Fish-isolated *Naegleria* strains and their phylogeny inferred from ITS and SSU rDNA sequences

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Abstract. Effort was made to identify *Naegleria* strains isolated from organs of fish, using phylogenetic analyses of SSU rDNA and ITS sequences. Eighteen fish-isolated strains studied enlarged substantially the so far available set of *Naegleria* strains characterized by both molecular markers. The phylogenetic analyses of separate and concatenated SSU rDNA and ITS sequences revealed phylogenetic relationships of strains under study; however, they failed to solve classification of fish-isolated strains into species. The sequence similarity of strain-representatives of *Naegleria* species as well as data obtained on intragenomic variation of ITS sequences discouraged the authors from the definition of new species. The results of the present study provide evidence of a need to re-evaluate the current practice of setting boundaries between species of the genus *Naegleria*. Sequences obtained in this study have been deposited in GenBank with accession numbers DQ768714–DQ768743.

The biomedical importance of free-living amoebae of the genus *Naegleria* Alexeieff, 1912 has been widely recognized and stressed in many original papers published in the field. The research history and the development of methods to identify strains and species of the genus were detailed in a comprehensive review article by De Jonckheere (2002). In this, as well as in a following paper, De Jonckheere (2004) noted that with the exception of the pathogenic species *N. fowleri*, very little had been known about the degree of variation within individual *Naegleria* species and that there had been still many species represented by one strain only. He called for comparison of more isolates of different origin. The summary of data available on the type and other *Naegleria* strains representing 40 species of the genus recorded to date (Table 1) clearly shows that definitions of individual species are based on different molecular markers. Sequences of the small subunit of the nuclear ribosomal DNA (SSU rDNA) and internal transcribed spacer (ITS) are known for approximately one half of the named *Naegleria* species. Other species were defined using sequences of the ITS region only.

Our first screening for the presence of free-living amoebae in organs of fish resulted in isolation of 18 *Naegleria* strains. Six of them were subjected to sequence and ribotyping analyses of SSU rRNA genes (Dyková et al. 2001, Dyková and Lom, 2004). Phylogenetic analyses resulted in identification of one strain from the brain of a fish with *Naegleria australiensis* and another five strains as closely related to *N. clarki*.

Since ITS sequences have been introduced as a tool to resolve phylogenetic relationships between closely related *Naegleria* species and 11 new species have been

recently defined using this tool (De Jonckheere 2004), we have decided to subject a new set of *Naegleria* strains of fish origin to sequencing of both SSU rDNA and ITS and to compare the results of their phylogenetic analyses.

MATERIALS AND METHODS

Strain origin, culturing and morphological studies

Fish-isolated *Naegleria* strains used in the study are listed in Table 2 along with details of their origin. Cryopreserved clonal cultures of these strains have been stored in the culture collection maintained in the Institute of Parasitology (Biology Centre, Academy of Sciences of the Czech Republic).

The assemblage of *Naegleria* strains included in the study consists of 12 novel and 6 strains that were partly characterized in a previous study (Dyková et al. 2001). Except for two strains (CL/I and RR13Z/I) cultured in Bacto Casitone medium enriched with calf serum and antibiotics and changed every other day (Červa 1969), all other strains were grown on non-nutrient agar supplemented with malt and yeast extracts (0.1 g of each per litre) that, together with the bacteria brought by individual cell populations to cultures, served as a food supply. The growth of bacteria and amoebae was balanced during a long period of subculturing.

Clonal cultures of individual strains (maintained at 20°C and subcultured once a week) were used for light microscopic and ultrastructural observations as well as DNA extractions. The maximum temperature limit for culture growth was determined by incubation at 30°C and 37°C. A single attempt was made to establish the influence of low temperature.

Hanging drop preparations and standard light microscopy approaches, including translucent light and Nomarski differential interference contrast (DIC), were used to characterise and

Table 1. *Naegleria* strains assigned to defined species and their molecular characteristics available to date. Species with authorships are listed below the table. Shaded areas indicate the sequences that were used to identify the respective amoebae.

<i>Naegleria</i> species	Strain	SSU rDNA	ITS	Intron
<i>N. americana</i>	CCAP1518/1G			
<i>N. andersoni</i>	A2			
	PPMFB-6			
<i>N. angularis</i>	T692			
<i>N. antarctica</i>	NG409			
<i>N. australiensis</i>	TT			
	Yodo5			
	Yodo6			
	PP397			
	LSR34			
	CB2B/I			
<i>N. byersi</i>	NG597			
<i>N. canariensis</i>	TE1(37)			
<i>N. carteri</i>	NG055			
<i>N. chilensis</i>	NG946			
<i>N. clarki</i>	Pd56Z/I			
	Pd72Z/I			
	4709/I			
	4564/IV			
	4177/I			
	BG6			
	RU30			
	RU42			
<i>N. dobsoni</i>	NG257			
<i>N. dunnebackei</i>	CDCV419			
<i>N. endoi</i>	EDF6			
<i>N. fowleri</i>	0360			
	MCM			
	Northcott			
	Mst1			
	M4E			
	LEE			
	KUL			
	AR12			
	J/16/1/42E			
<i>N. fultoni</i>	NG885			
<i>N. galeacystis</i>	AV500			
<i>N. gallica</i>	Capd60			
<i>N. gruberi</i>	NEG-M			
	AUD1			
<i>N. italica</i>	AB-T-F3			
<i>N. indonesiensis</i>	NG945			
<i>N. jadini</i>	0400			
<i>N. jamiesoni</i>	T56E			
<i>N. johanseni</i>	NG874			
	NG927			
<i>N. laresi</i>	EDF147			
<i>N. lovaniensis</i>	Aq/9/1/45D			
	F9			
	C-0490			
<i>N. martinezi</i>	NG872			
<i>N. mexicana</i>	Mx6J			
<i>N. minor</i>	WTO43			
<i>N. morganensis</i>	NG236			
<i>N. niuginiensis</i>	NG427			
<i>N. pagei</i>	CCAP1518/1E			
<i>N. peruana*</i>	N280			
	NG206			
	RJTM			
<i>N. philippinensis**</i>	RITM-1			
<i>N. pringsheimi</i>	CCAP1518/1D			
	1D			
<i>N. pussardi</i>	EDF258			
	NG260			
<i>N. robinsoni</i>	NG944			
<i>N. schusteri</i>	CCAP1518/1F			
	NG325			
<i>N. sturti</i>	NG334			
<i>N. tenerifensis</i>	TE3(37)			
<i>N. tihangensis</i>	T2A			

Naegleria americana De Jonckheere, 2004; *N. andersoni* De Jonckheere, 1988; *N. angularis* De Jonckheere et Brown, 2005; *N. antarctica* De Jonckheere, 2004; *N. australiensis* De Jonckheere, 1981; *N. byersi* De Jonckheere, 2004; *N. canariensis* De Jonckheere, 2006; *N. carteri* Dobson, Robinson et Rowan-Kelly, 1997; *N. chilensis* De Jonckheere, Brown, Dobson, Robinson et Pernin, 2001; *N. clarki* De Jonckheere, 1994; *N. dobsoni* De Jonckheere, 2004; *N. dunnebackei* Visvesvara, De Jonckheere, Marciano-Cabral et Schuster, 2005; *N. endoi* De Jonckheere, 2004; *N. fowleri* Carter, 1970; *N. fultoni* De Jonckheere, Brown, Dobson, Robinson et Pernin, 2001; *N. galeacystis* De Jonckheere, 1994; *N. gallica* De Jonckheere, 2004; *N. gruberi* Schardinger, 1899; *N. italica* De Jonckheere, Pernin, Scaglia et Michel, 1984; *N. indonesiensis* De Jonckheere, Brown, Dobson, Robinson et Pernin, 2001; *N. jadini* Willaert et Le Ray, 1973; *N. jamiesoni* De Jonckheere, 1988; *N. johanseni* De Jonckheere, 2004; *N. laresi* De Jonckheere, 2004; *N. lovaniensis* Stevens, De Jonckheere et Willaert, 1980; *N. martinezi* De Jonckheere, 2004; *N. mexicana* De Jonckheere, 2004; *N. minor* De Jonckheere et Brown, 1995; *N. morganensis* Dobson, Robinson et Rowan-Kelly, 1997; *N. niuginiensis* Dobson, Robinson et Rowan-Kelly, 1997; *N. pagei* De Jonckheere, 2002; *N. peruana** De Jonckheere; *N. philippinensis*** De Jonckheere, 2002; *N. pringsheimi* De Jonckheere, 2002; *N. pussardi* Pernin et De Jonckheere, 1996; *N. robinsoni* De Jonckheere et Brown, 1999; *N. schusteri* De Jonckheere, 2004; *N. sturti* Dobson, Robinson et Rowan-Kelly, 1997; *N. tenerifensis* De Jonckheere, 2006; *N. tihangensis* De Jonckheere, 2002.

*Species not defined formally, the name is first mentioned in GenBank for the sequence AJ785757.

**Species name first mentioned in Matias et al. (1991), after that as “in preparation” by De Jonckheere (2002) (strain RJTM). Sequences of another two strains are available in GenBank – Wikmark et al. (unpubl.).

The following eight species, *Naegleria arctica*, *N. neoantarctica*, *N. neochilensis*, *N. neodobsoni*, *N. neopolaris*, *N. paradobsoni*, *N. polaris*, and *N. spitzbergenensis*, had been named in GenBank but not formally published before this paper was accepted for print. Recently, these amoebae have been described as eight new species by De Jonckheere (2006).

Table 2. Origin of fish-isolated *Naegleria* strains included in the study.

Strain/ clone	Fish species and organ sampled	Local origin of fish	Strain isolated/ cloned
BCZ4/I	<i>Bunocephalus coracoideus</i> (Cope); gills	upper Amazon River (import)	Feb 1999/Feb 1999
CL/I	<i>Corydoras leucomelas</i> Eigenmann et Allen; brain	upper Amazon River (import)	Mar 2001/Dec 2002
S1Z/I	<i>Esox lucius</i> Linnaeus; gills	Rožmberk pond, Czech Rep.	Apr 1998/Feb 2002
O3Z/I	<i>Perca fluviatilis</i> Linnaeus; gills	Svět pond, Czech Rep.	Apr 1998/ Feb/ 2002
4796/I	<i>Rutilus rutilus</i> Linnaeus; liver	Lužnice River, Czech Rep.	Nov 1991/May 1999
RR13Z/I	<i>R. rutilus</i> ; gills	Novohradský pond, Czech Rep.	Feb 2000/May 2000
4830/I	<i>Salmo trutta fario</i> Linnaeus; kidney	Liběchovka brook, Czech Rep.	Dec 1991/Jun 1997
RR11Z/I	<i>Rutilus rutilus</i> ; gills	Novohaklovský pond, Czech Rep.	Feb 2000/Apr 2000
SUM3V/I	<i>Silurus glanis</i> Linnaeus; skin lesions	Elbe River tributary, Czech Rep.	May 2000/Feb 2002
GP3/III	<i>Gnathonemus petersii</i> (Günther); gills	Congo River (import)	Mar 2002/Apr 2002
A22/I	<i>Abramis brama</i> (Linnaeus); liver	Vyšatov pond, Czech Rep.	Apr 1994/Sep 2004
4564/IV	<i>Perca fluviatilis</i> ; kidney	Černovický potok brook, Czech Rep.	Mar 1991/Jan 1995
Pd72Z/I	<i>Oncorhynchus mykiss</i> (Walbaum); gills	Černá farm, Czech Rep.	Apr 1998/May 1998
CB2B/I	<i>Clarias batrachus</i> (Linnaeus) hybrid; brain	Thailand, Bangkok market	Nov 1997/Dec 1997
CB1S/I	<i>C. batrachus</i> hybrid; spleen	Thailand, Bangkok market	Nov 1997/May 2005
4709/I	<i>Perca fluviatilis</i> ; spleen	Černovický potok brook, Czech Rep.	Jul 1991/Mar 1997
Pd56Z/I	<i>Oncorhynchus mykiss</i> ; gills	Černá brook, Czech Rep.	Apr 1998/Jul 1998
4177/I	<i>Blicca bjoerkna</i> (Linnaeus); brain	Dvořiště pond, Czech Rep.	May 1990/Mar 1997

document morphological features of living amoebae. All clones were tested for production of temporary flagellated stages. The study of ultrastructure was an integral part of strain characterisation. Fixation and processing of material for electron microscopy followed procedures described elsewhere (Dyková et al. 2005).

Molecular characterisation of strains under study

Genomic DNA was extracted exclusively from clonal cultures of strains thoroughly washed to remove contaminating bacteria. When a relatively small amounts of cultured cells were available, the DNA was extracted using DNeasy™ Tissue Kit (Qiagen, Germany) according to instructions of the manufacturer. Phenol/chloroform extractions of cell lysates as described by Maslov et al. (1996) were used in six strains due to a large number of cells harvested. Sequences of SSU rDNA and ITS were used as diagnostic tools to identify fish-isolated *Naegleria* strains.

SSU rRNA gene sequences

The SSU rDNA was amplified by PCR using universal eukaryotic primers (forward ERIB1 5'-ACCTGGTTGATCCTG CAG-3' and reverse ERIB10 5'-CTTCCGCTGGTTCACCT ACGG-3') (Barta et al. 1997). Approximately 50 ng of genomic DNA template, 250 µM of dNTPs, 10 pM of each primer, 2.5 µl 10 × PCR buffer (Takara Bio, Otsu, Shiga,

Japan), and 1 unit of TaqDNA polymerase (Takara) were used in a 25-µl reaction. The PCR amplification was performed with a thermal cycler T3 (Biometra Goettingen, Germany). The cycling conditions included an initial 5 min denaturation at 95°C, then the SSU rRNA gene was amplified in 5 cycles (94°C/1 min, 44°C/1.5 min, and 72°C/2 min) followed by 25 cycles (94°C/1 min, 48°C/1.5 min and 72°C/2 min) and a final extension at 72°C/10 min. The amplified products were gel-purified and cloned into pCR® 2.1 TOPO cloning vectors (Invitrogen Carlsbad, California, USA) using the suggested protocol. Positive clones were sequenced in both directions using the CEQ DTCS Dye Kit (Beckman Coulter, Krefeld, Germany) with a combination of flanking and internal primers on an automatic sequencer CEQ™ 2000 (Beckman Coulter).

Internal transcribed spacer sequences

The forward primer G-FOR 5'-GGGATCCGTTTCCGT AGGTGAACCTGC-3' and reverse G-REV 5'-GGGATCCAT ATGCTTAAGTTCAGCGGGT-3' (Coleman and Vacquier 2002) were used for the selective PCR amplification of the whole ITS region (ITS1, 5.8S, and ITS2). Except for primers, the other components of amplification mixture were the same as in the SSU rDNA amplification. The cycling conditions included an initial 5 min denaturation at 95°C followed by 30 cycles of amplification with a 1 min denaturing at 95°C, a 2

min annealing at 46°C, a 1 min extension at 72°C and a final extension of 10 min at 72°C.

Sequence-based phylogenetic analyses

To construct phylogenetic trees, the following sets of sequences were aligned using the Clustal_X program (Thompson et al. 1997) applying various alignment parameters.

a) SSU rRNA gene sequences of 18 fish-isolated *Naegleria* strains (Table 2) supplemented with all corresponding sequences of type and other strains of *Naegleria* species available in the GenBank database to date. Sequences of *Vahlkampfia inornata* and *V. avara* served as outgroup.

b) ITS sequences of fish-isolated *Naegleria* strains supplemented with corresponding sequences of selected *Naegleria* strains, which as well as fish-isolated strains are characterized by both ITS and SSU rDNA sequences. ITS sequences consisted of ITS1, 5.8S and a portion of ITS2. The most variable segment of sequences, the fourth stem-loop of ITS2, was excluded from alignment. The sequences of *Naegleria andersoni*, *N. jamiesoni* and *N. minor* served as outgroup because ITS sequences of *V. inornata* and *V. avara* differed substantially and could not be aligned with this data set.

c) Concatenated data set of the SSU rDNA and ITS sequences.

To estimate the intragenomic variation at ITS loci, a total of eight subcloned PCR products (two clones of each of four independent PCR products) from the same sample of DNA (extracted from clonal culture of *Naegleria* strain Pd72Z/I) were sequenced and compared.

Two types of analyses were conducted to assess phylogenetic relationships within *Naegleria* strains. Maximum parsimony method (MP) used as implemented in the program package PAUP*, version 4.0b10 (Swofford 2001) was done using heuristic search with random addition of taxa (10 replications). Gaps were treated as missing data. The MP analyses were performed using transition/transversion (Ts:Tv) ratio 1:2. The semistrict consensus tree was made from the resulting topologies. The confidence of branching was assessed using 1,000 bootstrap resamplings of the data sets.

Bayesian inference of phylogeny (BI) was estimated from concatenated SSU rDNA and ITS sequences using MrBayes programme (version 3.0) and Markov Chain Monte Carlo Methodology (MCMC) (Ronquist and Huelsenbeck 2003). BI was performed with parameters (rates = invgamma, NST = 6, ncat = 4) corresponding to the model (GTR + I + Γ) estimated by MrModeltest v. 2.2 (Nylander et al. 2004). No a priori assumptions about the topology of the tree were made and all searches were provided with a uniform prior. The MCMC processes were set so that four chains were run simultaneously for 500,000 generations, with trees being sampled every hundredth for a total of 5,000 trees. Burn-in was determined when visual inspection indicated that the log-likelihood values achieved an asymptote over a large number of generations. The length of burn-in period was 100,000 generations.

Putative secondary structure prediction for ITS2 sequence of arbitrarily chosen fish-isolated strain was obtained using the programme Mfold version 3.2 (Zuker 2003). Sequences corresponding to the fourth stem-loop were then compared within the whole data set.

RESULTS

Safe assignment of fish-isolated strains to the genus *Naegleria* resulted from light and electron microscopic observations. All fish-isolated strains included in the study were able to transform to the flagellated stages and form cysts characteristic of *Naegleria* spp. The morphology of our set of strains was rather uniform. Subtle morphological and size differences of trophozoites of individual strains were not permanent. They were observed on a temporary basis, being provoked mostly by load of bacteria that especially in early phases of subculturing sometimes changed culture conditions. The clonal cultures of two strains (CB2B/I and CL/I) tolerated 37°C, while two other strains (4564/IV and 4171/I) were successfully grown at 30°C. The latter two plus another two strains (4830/I, 4709/I) tolerated a wide range of temperatures (6–30°C). The division of flagellated stages was not observed in any of our fish-isolated strains.

Sequence data

Twelve new SSU rDNA sequences (DQ768714 to DQ768725) and 31 ITS sequences characterizing 18 fish-isolated *Naegleria* strains (DQ768726–DQ768743) were obtained in this study. The size of sequences and also presence or absence of the group I intron are summarized in Table 3. The lengths of SSU rDNA sequences range from 1,980 to 3,359 bp within the set of 18 strains (Table 3). The GC content ranges from 44.26 to 47.99%. The mean GC content in strains that have group I intron located within the SSU rRNA gene corresponds to 44.46, in intronless strains to 47.74%. Seventeen strains have identical length of ITS1 and 5.8S gene (33 and 175 bp, respectively). One only strain of this assemblage differs in having ITS1 34 bp long. The ITS2 sequences of fish-isolated strains are 100–207 bp in length. The set of strains with identical ITS1 and 5.8S is divided into eight subsets depending on identical ITS2 lengths (Table 3). Ten of 18 strains were found to have the group I intron located within the SSU rRNA gene.

Phylogeny and taxonomy of fish-isolated *Naegleria* strains

Congruent results predominated in the sequence-based phylogenetic analyses of our data sets (Figs. 1–4). The branching pattern, basically the same in all analyses performed, was supported with slightly different bootstrap values (higher values were computed in MP analysis of concatenated data for most nodes). Bayesian approach to estimate posterior probability of phylogeny based on concatenated sequences of SSU rDNA and ITS region (Fig. 4) supported branching pattern of MP analyses. The minimal lengths of branches characterizing the third clade of strains analysed in the study stressed their remarkable phylogenetic relatedness.

Table 3. Fish-isolated *Naegleria* strains ordered by bp length of their ITS regions, with bp length of their SSU rDNA sequences and presence/absence of the group I intron.

Strain/clone	ITS1	5.8S	ITS2	Total ITS	SSU rDNA	Intron
CB2B/I	33	175	100	308	1,980*	–
4796/I	34	175	106	315	2,011	–
4830/I	33	175	165	373	2,011	–
A22/I	33	175	165	373	2,011	–
CL/I	33	175	170	378	3,317	+
GP3/III	33	175	170	378	3,317	+
Pd72Z/I	33	175	170	378	3,285	+
Pd56Z/I	33	175	170	378	3,285	+
BCZ4/I	33	175	177	385	3,318	+
S1Z/I	33	175	185	393	2,013	–
O3Z/I	33	175	185	393	2,013	–
RR11Z/I	33	175	192	400	2,021	–
SUM3V/I	33	175	192	400	3,359	+
4177/I	33	175	200	408	3,285*	+
4564/IV	33	175	200	408	3,285*	+
4709/I	33	175	200	408	3,285*	+
CB1S/I	33	175	200	408	3,315	+
RR13Z/I	33	175	207	415	2,021	–

* Sequences reported in previous study (Dyková et al. 2001).

Phylogenetic analyses have divided *Naegleria* strains under study into three distinct clades (Figs. 1–4) regardless of data set utilized. When two *Vahlkampfia* species were used as outgroup (in MP analyses of SSU rDNA and concatenated sequences of SSU rDNA and ITS) the first, most basal and well-supported clade of analysed strains contained those representing *N. minor*, *N. jamiesoni* and *N. andersoni*.

The second, moderately supported clade contained strain-representatives of six named species of the genus (*N. fowleri*, *N. martinezi*, *N. lovaniensis*, *N. sturti*, *N. niuginiensis*, *N. morganensis*) and three fish-isolated strains. As documented in Figs. 1–4, i.e., in all analyses performed, strains RR13Z/I, RR11Z/I and SUM3V/I form a common, well-supported cluster of closely related strains. The lengths of ITS2 sequences differ in having 192 bp in RR11Z/I and SUM3V/I, and 207 bp in RR13Z/I (Table 3). The highly variable nucleotide sequence corresponding to the fourth stem-loop of ITS2 secondary structure is identical in strains RR11Z/I and SUM3V/I, while in RR13Z/I it matches *N. dobsoni*. Unfortunately, SSU rDNA sequence of *N. dobsoni* is not available.

All other fish-isolated strains branched within the third, most numerous clade that integrated 25 strains, subdivided into 6 clusters (Fig. 3). Their close relationships can be documented by the fact that the sequence similarity of two distant strains of this clade

Fig. 1. Maximum parsimony tree (strict consensus of 132 trees) for SSU rDNA sequences from fish-isolated *Naegleria* strains (bold-faced) and strain-representatives of defined *Naegleria* species. Bootstrap values (1,000 replicates) are indicated for the nodes gaining more than 50% support. *Vahlkampfia* spp. were set as outgroup. GenBank accession numbers are in parentheses.

(*N. robinsoni* and *N. australiensis*) is much higher (94.75%) than the similarity of most species within the first and second clade (e.g., *N. jamiesoni* and *N. minor* reveal 89.04% similarity).

The cluster “a” with basal position within the third clade contains only sequences of *N. robinsoni* and *N. indonesiensis*. Newly characterized fish-isolated strains S1Z/I and O3Z/I form cluster “b”. They have the same lengths of ITS sequences as recorded in *N. americana* and also identical is the sequence of the fourth stem-loop in secondary structure of ITS2. The sequence of SSU rDNA of *N. americana* is not available.

Strain 4796/I together with strains representing *N. fultoni* and *N. pringsheimi* form cluster “c”. The former strain, unique in our assemblage because of the length of ITS1 (34 bp), has the whole ITS sequence as well as the fourth stem-loop in the secondary structure of ITS2 identical with *N. fultoni*.

Closely related fish-isolated strains 4830/I and A22/I join the strain of *N. pagei*, forming cluster “d”. Their

Fig. 2. Maximum parsimony tree (strict consensus of two trees) for ITS sequences of fish-isolated *Naegleria* strains (bold-faced) and strain-representatives of defined *Naegleria* species. Bootstrap values (1,000 replicates) are indicated for the nodes gaining more than 50% support. GenBank accession numbers are in parentheses.

close phylogenetic relationship documented in Figs. 1–4 is supported by the sequence identity in fourth stem-loop of the secondary structure of ITS2.

Due to unstable phylogenetic position of strain BCZ4/I, its relatedness to defined *Naegleria* species was difficult to infer from analyses performed in this study. Nevertheless, the length of ITS2, unique among 48 named *Naegleria* species (of which 8 have been named in GenBank only but not formally published yet), is worth mentioning.

Three strain-representatives of *N. australiensis* together with *N. tihangensis* form cluster “e”. The previous assignment of fish-isolated strain CB2B/I to *N. australiensis*, which was based on phylogenetic analysis of SSU rDNA sequences, has been confirmed in this study. In addition to identity of ITS sequences, also the sequence of the fourth stem-loop in secondary structure of ITS2 was found identical with *N. australiensis*.

The sixth cluster (“f”) contains eight fish-isolated strains, among them three newly introduced (*GP3/III*, *CL/I* and *CB1S/I*) and five that on the basis of SSU

Fig. 3. Maximum parsimony tree (strict consensus of 13 trees) that includes the concatenated SSU rDNA and ITS sequences of fish-isolated *Naegleria* strains (bold-faced) and strain-representatives of defined *Naegleria* species. Bootstrap values (1,000 replicates) are indicated for the nodes gaining more than 50% support. *Vahlkampfia* spp. were set as outgroup. GenBank accession numbers are in Figs. 1 and 2.

rDNA sequences were assigned previously to *N. clarki* (Dyková et al. 2001). Their SSU rDNA sequences were supplemented with sequences of ITS region in this study. Strains of this cluster changed their mutual position depending on data set of sequences analysed. Strains *GP3/III*, *CL/I*, *Pd72Z/I* and *Pd56Z/I* have identical lengths of ITS1 and 5.8S and ITS2, the same as in *N. canariensis*, *N. dunnebackei* and *N. gallica*. The relatedness of this group of strains is reflected in identity of sequences of the fourth stem-loop in the ITS2 secondary structure. Unfortunately, SSU rDNA sequences of *N. canariensis*, *N. dunnebackei* and *N. gallica* are not available. ITS sequences of strains *CB1S/I*, *4177/I*, *4709/I* and *4564/IV* that branch within cluster “f” are of the same length and identical with ITS sequence of reference strain of *N. clarki* (RU30).

Only eight fish-isolated strains could be assigned to species already defined, if both criteria, the recent ITS approach in defining *Naegleria* species (De Jonckheere 2004) and SSU rDNA sequence data were applied.

Fig. 4. Molecular phylogeny of fish-isolated *Naegleria* strains (bold-faced) and strain-representatives of defined *Naegleria* species (including *Vahlkampfia* spp. as outgroup) inferred from Bayesian analysis of the combined data (SSU rDNA and ITS sequences) using general-time-reversible model of nucleotide substitution. The branch leading to *Naegleria* spp. was shortened six times to demonstrate branching pattern within the third clade consisting of sequences of 25 strains.

Strain CB2B/I could be identified with *N. australiensis*; strains 4830/I and A22/I with *N. pagei*, strain 4796/I with *N. fultoni* and strains CB1S/I, 4177/I, 4709/I and 4564/IV with *N. clarki*.

Intragenomic variation of ITS

The comparison of ITS sequences obtained from the eight subcloned PCR products (two clones of each of four PCR products from the same sample of DNA extracted from clonal culture of the fish isolated strain) revealed nucleotide diversity ranging from 0 to 8 bp substitutions (the sequence data are available on request).

DISCUSSION

The aim of the present study, i.e., possible identification of fish-isolated *Naegleria* strains with species already defined, using the novel data obtained in this

study and data recorded before, could not be fully accomplished. However, several topics related to molecular taxonomy of *Naegleria* species worth discussing resulted from this study.

Because of a rather uniform morphology, the introduction of molecular approaches became of fundamental importance in the history of species definitions within the genus *Naegleria* Alexeieff, 1912 (De Jonckheere 1994, 1998, 2002, 2004, Pélandakis et al. 2000).

The SSU rRNA gene-derived phylogeny was used as a basis for improvements in taxonomy of many eukaryotic genera and for assessment of relatedness of organisms at higher taxonomic levels as well. However, the evolutionary conserved nature of these genes that makes them relatively uninformative at the generic and species levels in eukaryotes has been stressed several times (Sogin 1991, Coleman et al. 1998, Sogin and Silberman 1998, Pélandakis et al. 2000). The spacer regions of nuclear ribosomal genes have generally been found more variable and useful in comparing closely related species or populations (Felleisen 1997, De Jonckheere 1998, Pélandakis et al. 2000).

Since the time that sequences of genes were introduced to taxonomy, the question of “how much of a difference in base pairs of these sequences justifies recognition of a new species” has undoubtedly been asked and discussed many times. The answer given by De Jonckheere (2004) was, contrary to inconclusive outcomes of such discussions or analyses, concrete and taken as the usable rule in defining *Naegleria* species (Visvesvara et al. 2005). The currently accepted molecular definition of species within the genus *Naegleria* is primarily based on differences in the 5.8S and ITS sequences, but it is ITS2 sequence that defines species among the strains that have identical ITS1 and 5.8S: “any strain that has an ITS2 sequence that differs by more than one nucleotide bp can be considered as another species” (De Jonckheere 2004).

Out of 40 *Naegleria* species introduced with names to date, 22 were characterized by sequences of SSU rDNA with 15 of them, in addition, also by sequences of ITS. Definitions of 18 *Naegleria* species published to date plus another 8 named species whose existence has so far only been announced in GenBank (see Table 1) are based on ITS sequences only.

ITS2 sequences of several defined species actually differ in two nucleotide bp, (e.g., *N. canariensis* and *N. gallica* that have the same length of ITS2 sequence, or *N. australiensis* and *N. tihangensis* that differ just in two nucleotide bp that at the same time increase the length of ITS2). Two strains (N280 and AB-T-F3) recorded in GenBank as representatives of two species (*N. peruana* and *N. italica*) have absolutely identical ITS sequences. Minute differences in ITS2 sequences can be demonstrated in strains assigned to the same species: in one (NG434) out of three strains of *N. byersi*, and in one (A2) out of six strains of *N. andersoni*, the ITS2 se-

quences differ in one nucleotide bp. Pronounced differences (up to 8 bp) were found in sequences of subcloned PCR products from one sample of DNA of our fish-isolated strain.

Problems encountered in comparing nucleotide bp differences or genetic distances while intending to delineate species have usually been concluded by the statement that from purely taxonomic standpoint no established criteria exist to recognise species on the basis of nucleotide sequence data. For this reason, divergent sequences or genetic distances between analysed organisms must be interpreted with caution, as stressed by Sogin (1990, 1991) especially for protozoans.

When species concepts were analysed in relation with parasites, the hope was expressed that the habit to identify new species just on the basis of a certain number of base changes within the spacer sequences of rDNA will stop and the search for new criteria for species definitions will continue according to requirements of different phyla (Kunz 2002).

Since we felt that the potential power of SSU rDNA sequences has not been fully exploited in taxonomy of *Naegleria* and the information obtained on variations in the ITS sequences of subcloned PCR products of the same sample of DNA (strain Pd72Z/I) contravened De Jonckheere's (2004) guidelines for *Naegleria* species definition, we have tried to combine SSU rDNA and ITS sequence data. Unfortunately, heterogeneity in molecular characterisation of *Naegleria* species, i.e., the lack of corresponding SSU rDNA and ITS sequences, did not allow analysis of more than 38 *Naegleria* strains, which represent only half of the defined (named) species.

The predominating congruence of phylogenetic relationships found within three data sets of *Naegleria* se-

quences was a promising result of this study, but in the light of the number of defined species it still is not convincing enough for us to restrict definition of new species to phylogenetic analysis of relatively short ITS regions.

Considering the degree of sequence similarity of strain-representatives of many denominated *Naegleria* species on the one hand and the intragenomic variations of ITS sequences established in our screening on the other, one can expect that criteria for species definitions might require a re-evaluation in the future. The intragenomic as well as interstrain polymorphism exhibited at ITS loci should be clarified using goal-directed selection of strains, i.e., using samples of DNA from clonal cultures of type or reference strains of phylogenetically close as well as distantly related species. The range of ITS sequence variation should be estimated also for strain-representatives of the presumably same species reported from far distant localities. In addition, direct sequencing of PCR products and comparison of sequences with those obtained from subcloned PCR products might be useful in striving for relevant molecular markers of species within the genus *Naegleria*.

The data accumulated on ITS sequences of *Naegleria* strains are of a great value; nevertheless, we think they should not be taken as the exclusive markers of species boundaries. In the future, they could characterise types of ITS sequences within species redefined on the basis of multiple gene analysis.

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