

# Identity of *Naegleria* strains isolated from organs of freshwater fishes

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**ABSTRACT:** Eighteen *Naegleria* strains were isolated from organs of freshwater fishes belonging to 5 species. Morphometric study allowed the separation of the *Naegleria* strains from the non-vahlkampfiid amoeboflagellates, but was inadequate for species determination. Six strains, representatives of groups that had a slightly different cyst size, were selected and corresponding derived clones were subjected to sequence analysis and riboprinting restriction fragment length polymorphism (RFLP)-PCR analysis of the small subunit (SSU) rRNA genes. One strain isolated from the brain of a fish with systemic infection was characterised by an intronless 2 kb long SSU rRNA gene and was identified as *N. australiensis*. Another 5 strains had a 1.3 kb long group I intron in their SSU rRNA gene and, based on the SSU rRNA sequences and riboprints, RFLP-PCR patterns appeared in phylogenetic trees to be closely related to *Naegleria clarki*.

**KEY WORDS:** *Naegleria* spp. · Fish strains · Molecular taxonomy · SSU rRNA

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## INTRODUCTION

The genus and species composition of amoebae that invade fish as well as their biology and host-parasite relations are of fundamental interest to fish pathologists because mortalities attributed to free-living amoebae have been reported in various species of cultured fishes. Unfortunately, even amoebae reported as the proven or the contributing factor of mortalities have not always been exactly determined, or adequately documented or stored for future identification. This also applies to species of the genus *Naegleria* Alexeieff, 1912, diagnosed in fish. Contrary to the considerable number of environmental *Naegleria* strains isolated since pioneering workers raised interest in the water-related human infections (primary amoebic meningoencephalitis), *Naegleria* strains isolated from organs of fish have only been recorded a few times. Taylor (1977), who screened organs of 23 species of freshwater fishes for the presence of small free-living amoebae, found amoebae of the genus *Naegleria* in 11 fish

hosts. *Naegleria gruberi* was isolated along with other species of free-living amoebae from the intestinal contents of white suckers *Catostomus commersoni* and common shiners *Notropis cornutus* (Franke & Mackiewicz 1982). Webb et al. (1998) tentatively identified a *Naegleria* sp. in fishes from the tidal James River, Virginia, USA. *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis in humans, was described in 1970 (Carter 1970). Until that year any amoeba that had characteristics of the genus *Naegleria* was determined as *N. gruberi* Schardinger, 1899. As late as the 1980s any isolate that did not cause disease in mice was considered to be *N. gruberi* (De Jonckheere 1987). In the first taxonomic revision of free-living amoebae, 5 species of the genus *Naegleria* were defined using morphology and isoenzyme analysis (De Jonckheere 1987). Since that time, molecular criteria, mainly partial small subunit (SSU) rRNA sequences, enabled the revision of species names and the establishment of new species of the genus *Naegleria* (De Jonckheere 1988, 1994a, 1998, Pernin & De Jonckheere 1996, De Jonckheere & Brown 1999). Recently the genus *Naegleria* was divided into 17 species (De Jonck-

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heere & Brown 1999). Prior studies have not subjected *Naegleria* strains isolated from fish organs to molecular analyses. Several strains designated as isolated from tropical fish imports (De Jonckheere 1988) were actually isolated from aquarium water. One strain was isolated from the kidney of goldfish (see 'Discussion') but its molecular characteristic was not presented.

The intent of the present study was to determine selected *Naegleria* strains that were isolated from fish organs during our long-term study of free-living amoebae capable of colonising fish.

## MATERIALS AND METHODS

Eighteen *Naegleria* strains isolated from organs of 18 freshwater fish belonging to 5 species and 1 hybrid were included in the study. Most of the fish (*Blicca bjoerkna*, *Oncorhynchus mykiss*, *Perca fluviatilis*, *Salmo trutta* and *Salvelinus fontinalis*) were collected in South Bohemia, Czech Republic, while the hybrid of *Clarias macrocephalus* × *garipepinus* was collected from a fish farm in Thailand. The agar-plate isolation, culturing, sub-culturing and cloning of amoebae were as described previously (De Jonckheere 1980, Dyková et al. 1997). All observations and measurements of living trophic amoebae were made in hanging drops. Cysts were observed and measured in wet mounts on an agar layer to fix them in a steady position, and in permanent mounts after impregnation using the method by Pussard & Pons (1979). One clone was derived from each of the strains and used in further work. All clones were tested for production of temporary flagellated stages and subjected to morphological and morphometric analysis including observation in the transmission electron microscope. Six clones that had slight differences in cyst size were selected for molecular taxonomy. They are listed in Table 1 along with their origin and morphometric characteristics. Clones were grown in Bacto Casitone (BCS) medium that was changed daily (Červa 1969) enriched with ram serum and antibiotics (penicillin 100 U, streptomycin 100 µg and neomycin 200 µg ml<sup>-1</sup>). In this medium the axenisation of clonal cultures was not achieved in fish clones (e.g., in clone CB2B/I not even after 53 passages). In Chang's

serum casein glucose yeast extract medium, the growth of bacteria contaminating clonal cultures of naegleriae was even more massive. The *Naegleria fowleri* strain 0360 (obtained through the courtesy of Dr L. Červa) used as a control was maintained in axenic culture. Amoebae suspensions containing 1 to 3 × 10<sup>8</sup> cells ml<sup>-1</sup> were used for DNA extraction.

The method of DNA isolation followed basically the protocol by Maslov et al. (1996). Pelleted cells were suspended in NET 50 (10 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0) and lysed with Pronase E (Sigma, Aldrich, Prague) at the final concentration of 0.35 mg ml<sup>-1</sup> and 3% N-lauryl-sarcosyl (Sigma). The lysates were phenol/chloroform extracted and DNA was precipitated with ethanol, dissolved in distilled water and stored at -20°C. The SSU rRNA gene was amplified by PCR using the 5' and 3'-end specific primers XY2 (TACCTGGTTGATCCTGCCAG) and Y25 (AAATGATCCCTACGCAGGTT) (Clark & Cross 1988). PCR reactions were performed in a total volume of 25 µl with 25 pmol of each primer, reaction buffer, 10 nmol of deoxyribonucleoside-5'-triphosphates and 1 U of Taq polymerase (TaKaRa, Prague) according to the manufacturer's instructions. The PCR conditions were as follows: DNA predenaturation at 95°C for 5 min was followed by 30 cycles of amplification that consisted of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min, and ended by an extension at 72°C for 8 min. The PCR products were cloned into the pCR<sup>®</sup> 2.1-TOPO cloning vector (Invitrogen, Groningen) and sequenced using the Big Dye kit (Perkin Elmer Applied BioSystems, Boston) on the ABI 377 automatic DNA sequencer. Parallel extraction, amplification and sequencing were performed on the *Naegleria fowleri* strain 0360. Sequences were assembled using the program SeqMan II (DNASTAR Inc., Madison, WI, USA) and aligned using the program CLUSTAL X (Thompson et al. 1997) with other available SSU rRNA sequences of amoebae of the genus *Naegleria* and family Vahlkampfiidae obtained from GenBank and analysed using the program PAUP (version 4.0b4, Sinauer Associates, Sunderland, MA, USA). The GenBank accession numbers for our sequences are as follows—clone Pd72Z/I: AF338417; 4177/I: AF338418; 4564/IV: AF338419; 4709/I: AF338420; CB2B/I: AF338421;

Table 1. Origin of *Naegleria* clones subjected to molecular analyses

Clone	Fish host	Organ	Locality	Cysts (µm)
4709/I	<i>Perca fluviatilis</i>	Spleen	Černovický potok brook, Czech Rep.	8.6 (5.0–12.5)
4177/I	<i>Blicca bjoerkna</i>	Brain	Dvořiště pond, Czech Rep.	9.1 (7.0–12.5)
Pd56Z/I	<i>Oncorhynchus mykiss</i>	Gills	Černá brook, Czech Rep.	9.8 (8.0–12.5)
4564/IV	<i>Perca fluviatilis</i>	Kidney	Černovický potok brook, Czech Rep.	10.6 (8.0–13.0)
Pd72Z/I	<i>Oncorhynchus mykiss</i>	Gills	Černá farm, Czech Rep.	10.7 (8.7–14.5)
CB2B/I	<i>Clarias</i> (hybrid)	Brain	Fish farm, Thailand	10.9 (8.0–12.5)

Pd56Z/I: AF338422; and 0360: AF338423. For compared sequences, the accession numbers are *N. lovaniensis* U80062, *N. minor* X93224, *N. jamiesoni* U80061, *N. andersoni* U80057, *N. gruberi* M18732, *N. italica* U80060, *N. australiensis* U80058 and *N. fowleri* U80059.

The restriction fragment length polymorphism-PCR analysis of SSU rRNA genes (riboprints) was performed using the enzymes *AluI* and *HinI*. The SSU riboprints recorded for *Naegleria* species by De Jonckheere (1994b) were used for comparison.

All strains and clones used in this study were cryopreserved and are stored in the culture collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

## RESULTS

### Morphological analysis

The strains belonging to the genus *Naegleria* were isolated from the gills of 11 specimens of *Oncorhynchus mykiss*, 1 specimen of *Salvelinus fontinalis* and various organs of the body cavity of 3 specimens of *Perca fluviatilis* and 1 specimen each of *Blicca bjoerkna*, *Salmo trutta* and *Clarias* hybrid. All strains were isolated from clinically healthy adult fishes. A systemic infection was diagnosed in the *Clarias* hybrid.

The shapes of moving and resting trophozoites of all *Naegleria* clones under study (Figs 1 to 7) were identical and corresponded to the taxonomic criteria set by Page (1967), who summarised several extensive descriptions of *Naegleria* spp. Amoebic trophozoites transformed into flagellated swimming stages within 30 min to 2 h after the agar plate cultures had been overlaid with water. In the majority of the flagellated forms 2 flagella were observed (Fig. 12). Examination of trophozoite ultrastructure revealed mitochondria with the discoid type of cristae (Figs 11 & 14). This feature supported the generic diagnosis. All clones produced spherical cysts of identical type with a smooth, thick, refractile wall and a perinuclear layer of granules (Figs 8 & 9). The wall consisted of a thick inner layer and a thin outer layer and contained pores with plugs (Figs 13 & 15). Statistical analysis of cyst diameter revealed that only in cysts at least 6 d old did their diameter reach a constant value that could be used as a clonal characteristic. The comparison of cyst diameters provided the only criterion to sort our assemblage of morphologically similar *Naegleria* clones. The average diameters of cysts ranged from 8.6 to 14.2  $\mu\text{m}$  in individual clones. Six clones (Table 1), the average cyst diameters of which covered the range of our assemblage, were selected and determined as

described below. Strains under study were isolated and cultured at 20°C. With the exception of the clone CB2B/I they did not tolerate 37°C. The clone CB2B/I was grown in 3 consecutive passages at 37°C. It did not tolerate higher temperatures.

### Molecular analysis

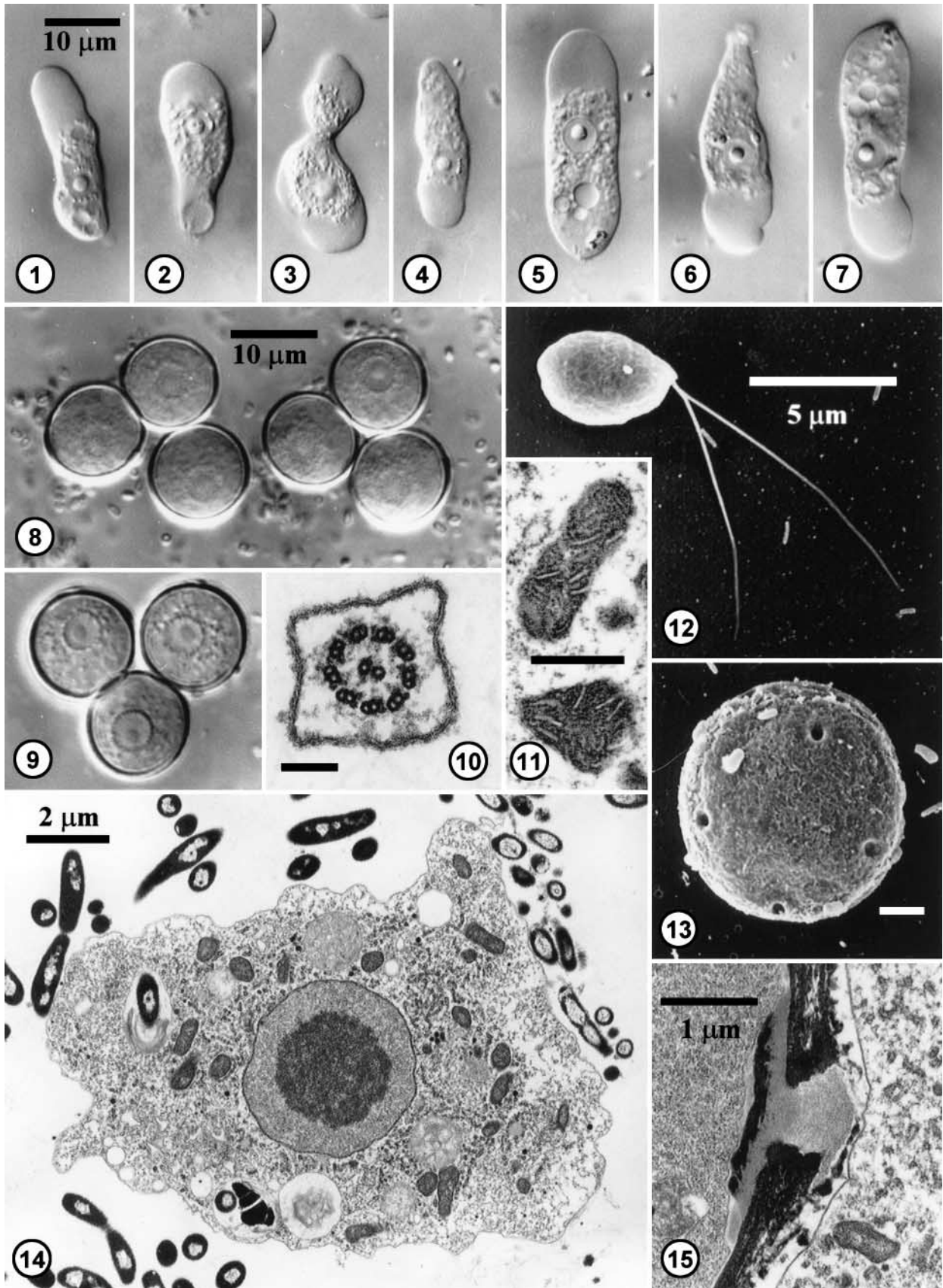
PCR amplification of the SSU rRNA genes divided the analysed assemblage of clones into 2 groups (Fig. 16). The first group, which consisted of 5 clones listed in Table 1 (4709/I, 4177/I, Pd56Z/I, 4564/IV and Pd72Z/I), was characterised by the SSU rRNA gene that was 3.3 kb long and contained an intron. Sequence analysis revealed that the introns are invariably 1305 bp long and situated between nucleotide positions 630 and 631, and belong to the group I introns as described by Embley et al. (1992) and De Jonckheere (1993). The group I intron sequences in 5 clones were identical. They differed from group I intron in *Naegleria clarki* (EMBL No. X78281) in 3 bases out of 1305 nt. In all 5 clones the SSU rRNA gene is 1980 bp long. The sequences differ from each other on the average in 4 bp, most of which were transitional. Except for clone 4177/I, the riboprinting patterns obtained using the restriction enzymes *AluI* and *HinI* (Fig. 17) were almost identical and were homologous to the riboprint of *N. clarki* (strain RU42) (De Jonckheere 1994b). In the 4177/I riboprint there was partial digestion with *AluI* (Fig. 17).

In the remaining clone CB2B/I as well as in *Naegleria fowleri* strain 0360, a 2 kb band was amplified with the SSU rRNA specific primers. The gene was 1980 and 1988 nt long in CB2B/I and 0360, respectively.

A search of GenBank identified *Naegleria australiensis* as the closest match to CB2B/I, and the strain 0360 was identical to *N. fowleri*. Phylogenetic analysis using maximum likelihood and maximum parsimony methods showed a similar result (Fig. 18). We confirmed a monophyly of the genus *Naegleria* within the family Vahlkampfiidae (data not shown). Within the genus *Naegleria* at least 3 monophyletic clades can be observed: *N. fowleri* and *N. lovaniensis*, *N. andersoni* and *N. jamiesoni*, and the biggest group involving *N. clarki*, *N. gruberi*, *N. italica* and *N. australiensis*. The 5 SSU rRNA intron-containing strains form a coherent group at the top of the third clade.

## DISCUSSION

Discussing our results and those achieved by other authors, we can hardly avoid frequent use of 2 terms: strain and clone. The term 'strain' was used mostly in



Figs 1 to 15. Morphology of *Naegleria* strains under study. Figs 1 to 7. Representative trophozoites of 4 *Naegleria* strains isolated from organs of fishes as seen in hanging drops. Nomarski differential interference contrast. Scale bar in Fig. 1 applies to Figs 1 to 7. Figs 1 & 2. Clone 4709/I. Fig. 3. Clone 4177/I. Fig. 4. Clone Pd56Z/I. Figs 5 to 7. Clone CB2B/I. Figs 8 & 9. Cysts of *Naegleria* clones 4709/I and 4564/IV, respectively. Nomarski differential interference contrast. Scale bar applies to both figures. Fig. 10. Transverse section of the flagellum of the temporary flagellated stage (clone CB2B/I). Scale bar = 100 nm. Fig. 11. Mitochondria containing discoid cristae typical of the genus *Naegleria*. Scale bar = 500 nm. Fig. 12. Scanning electron microscopy showing a trophozoite of the clone 4564/IV transformed into the flagellated form. Scale bar = 5  $\mu$ m. Fig. 13. Scanning electron microscopy showing a cyst of the *Naegleria* strain 4709/I with 4 pores. Scale bar = 2  $\mu$ m. Fig. 14. Transmission electron microscopy showing a trophozoite of the clone Pd72Z/I. Fig. 15. Transmission electron microscopy showing the cyst wall sectioned at the level of a plugged pore (clone Pd72Z/I)

previous papers, although actually clones derived from strains were used in those studies.

Trying to determine the isolated strains we faced the problem of species delineation in the genus *Naegleria*, which has a long history with many naegleriologists involved (De Jonckheere 1994a). Recently, using molecular techniques, the validity of 17 species was confirmed (De Jonckheere 1994a,b, De Jonckheere & Brown 1999) including all upgraded subspecies. Among *Naegleria* strains studied and recorded in the literature thus far (De Jonckheere 1987, 1988, 1993, 1994a,b, De Jonckheere et al. 1984, Baverstock et al. 1989), our assemblage of strains isolated from fish organs is unique. Since amoebae isolated from gills of clinically healthy fish may be considered temporarily epizootic and strains derived from such isolates as belonging to the water environment, isolation of strains from liver, spleen, kidney and brain of fish shows the capability of *Naegleria* strains for an endozoic way of life in the heterotherm organisms. This stresses the necessity to clarify the pathogenic potential of *Naegleria* spp. for fish.

Sequence analysis of the SSU rRNA gene of 5 strains revealed a slight difference (4 bp) mostly considered as transitional. It is interesting to note that 5 strains isolated from different organs of different fish species collected in different localities showed the same restriction pattern and form a coherent group embracing

*Naegleria clarki*. This species was established by De Jonckheere (1994a) for 2 strains (RU30 and RU42) as the only representatives of the species. Both strains were isolated from a hot spring in Rotorua, New Zealand, and tested as non-pathogenic for mice. Except for the type of culture medium we have no data on culturing conditions, including the temperature that was required by strains RU30 and RU42, to compare with the culture condition of the 5 clones that we assigned to the same species (*N. clarki*). Therefore, we can only speculate that a *Naegleria* species might embrace strains that differ in their temperature tolerance. This assumption appears to be supported by data mentioned by De Jonckheere (1988) for *N. australiensis*, *N. italica* (formerly *N. australiensis australiensis* and *N. australiensis italica*, respectively) and *N. gruberi*. After his strains had been adapted to axenic culturing, they grew very well at temperatures that were lower than the highest temperature tolerated in monoxenic culture.

Since clone CB2B/I, which is the agent of systemic infection diagnosed in the *Clarias* hybrid, was identi-

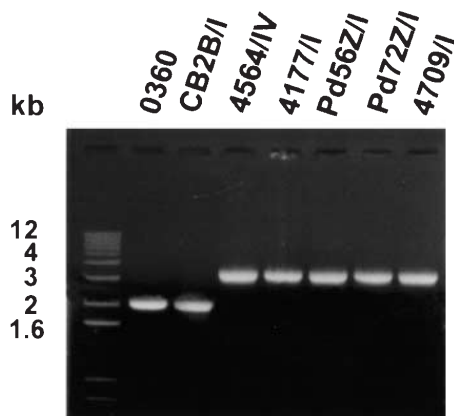


Fig. 16. PCR amplified small subunit rRNA gene of the *Naegleria* strains under study. Size marker on the left

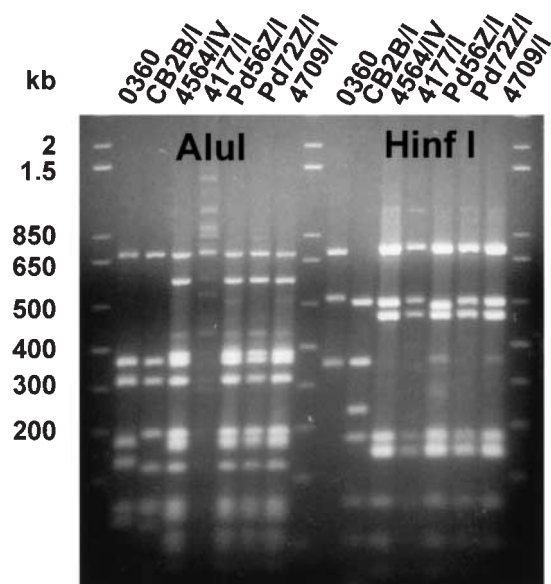


Fig. 17. *AluI* and *HinfI* riboprint patterns of *Naegleria* strains

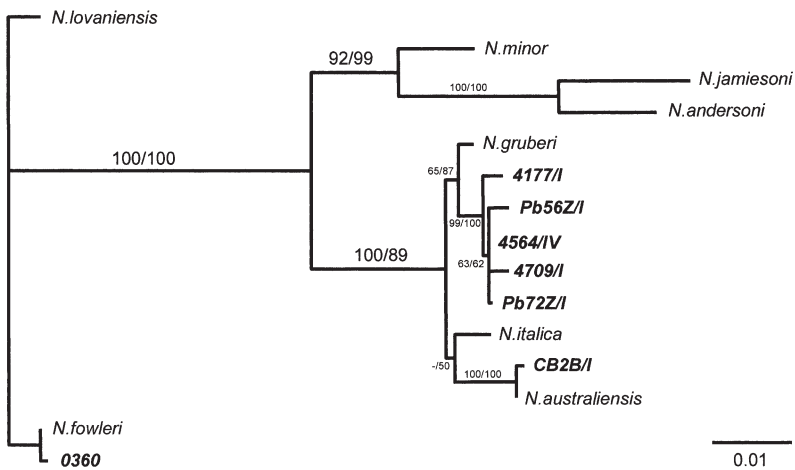


Fig. 18. Maximum likelihood phylogram ( $-\ln = 4660.8$ ) based on 1990 character long alignment (170 informative sites). The tree was rooted using *Naegleria fowleri* strain 0360 and *N. lovaniensis* as an outgroup. Newly obtained sequences are in bold. Maximum parsimony produced the 3 most parsimonious trees with a length of 315 steps and consistency index of 0.8. The tree calculated by the distance method (general time reversal) had the best score of 0.152. Numbers on the branches indicate nodal support for 1000 replicates calculated by the maximum parsimony/distance method (general time reversal)

fied as *Naegleria australiensis*, the ability of the clone to colonise both hetero- and homoiotherm organisms along with its pathogenicity for mice have to be tested experimentally. The virulence and pathogenicity of the type strain of *N. australiensis* (PP 397) were tested repeatedly and the pathogenicity was proved after intracerebral inoculation of axenically grown amoebae (De Jonckheere 1981). There was also 1 strain isolated in 1989 from kidney of goldfish *Carassius auratus* deposited in ATCC (50387) and identified later as *N. australiensis* (De Jonckheere pers. comm.).

Surprisingly, none of our determined clones were grouped with *Naegleria gruberi*, considered for a long time to be widely distributed. Although the attempts at separating *Naegleria* species or morphotypes on the basis of cyst morphology (Page 1975, Pussard & Pons 1979) have proved unreliable (De Jonckheere 1987, 1988, Pernin & de Jonckheere 1996), the differences in average cyst diameter were used in our study as an arbitrary method for clone selection. However, our results confirmed that the morphometric characteristics of individual clones, which were extremely variable, could not be aligned with the results based on molecular criteria (see, for example, the difference in average cyst diameters in strains related to *N. clarki*; Table 1).

Phylogenetic analysis based on the SSU rRNA sequences showed results similar to those given by Pélandakis et al. (2000). It has been shown previously that the results of phylogenetic analysis depend on the *Naegleria gruberi* strains used. It is known that there are 4 clusters within the *N. gruberi* species that branch in

different positions. In this study we used NEG-M *N. gruberi*. Five strains with intron branched in the same position as *N. clarki* and a sister group of *N. gruberi*, *N. italica* and *N. australiensis*, in line with the results of Pélandakis et al. (2000). There is no sequence of *N. clarki* in GenBank, but our riboprint results permitted us to compare the restriction pattern with that of *N. clarki* (RU-42), which helped us to identify our strains and to compare group I intron sequences.

The results obtained thus far suggest the need for further study of fish isolates and indicate that fish hosts may yield important material for research on *Naegleria* spp. and for studies on their distribution in the aquatic environment.

**Acknowledgements.** This research was funded by the Grant Agency of the Academy of Sciences of the Czech Republic (Project No. A6022802) and by the Ministry of Education (Project No. MSM123100003).

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Editorial responsibility: Wolfgang Körting,  
Hannover, Germany

Submitted: February 3, 2001; Accepted: April 4, 2001  
Proofs received from author(s): August 15, 2001