Pernin in Lyon, France, and the Protozoology Section of the Australian Water Quality Centre of Bret Robinson in Adelaide, South Australia. In these three laboratories some interesting results concerning the presence of thermophilic *Naegleria* spp. were obtained.

Although good species identification methods are available now for *Naegleria*, it seems that there is still a problem with isolation methods. The recovery rates of *Naegleria* from water is very low by filtration. Although the recovery by centrifugation is much better, it is still only 22% and 57% for trophozoites and cysts, respectively (Pernin *et al.* 1998). I have used the centrifugation method for concentrating water samples since the severities (De Jonckheere 1978) as it is more efficient, diminishes competition between different species and is less work to quantitate the amoebae.

The Australian Water Quality Centre has a lot of data and a huge collection of strains (Dobson personal communication) but unfortunately, limited data have been published. It was observed in Australia that the upper temperature limit for growth of each described *Naegleria* sp. varies narrowly (<2°C) over many isolates (Robinson *et al.* 1996a). The temperature optimum for each species is approximately 4°C lower than the upper temperature limit. Natural populations of *Naegleria* spp. display temperature-correlated seasonal successions of species occupying different temperature frames. The Australian Water Quality Centre found, for example, that Hope Valley Reservoir in South Australia supports 12 *Naegleria* spp. at densities up to 320 l⁻¹ (Robinson *et al.* 1996a). At this place in South Australia, *N. fultoni* (not thermophilic), *N. australiensis* and the WA variant of *N. lovaniensis* were found, but never *N. fowleri* nor *N. lovaniensis*, although these two are closely related to the latter one. The recently described species, *N. carteri*, *N. morganensis*, *N. sturti* and *N. niuginensis*, seem to colonise artificial environments, such as water supplies, rather poorly, in contrast to *N. fowleri* and *N. lovaniensis* (Dobson *et al.* 1997). The colonisation of *N. fowleri* in different water supply pipelines in four Australian states occurs as discrete, infrequent events, rather than as continuous contamination, reflecting increased temperature and poor distribution of chlorine (Robinson *et al.* 1996b).

This discontinuous presence of *N. fowleri* is also observed in the cooling water of industries in Belgium (De Jonckheere and van de Voorde 1977a) and in fish farms using heated waters (De Jonckheere *et al.* 1983b). During isolation campaigns over the last two years I found that none of the described thermophilic *Naegleria* spp. apart from *N. fowleri*, *N. lovaniensis*, *N. jamielsoni*, *N. australiensis* and *N. tihangensis*, are encountered in Belgium (De Jonckheere unpublished). When the water temperature drops below 24°C no thermophilic *Naegleria*

are detected. The latter seems to be in contradiction with previous reports in Belgium (De Jonckheere and van de Voorde 1977a, De Jonckheere *et al.* 1983b), however, during the recent campaigns no sediments were sampled. In lakes in Florida pathogenic *Naegleria* were not detected in water samples when the water temperature dropped below 26.5°C, although the organism remained present in sediment samples (Wellings *et al.* 1977). Also, the cooling waters were disinfected with chlorine and, consequently, the *Naegleria* cannot use spots with higher water temperature within the cooling system to proliferate. In previous investigations the temperature of the water where the amoebae proliferated was probably higher than that of the water sampled.

Although the laboratory of Pernin in France has vast information on the occurrence of the other *Naegleria* spp. typed by allozyme, little of it has been published. In France up to 3,000 *N. fowleri* are detected per liter in cooling water and 80 l⁻¹ in the river downstream from the electricity power plant (Pringuez *et al.* 1998). In that study it was not indicated how far downstream sampling was conducted. In Poland, pathogenic *N. fowleri* were detected repeatedly up to 13 km downstream from the warm discharge of an electric power station (Kasprzak *et al.* 1982).

There is only minimal information on the occurrence of *Naegleria* spp. other than *N. fowleri* in continents besides Europe and Australia. A study by John and Howard (1995) indicates that *N. australiensis* seems to be prevalent in the USA. Attempts in my laboratory to isolate thermophilic *Naegleria* spp. from African mud samples yielded many strains that did not correspond to species that were described prior to 1990. While no *N. fowleri* could be isolated from the samples, several strains of *N. australiensis* were identified, as well as *W. magna* (De Jonckheere and Bafort 1990). Also, the Australian Water Quality Centre isolated many *Naegleria* strains from all over the world (Dobson personal communication) and some of the newly-described *Naegleria* spp., such as *N. niuginensis*, *N. chilensis* and *N. indonesiensis*, are actually based on unique strains from other continents (Dobson *et al.* 1997). Also, the descriptions of *N. andersoni* and *N. jamiesoni* were based on strains that I had isolated from waters with fish imported from other continents (De Jonckheere 1988a).

Several descriptions of *Naegleria* sp. are based on only one known strain or on strains from only one place, such as *N. jadini*, *N. pussardi*, *N. niuginensis*, *N. galeacystis*, *N. minor*, *N. robinsoni*, *N. chilensis*, *N. indonesiensis*, *N. italica*, *N. morganensis* and

N. fultoni. The most notoriously restricted occurrence of a *Naegleria* sp. is *N. italica*, which has only been detected in one place in Italy until recently, although the Australian Water Quality Centre, especially, searched for it all over the world (Dobson and Robinson personal communication). These observations on the restricted occurrence of certain *Naegleria* spp. are in conflict with the idea of ubiquitous dispersal of microbial species (Finlay and Clarke 1999). I believe this conflict is caused by the fact that these authors, who support this ubiquitous dispersal of species, use morphotypes as the basis of species, while morphotypes might actually constitute genetically different species. The different morphotypes these authors refer to are far more different in the SSU rDNA sequences than species of *Naegleria*. In this hypothesis most *Naegleria* spp. would be treated as one morphotype and, therefore, it is actually the genus that is ubiquitous, not the different species.

It is sure many more *Naegleria* spp. are awaiting species description and it will be important to keep the type strains in culture collections. As can be noticed in the species descriptions above, not all type strains have been submitted in the past. This is a pity because strains of *Naegleria* can easily be cryopreserved (Simione and Daggett 1976, Kilvington and White 1991, Brown and Day 1993, Menrath *et al.* 1995). The Australian Water Quality Centre keeps a large collection of their isolates cryopreserved and these collections are a goldmine for people like me who investigate the biodiversity of the genus *Naegleria*.

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