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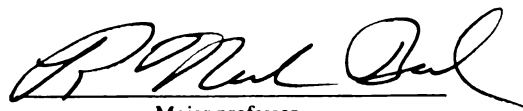
A STUDY OF THE EVOLUTION AND TAXONOMY OF
NAEGLERIA AND VAHLKAMPFIA AMOEBAE (SCHIZOPYRENIDA,
VAHLKAMPFIIDAE) UTILIZING MITOCHONDRIAL DNA

presented by

STEPHEN MARK MILLIGAN

has been accepted towards fulfillment
of the requirements for

Doctoral degree in Zoology


Major professor

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MITOCHONDRIAL DNA

By

Stephen Mark Milligan

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ABSTRACT

A STUDY OF THE EVOLUTION AND TAXONOMY OF NAEGLERIA AND VAHLKAMPFIA
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Stephen Mark Milligan

Using restriction enzyme analysis, mitochondrial DNA fragment patterns from seven strains of pathogenic and nonpathogenic Naegleria and one strain of Vahlkampfia were compared to estimate nucleotide sequence divergence. High levels of estimated genetic variation (>12% estimated nucleotide substitutions) between morphologically-identical strains of N. gruberi, N. fowleri and N. jadini support the current taxonomic level of the individual Naegleria species and suggests a distinct phylogeny for each group. Naegleria lovaniensis, strain TS, was shown to have significant nucleotide sequence homology (7% estimated nucleotide mismatches) with N. gruberi, strain EGs, suggesting that the two groups share a close taxonomic relationship. The pathogenic strain MB-41 of N. fowleri exhibited distinct genetic divergence from the highly homologous, pathogenic strain Nf66 and the drug-cured strain 6088. Morphologically distinct strains EGs and 1518/1a of N. gruberi exhibited significantly large sequence divergence (11% estimated nucleotide substitutions) consistent with a more distant taxonomic relationship. Amoebae from the genus Vahlkampfia expressed genetic similarity with strains of N. gruberi. A 12.9-16.3 kilobase-pair circular, non-mitochondrial, extrachromosomal DNA element was discovered in all strains of Naegleria and Vahlkampfia.

DEDICATION

To
Dad and Mom,
and
Valarie

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¹ INTRODUCTION

Amoebo-flagellates are wide-spread protozoa commonly found in soil and freshwater (Fulton, 1970). Some species are also pathogenic to man and animals. The genus Naegleria consists of free-living, small, naked amoebae, all of which can transform from amoebae to flagellates. There are currently five recognizable species of Naegleria, each containing a large number of strains. Naegleria gruberi is a common species in soil and freshwater, with some high temperature-tolerant strains potentially pathogenic to man (Fulton, 1970). In soil, this species is an important micropredator, feeding on bacteria, yeast and fungi. There are approximately 1,000 to 4,000 N. gruberi amoebae per gram soil. Naegleria fowleri is a virulent pathogen and the causal agent of primary amoebic meningo-encephalitis (PAM) in man (Carter, 1970). Eighteen strains of N. fowleri have been isolated from humans in addition to a large number of strains isolated from soil and water. Strain 6088, isolated from the cerebrospinal fluid of a 9 year old female, represents the only known strain of N. fowleri to be successfully drug-cured. Strains of Naegleria which are biologically and antigenically distinct from N. gruberi and N. fowleri are considered to be the parental strains of three newly

¹
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formed species, N. jadini, N. australiensis, and N. lovaniensis (Carosi et al., 1976; De Jonckheere, 1981; Stevens et al., 1980). Other species of small amoebae in the Family Vahlkampfiidae that morphologically resemble Naegleria, but cannot be transformed into flagellates are placed in the genus Vahlkampfia (Page, 1976). It is assumed that the genus Vahlkampfia is a taxonomic convenience for amoeb-flagellates that cannot be transformed to flagellates in vitro or are incapable of transformation through mutation. The genus Vahlkampfia consists of several isolates which have not been investigated to the extent seen in Naegleria.

The study of taxonomic relationships among members of this group has been based upon morphological and physiological criteria (Schuster, 1979). Previous attempts to examine the phylogenetic relationships of these organisms using morphological characters such as organelle shape/organization and cyst structure have not been successful (Carosi et al., 1976; Carter, 1970; De Jonckheere and Van De Voorde, 1977; Page, 1975; Visvesvara and Callaway, 1974). Other species differences collectively provide useful criteria for taxonomic classification, but no single characteristic is species specific: 1) effect on cell cultures (De Jonckheere and Van De Voorde, 1977; Dunnebacke and Schuster, 1985; Marciano-Cabral and Fulford, 1986; Stevens et al., 1980), 2) pathogenicity to mice (De Jonckheere and Van De Voorde, 1977; Marciano-Cabral and Fulford, 1986; Stevens et al., 1980), 3) temperature tolerance (Aufy et al., 1986; De Jonckheere

and Van De Voorde, 1977; Griffin, 1972; Stevens et al., 1980),
 4) nutritional requirements (De Jonckheere, 1977; John, 1982),
 5) drug and chemical resistance (De Jonckheere and Van De Voorde,
 1976; De Jonckheere and Van De Voorde, 1977; Schuster and Rechthand,
 1975), 6) lectin agglutination (Stevens et al., 1980), and 7) antigenic
 variation (De Jonckheere and Van De Voorde, 1977; Stevens et al.,
 1980). In addition, previous studies could not provide quantification
 of genetic divergence and evolutionary relationships between these
 group members as can be currently obtained using DNA sequence analysis.
 The biochemical identification and differentiation of suspected
 pathogenic and nonpathogenic vahlkampfiid amoebae using isoenzyme
 electrophoretic analysis (Daggett and Nerad, 1983) or isoenzyme
 isoelectric focusing (Pernin, 1984) has proven to be a useful tool in
 that genetic distances (i.e. the average number of gene substitutions
 per locus) between the various species and strains can be estimated
 (from allele frequencies) and possible relationships drawn
 (Pernin et al., 1985). Large scale surveys of interstrain isoenzyme
 variation are feasible using these molecular techniques, but
 classification schemes for Naegleria as well as for other small soil
 amoebae (Costas and Griffiths, 1980; Daggett et al., 1985) based on
 zymogram patterns have not been found to be entirely consistent with
 previous morphologically and/or biologically defined boundaries. In
 the genus Naegleria, with the exception of N. gruberi, species
 specificity has been demonstrated using three isoenzymes (Daggett and

Nerad, 1983; Nerad and Daggett, 1979) thereby improving the taxonomic classification scheme used for the identification of future suspected vahlkampfiid amoebae.

In phylogenetic investigations, a study of DNA sequence variation is much more informative than protein sequence variation since a large part of DNA sequences are not encoded into proteins as well as the fact that there is degeneracy of the genetic code (Nei, 1987). Four molecular approaches currently exist for the measurement of overall genotypic variation at the genetic level. Technical limitations such as ability to clone and directly sequence only small portions of the total nuclear genome make these techniques impractical for use in estimating overall nucleotide sequence differences in large taxonomic surveys. DNA hybridization is more suitable for testing overall nuclear sequence homology among large numbers of organisms, but it has been shown to lack the desired sensitivity necessary to examine significant genetic differences among closely-related species and strains (Ferris et al., 1981; Nei, 1987). Most recently, full, direct sequencing of bacterial and eukaryotic small subunit ribosomal ribonucleic acids (rRNAs) have allowed intermediate to distant phylogenetic relationships to be rapidly measured (Woese, 1987). However, their effective usefulness as molecular chronometers among closely-related, eukaryotic species and strains has yet to be explored. A fourth method for studying evolutionary relationships among closely-related organisms involves the comparison of organelle DNA

sequence differences. Unlike nuclear genes, mitochondrial and chloroplast genomes can be extracted and purified relatively easily. Because of a low rate of evolution, chloroplast DNA (cpDNA) is not very suitable for studying the genetic relationship of closely-related species (Nei, 1987). Recent methodological advances, which center on restriction endonuclease analysis, DNA cloning, and nucleotide sequence analysis, have proven quite powerful in revealing mitochondrial DNA (mtDNA) organization, function, and expression among a wide variety of organisms.

Mitochondrial DNA (mtDNA) is a functionally conservative molecule that encodes for basically the same genes in all eukaryotes, yet shows great diversity in structural organization and expression (Gray, 1982). Small closed circular monomer forms of mtDNA predominate in unicellular eukaryotes as well as animal and plant cells. Linear conformations have only been reported in the yeast Hansenula mrakii, ciliate protozoa Tetrahymena pyriformis and Paramecium aurelia, and several species of the plant Class Angiospermae (Birley and Croft, 1986; Kroon and Saccone, 1980; Wallace, 1982). MtDNA is generally much smaller than the DNAs found in bacteria with ranges from 34.5 μ m long (108 kilobase pairs (kbp)) in the fungus Brettanomyces custersii to 4.5 μ m long (14.1 kbp) in the alga Chlamydomonas reinhardtii for unicellular eukaryotes (Clark-Walker and Sriprakash, 1982; Wallace, 1982). Certain Neurospora strains also contain highly-conserved 3.3 to 3.4 kilobase circular mitochondrial plasmids (Nargang, 1986;

Wallace, 1982). In addition, plasmid-like DNA has been isolated from mitochondria of the filamentous fungi Podospora, Aspergillus and Cochliobolus (Cummings et al., 1985; Garber et al., 1984; Lazarus and Kuntzel, 1981), all of which have been derived from and possess homology to sequences in their respective normal mitochondrial chromosomes. MtDNAs consisting of complex aggregates of large and small circles (maxicircles and minicircles) are found exclusively in the kinetoplasts of the Trypanosomatidae with size ranges of the transcribable maxicircles from 6.3 to 12 μm (19.7-37.6 kbp) for Trypanosoma brucei and Crithidia fasciculata, respectively (Wallace, 1982). Mitochondrial genome size variation was found to be much less extensive in multicellular animals (from flatworms to humans) with a contour length range from 4.7 to 6.2 μm (14.7-19.4 kbp) (Wallace, 1982). A large population of heterogeneous circular and/or linear DNA molecules, which vary in number and size between species, are found in the mitochondrion isolated from flowering plant species. Circular mtDNA with 30 μm lengths (94.0 kbp) seem to be a common element found to exist within many different plant species (Wallace, 1982). The summation of restriction endonuclease fragments yields large approximate mitochondrial genome sizes ranging from 136 to 348 kbp when overlapping heterologous fragment bands are ignored and from 350 to 750 kbp when multiple fragment bands are counted (Wallace, 1982). Renaturation kinetic analysis produced a wider size range from about 100 to as much as 2500 kbp (Wallace, 1982). Much of the size

difference found between the large mitochondrial genomes of yeast and plants and the small genome of animal mitochondria can be accounted for by the presence or absence of noncoding intragenic (introns) and intergenic sequences (Borst and Grivell, 1981).

A complete set of clustered transfer RNAs (tRNAs), a large subunit ribosomal RNA (rRNA), and a small subunit rRNA are universally encoded by mtDNAs from unicellular eukaryotic organisms. The arrangement and size of these genes varies greatly among species (Wallace, 1982). The type and arrangement of the seven known polypeptide-encoding genes also differs in all unicellular eukaryotic mtDNAs. However, common features of mitochondrial genome organization in this group include coding segments that are separated by long noncoding regions and several genes which are split to contain a number of intervening noncoding sequences (introns) (Gray, 1982). Those mitochondrial translated polypeptides identified to date include the three largest cytochrome c oxidase subunits (COI, COII, and COIII), the cytochrome b apoprotein, mitochondrial adenosine triphosphatase (mtATPase) subunits 6 and 9 (mitochondrial gene origin only in Saccharomyces cerevisiae and higher plants), and mitochondrial ribosomal protein (var 1) associated with the small ribosomal subunit (Birley and Croft, 1986; Gray, 1982; Wallace, 1982).

In contrast to these unicellular eukaryotic mtDNAs, all multicellular animal mtDNAs are very compact and have essentially the

same gene organization with identical ribosomal gene order. A maximum of twenty-five polypeptides have been identified from mammalian mitochondrial ribosome synthesis including subunits COI, COII, COIII, mtATPase subunit 6, cytochrome b, and another mtATPase subunit which is different from ATPase subunit 9. ATPase subunit 9 is synthesized in the cytosol and the mtATPase subunit 9 gene is not found in the mtDNA (Wallace, 1982). In addition, two rRNAs (16S and 12S) and all twenty-two tRNAs (which appear to be randomly interspersed), are encoded by the mtDNA genes. Most significant is the finding that animal mtDNAs have a highly efficient organization which lacks sequences without coding functions yet contains few overlapping genes. The extremely variable 1-5.1 kbp A + T - rich region around the origin of replication of Drosophila mtDNA is the most prominent exception to this efficient genetic arrangement (Birley and Croft, 1986). The variation found in the D-loop region of mammals containing the origin of replication is less extensive with significant restriction site homology found to exist among most primate species.

A uniform mitochondrial gene organization has been shown to exist in vascular plant cells as well. In contrast to gene organization in animal cells, three mtDNA rRNA genes (one large, one small, and a 5S rRNA) have been identified, one of which has significant homology to the bacterial 16S rRNA, and some of the tRNA genes have been shown to form clusters in the genome (Birley and Croft, 1986; Wallace, 1982). At least twenty polypeptides have been

shown to be synthesized in the mitochondria of vascular plants which is a substantially greater number than the nine polypeptides found in yeast (Stephenson et al., 1980). The basic structural organization of the plant mitochondrial genome is not yet clearly understood with the relationships between the various observed mtDNA molecules and plasmid-like DNAs still in need of clarification (Wallace, 1982).

In addition to the above mentioned gross structural variations resulting from extensive sequence rearrangements and additions and/or deletions of noncoding introns or intergenic regions, mitochondrial genomes are characterized by an unusually rapid divergence at the level of the primary sequence. Restriction endonuclease analysis of mtDNA (Brown et al., 1981; Brown et al., 1979) and sequence analysis of homologous mitochondrial genes/gene products (Gray, 1982) from unicellular eukaryotes and higher animals provide the most convincing evidence for rapid evolution of mtDNA sequences. In multicellular animals, evolutionary change of mtDNA occurs mainly through nucleotide substitution, although deletions and insertions are known to occur frequently in noncoding regions (Brown and Simpson, 1981).

Brown et al. (1982) have estimated the mitochondrial nucleotide substitution rate to be 10^{-8} per site per year which is 5 to 10 times higher than the average rate for single-copy nuclear DNA in primates. The high rate of animal mtDNA evolution is likely to be a consequence of a higher mutation rate for the mitochondrial genome. However, preliminary studies estimating rates for Drosophila and plant mtDNAs

have suggested that the pattern of rapid substitution in mammalian mtDNA is exceptional and that the mitochondrial DNAs in nonmammalian or nonvertebrate species evolve in the same fashion as that of nuclear genes (Nei, 1987). Analysis of mtDNAs with high rates of evolution may result in a classification scheme based on organelle DNA rather than for the organism (Byers et al., 1983) and phylogenetic relationships based on an underestimated number of base pair substitutions between distant species (Birley and Croft, 1986). However, the interdependence of nucleus and mitochondria suggests that the evolution of the two types of DNA should be highly correlated. In fact, classification schemes based on organelle DNA were found to be similar to conventionally based schemes (Byers et al., 1983).

In higher eukaryotes, this high level of genetical variation between even closely-related species coupled with the property of a maternal mode of mitochondrial DNA (mtDNA) inheritance (i.e. absence of mitochondrial recombination) makes the mitochondrial genome especially well suited for the study and construction of phylogenies at the within-species level (Birley and Croft, 1986). All progeny of a single female share a common mtDNA clone. In the pocket gopher (Geomys pinetis), distinctively different clones are found in neighboring geographical regions of a population with extremely similar subclones present within each region (Awise et al., 1979). Since sexual stages or reproduction have not been observed in

Naegleria (Schuster, 1979), clones of the mitochondrial genome should serve as an equally appropriate source for obtaining phylogenetic information among closely-related strains within natural populations.

Even when utilizing relatively small organelle genomes, a lack of efficiency and sensitivity make direct nucleotide sequencing and DNA/DNA hybridization, respectively, impractical for large-scale genetic studies (Byers et al., 1983; Nei, 1987). Restriction endonuclease analysis has proved quite powerful in revealing mitochondrial DNA (mtDNA) phylogenetic relationships among closely-related organisms (Brown and Simpson, 1981; Brown, 1980; Kozlowski and Stepien, 1982; Prunell et al., 1977). Type II restriction endonucleases recognize and cleave specific sequences (restriction sites) of 4 to 6 nucleotide pairs long. The number and locations of restriction sites vary with each nucleotide sequence so that restriction enzyme cleavages (digestions) of highly similar DNA sequences yield a similar number of fragments of comparatively close sizes. Hence, by comparing the locations of restriction sites, it is possible to estimate the number of nucleotide substitutions between two homologous DNAs (Nei, 1987). Although mapping of restriction sites on a DNA sequence may not be too difficult in order to compare and determine shared and nonshared restriction sites, it is often too labor intensive to be used for a large-scale population survey. The number of nucleotide substitutions may be estimated from the proportion of DNA fragments that are shared by the two DNA sequences.

This problem was first studied and mathematically interpreted by Upholt (1977). By comparing the electrophoretic fragment patterns of DNAs digested by a restriction endonuclease between two fixed length DNA sequences, an estimate of the number of nucleotide substitutions per site can be obtained. Two alternate mathematical methods have been developed to estimate the degree of genetic divergence between two DNA sequences based on the proportion of DNA fragments shared by them (Engels, 1981; Nei and Li, 1979). A comparative computer simulation study of the three length difference models (Kaplan, 1983) suggests that all of them give essentially the same result which generally possesses a much larger variance than that obtained by the more rigorous restriction site difference methods (Nei, 1987).

The approach suggested by Engels (1981) is the one of preference arising from its ease of application and possession of fewer assumptions regarding nucleotide sequence evolutionary patterns than other methods. This approach is based on the key assumption that cleavage of adjacent monomorphic restriction sites will produce fragments of equal size in the two compared digests, whereas cleavage at polymorphic sites will result in different sized fragments being produced. Appropriateness of this model depends on the validity of three key assumptions regarding changes in nucleotide sequences (Byers et al., 1983). First, evolutionary changes in fragment sizes results from the loss or gain of restriction sites due to single nucleotide changes rather than from deletions, additions, or major

rearrangements of nucleotides, or to nucleotide modifications such as methylation that make restriction sites resistant to digestion. Second, the frequency and distribution of restriction sites should be random. Third, similar sized fragments in different digests should indicate similar nucleotide sequences in the fragments.

This molecular approach has been used to examine the relatedness between various isolates of the soil amoebae, Acanthamoeba (Bogler et al., 1983; Costas et al., 1983). The present study of the phylogenetic relationships among vahlkampfiid amoebae uses this same approach of restriction enzyme analysis of mtDNA structural polymorphism. Detection and quantification of genetic variation between pathogenic and nonpathogenic strains of Naegleria may provide a molecular criterion for the identification and differentiation of future, potentially pathogenic isolates. Insight into pathogenicity determination may be gained through a comparison of genetic divergence between pathogenic strains and the successfully drug-cured strain of Naegleria fowleri. In addition, genetic diversity would provide a basis for taxonomic distinction between Naegleria and Vahlkampfia, thereby lending support to the current standing as separate, related taxonomic groups or suggesting that Vahlkampfia may be merely a mutant form of Naegleria unable to transform itself. This type of analysis also provides information regarding speciation and genetic diversity within a group of asexual organisms.

MATERIALS AND METHODS

Naegleria strains and cultivation. A description of the vahlkampfiid amoeba strains used in this work is given in Table I. The term "strain" denotes an independent isolate from nature. With the exception of Naegleria jadini, ITMAP 400, all strains of Naegleria were maintained axenically in liquid H-4 medium supplemented with 1 µg ml hemin (Sigma, St. Louis, Mo.) (Band and Balamuth, 1974). Strain ITMAP 400 was grown axenically in medium modified from Schuster & Mandel (1984) containing 0.25% yeast extract (Difco), 0.25% Proteose Peptone (Difco), and 0.5% Panmede (Harcros, Inc., Bronxville, N.Y.) in Modified Neff's Amoebae Saline (AS) (Page, 1976) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (Sigma). Vahlkampfiia lobospinosa was grown in a Low Salt saline (Sykes and Band, 1985) (LS; 4.6 mM MgSO₄·7H₂O, 0.36 mM CaCl₂, and 50 mM NaCl) suspension of Escherichia coli, strain K-12. All strains of N. fowleri and N. lovaniensis were grown at 35-37° C whereas N. gruberi and N. lobospinosa strains were cultured at 29.5° C and N. jadini at 25° C. For CsCl isolated, purified mitochondrial DNA preparations, axenically-cultivated amoebae were grown in 2.8 liter, silicone-coated (Band and Balamuth, 1974) Fernbach flasks (Bellco #2550) containing 500 ml medium incubated on a rotary shaker (100 rpm).

TABLE I. Description of vahlkampfiid amoeba strains.^a

Species-Strain	Source-Geographic origin	Reference(s)
<u>Naegleria gruberi</u>		
EGs	Soil; California, U.S.A.	Schuster, 1969
CCAP-1518/1a	Freshwater; Britain	Page, 1975
<u>Naegleria fowleri</u>		
MB-41 (HB-1)	Human spinal fluid; Florida, U.S.A.	Culbertson et al., 1968; Schuster & Dunnebacke, 1977
Nf66 (Carter)	Human cerebrospinal fluid; Australia	Carter, 1972
6088 (ATCC30896)	Human cerebrospinal fluid; California, U.S.A.	Center for Disease Control, 1978
<u>Naegleria jadini</u>		
ITMAP 400 (0400)	Swimming-pool; Belgium	Carosi et al., 1976
<u>Naegleria lovaniensis</u>		
TS (<u>N. gruberi</u>)	Vero cell culture; Ohio, U.S.A.	O'Dell & Stevens, 1973
<u>Vahlkampfia lobospinosa</u>		
ATCC 30298	Cattle feces; Tennessee, U.S.A.	Wilhelm & Anderson, 1971

^a

Naegleria strains provided by F. L. Schuster. Vahlkampfia lobospinosa, strain ATCC30298 provided by American Type Culture Collection.

Stationary monobacterial cultures (150 ml) of V. lobospinosa were maintained in 2.5 liter low form flasks (Corning #4422). Greater than 3×10^8 vegetative amoebae were used per DNA isolation (one shaker flask at 6×10^5 cells/ml for Naegleria strains; ten stationary flasks at 2×10^5 cells/ml for V. lobospinosa).

Enriched mitochondrial DNA fractions were obtained using single stationary cultures (100 ml) of axenically-cultivated amoebae (7×10^5 cells/ml) and a stationary bacterized culture (150 ml) of V. lobospinosa (6×10^5 cells/ml). Between $7 - 9 \times 10^7$ amoebae were used per mitochondrial DNA enrichment. One additional Naegleria strain studied using this DNA extraction procedure was obtained from G. S. Visvesvara (CDC): N. lovaniensis, type strain 76/25/15, isolated from water.

Mitochondrial DNA isolation.

(a) CsCl isolated, purified mitochondrial DNA. Using the UNSET procedure developed for the isolation of mtDNA from the small, soil amoeba, Acanthamoeba (Thomas J. Byers, personal communication), mtDNA was obtained and purified from Naegleria and Vahlkampfia strains as described. Amoeba cells were harvested in 500 ml bottles by centrifugation at 1000 g for 10 minutes using an RC-5 refrigerated centrifuge equipped with a GSA rotor (Ivan Sorvall Co., Norwalk, CT.) at 4° C. The resulting cell pellet was washed in cold phosphate buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4 , pH 7.5). Removal of bulk bacteria from V. lobospinosa prior to DNA isolation was achieved

by three successive low speed centrifugal washes (121 g, 77 g, 77 g; 5 minutes per sedimentation using an SS-34 rotor at 4° C) following the initial cell harvest. The washed cell pellet was rapidly mixed with 15 ml UNSET buffer (8 M Urea, 150 mM NaCl, 2% Sodium Dodecyl Sulfate (SDS), 1 mM EDTA, 100 mM Tris-HCl, pH 7.5) and extracted three times with an equal volume of phenol/CIA (25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol equilibrated with 50 parts H-NET buffer (150 mM NaCl, 1 mM EDTA, 100 mM Tris-HCl, pH 8.2)). After the final extraction, the recovered aqueous layer was made 0.3 M NaCl by the addition of 3.0 M NaCl and ethanol precipitated overnight at -20° C. The remaining isolation procedure was as described previously (Bogler et al., 1983) except for the following modifications. The precipitated DNA pellet (12,000 g for 10 minutes at 4° C an SS-34 rotor in an RC-5 centrifuge) was resuspended in 4 ml DNA buffer (150 mM NaCl, 20 mM NaH₂PO₄, 6 mM EDTA, pH 7.2) prior to saturation with solid CsCl (1.26 g ml⁻¹ of nucleic acid solution). Bisbenzimidide (Sigma, St. Louis; = Hoechst 33258) was added to a final concentration of 0.1 µg ml⁻¹ and the refractive index of the solution was adjusted to 1.390 by the addition of DNA buffer. Bisbenzimidide binds preferentially to A-T rich duplex DNA sequences, thereby enhancing base-composition-dependent buoyant density separations (Hudspeth et al., 1980). The mixture was centrifuged at 100,000 g for 70 hr at 20° C using a Beckman Type 40 rotor. A good separation into mitochondrial, nuclear, and bacterial (V. lobospinosa only) DNA

bands, visualized with long wavelength UV, was obtained for each of the strains. The mtDNA was collected from the side of the 16 X 76 mm stainless steel capped polyallomer ultracentrifuge tubes with a 1 ml disposable syringe equipped with a 22 gauge needle. A Centricon microconcentrator (Amicon Corp., Danvers, MA) was used to desalt the collected mtDNA fraction (upper band in CsCl gradient) after the bisbenzimidazole was removed by extracting the CsCl solution three times with equal volumes of CsCl-saturated isopropanol. The solvent was exchanged once with 1 X DNA buffer supplemented with 1M NaCl followed by four exchanges with 1 X DNA buffer only. After precipitation with ethanol, the sample was resuspended in 400 μ l H-NET buffer. Samples were sequentially digested with 20 μ l RNase solution (2 mg mT^{-1} RNase A (Sigma), 2.5% (v/v) RNase T1 solution (Sigma) in 1:1 H-NET/Glycerol) and 40 μ l Protease K (2 mg mT^{-1} Protease K (Sigma) in 1:1 H-NET/Glycerol) prior to a second triplet series of phenol/CIA extractions. The purified, ethanol precipitated, mtDNA pellet was resuspended in H-NET buffer at an approximate concentration of 0.5 $\mu\text{g } \mu\text{T}^{-1}$ and stored in liquid nitrogen. Concentration of the DNA sample was determined by absorbance at 260 nm (Schleif and Wensink, 1981). Purity of the sample was checked from absorbance at 260, 280, and 320 nm (Schleif and Wensink, 1981).

(b) Enriched mitochondrial DNA fraction. Direct extraction of mitochondrial and extrachromosomal DNA from Naegleria and Vahlkampfia amoebae was performed using a method modified from

Latorre et al. (1986). Amoeba cells were harvested in four 50 ml tubes by centrifugation at 1000 g for 10 minutes using an RC-5 refrigerated centrifuge equipped with an SS-34 rotor. The resulting pellets were washed 1 time with LS. Removal of bulk bacteria from V. lobospinosa prior to mtDNA enrichment was achieved by three successive centrifugal washes as described previously for CsCl isolated mtDNA. The washed cell pellets were each resuspended in 1 ml LS and pelleted in 1.5 ml sterile microcentrifuge tubes. Each of the four pellets was resuspended in 320 μ l of 10 mM Tris, 60 mM NaCl, 5% (w/v) Sucrose, 10 mM EDTA, pH 7.8. Four hundred microliters of 1.25% SDS, 300 mM Tris, 5% Sucrose, 10 mM EDTA, 0.8% diethyl pyrocarbonate (DEP), pH 9.0, was added to each tube and the mixture was incubated at 65° C for 30 minutes. One hundred twenty microliters of 3 M sodium acetate was added to all tubes and the mixture was cooled on ice for 45 minutes prior to microcentrifugation (Fisher) for 10 minutes at 4° C. Each supernatant was added to 750 μ l 2-propanol and kept at room temperature for 5 minutes. After microcentrifuging for 5 minutes at room temperature, the pellets were each thoroughly resuspended in 250 μ l sterile distilled water with 0.25% DEP and incubated for 30 minutes at the same temperature. Distilled water (250 μ l), 3 M sodium acetate (50 μ l) and ethanol (1000 μ l) were sequentially added to all tubes and the mixture was cooled on ice for 10 minutes. The precipitated DNA was pelleted (5 minutes, 4° C), washed with 70% ethanol, and re-pelleted. Residual ethanol was removed by drying the

precipitates for 30 minutes at 4 ° C. Ten microliters of cold TE buffer (10 mM Tris, 10 mM EDTA, pH 8.0) was used to resuspend each enriched mtDNA pellet. After pelleting any insoluble matter using the microcentrifuge (30 seconds, 4 ° C), the four supernatants were combined and stored in liquid nitrogen. Such DNA preparation is sufficient for preparing eight restriction digests. Actual DNA concentration could not be determined by absorbance at 260 nm resulting from protein/RNA contamination.

Restriction enzyme analysis.

(a) CsCl isolated, purified mitochondrial DNA. Restriction endonucleases Bcl I, Bgl II, Cla I, Eco RV, Hin dIII, and Sst I were purchased from Bethesda Research Laboratories (Gaithersburg, MD) or from International Biotechnologies, Inc. (New Haven, CT). Between 1-5 µg mtDNA was digested with an excess of enzyme (2-5 units of enzyme per µg of DNA) in a total volume of 10-20 µl using buffers suggested and supplied by the manufacturer. The digestion reactions were stopped after 4 hr of incubation by addition of 5 µl reaction stop mixture (5 M Urea, 10% glycerol, 0.5% SDS, 0.025% xylene cyanol, 0.025% bromphenol blue) (Rodriguez and Tait, 1983). The heat-dissociated samples (65 ° C, 15 minutes) were loaded onto 15.5 cm wide X 14.5 cm long horizontal 0.6% agarose gels (Bio-Rad, Ultra pure DNA Grade) and run in the submarine-mode at 18 volts for 18 hr in 1 X TBE buffer (90 mM Tris-HCl, 2.5 mM Na₂ EDTA, 89 mM boric acid, pH 8.2). After staining with ethidium bromide (0.4 µg ml⁻¹) in

1 X TBE for 30 minutes, the gel was briefly rinsed with water and the banding pattern visualized and photographed under short wave UV light (302 nm) using a Chromato-Vue transilluminator (Ultra-Violet Products, Inc.), Polaroid MP-3 camera and Wratten #22 filter.

Positive prints and negatives were obtained using Polaroid Type 665 film. Hin dIII digests of lambda phage were included in each gel run as fragment size standards. The sizes of Hin dIII fragments, measured in kilobase pairs, used for standard curves were 23.1 (#1), 9.4 (#2), 6.7 (#3), 4.4 (#4), 2.3 (#5), 2.0 (#6), and 0.56 (#7) as described in the 1985 BRL catalogue. Fragment size vs. distance migrated was plotted using 3 cycle semi-logarithmic graph paper. The region from ~1 to ~10 kbp was log-linear whereas the curve was non-linear for larger and smaller fragments. A least-squares linear regression was performed on the log-linear region whereas non-linear curves were fit to eye. Measurement of fragment sizes was performed as described previously (Bogler et al., 1983). Average fragment sizes and standard deviations were determined from three gel replicates. MtDNA conformation was determined by electrophoresis of undigested and Hin dIII digested DNA through 0.3% agarose gels in the presence of increasing quantities of ethidium bromide (Maniatis et al., 1982).

(b) Enriched mitochondrial DNA fraction. Approximately 2-3 μ g DNA (5 μ l DNA solution as determined by fluorescent intensity) was digested with 30 units of restriction enzyme in a final volume of 20 μ l as described for the CsCl isolated, purified mtDNA. One

microliter RNase stock solution ($40 \mu\text{g ml}^{-1}$ RNase A (Sigma) in 10 mM Tris HCl, 15 mM NaCl, pH 7.5) was added to the digestion mixture. The digestion reactions were stopped and heat-dissociated, as described for purified mtDNA, after 6 hr of incubation. The digestion fragments were electrophoretically separated in 0.6% agarose at 18 volts for 16 hr using 1 X TBE buffer. After electrophoresis, gels were stained and photographed as described for purified mtDNA. Measurement of fragment sizes was performed as described for purified mtDNA.

Undigested, enriched mtDNA fractions from Naegleria strains were electrophoretically migrated to identify and characterize additional extrachromosomal elements separated with the mitochondrial DNA using the enrichment extraction procedure. Horizontal, submarine-mode agarose gels (0.6%) containing RNase A digested ($2 \mu\text{g ml}^{-1}$) enriched mtDNA fractions were electrophoresed at 29 volts (2 v/cm) for 18 hr in 1 X TBE buffer.

Thermal denaturation temperature (T_m) determination. A

Centricon microconcentrator (Amicon Corp.) was used to desalt nuclear and mitochondrial DNA samples by exchanging the solvent three times with 0.1 X SSC (0.015 M NaCl, 0.0015 M Na citrate). The DNA was at a final concentration of approximately $1 \mu\text{g ml}^{-1}$, and was contained in a small (1.5 ml), closed quartz cuvette. Determination of the denaturation temperature was performed as described by Marmur & Doty (1962).

DNA-DNA hybridization studies.

(a) DNA isolation. Escherichia coli colonies containing a cloned Eco RI fragment (3.05 kbp) of mtDNA from Acanthamoeba castellanii (Neff strain) complementary to Cytochrome b gene probes isolated from both Neurospora and Saccharomyces, and a cloned Bam HI/Mbo I fragment (~2 kbp) of mtDNA from the yeast Saccharomyces cerevisiae containing the Cytochrome C oxidase gene were obtained from T. J. Byers. Plasmid DNAs (pBR322) were isolated from E. coli lysates using the large-scale plasmid purification procedure described by Rodriguez & Tait (1983). Naegleria mtDNAs were isolated and purified using the previously described UNSET procedure.

(b) Biotin labeling of plasmid and mitochondrial DNAs.

Plasmid and mitochondrial (Naegleria fowleri, strain Nf66) DNAs were biotin-labeled with biotin-11-dUTP by nick translation (Rigby et al., 1977) using the Nick Translation System (BRL).

(c) Restriction endonuclease cleavage and gel

electrophoresis of mitochondrial DNAs. Hin dIII digested samples of mtDNA (Naegleria fowleri (MB-41, Nf66, and 6088), Naegleria lovaniensis (TS), and Naegleria jadini (0400)) were electrophoresed in 0.6% agarose horizontal slab gels as previously described. Positive (Sau 3A digest of Saccharomyces clone, or Hin dIII digest of Acanthamoeba clone or Naegleria fowleri (Nf66) mtDNA) and negative (Hin dIII digest of lambda phage DNA) controls were included.

(d) Gel blotting. DNA restriction fragments were transferred

from agarose gels to Zeta-Probe nylon membranes using a Trans-Blot Cell System (Bio-Rad). Transfers were performed as described by the manufacturer and run at 60 volts for 5 hr. After blotting, the membranes were baked for 2 hr at 80 ° C.

(e) DNA-DNA hybridization. Blots of Naegleria mtDNA restriction fragments were hybridized in reaction mixtures containing denatured, biotin-labeled DNA probes at 15-36 ° C in 20 mM NaH₂PO₄ (pH 6.5), 1 X Denhardt's solution, 0.2 mg ml⁻¹ freshly denatured, sheared salmon sperm DNA, 2-5 X SSC, and 20-40% formamide so that the effective temperature (calculated from the formamide and salt concentration) was 25-45 ° C below the calculated T_m value (i.e. T_m - 25 ° C or T_m - 45 ° C). Each blot was sequentially washed in 2 X SSC and 0.2 X SSC, respectively, at the equivalent effective temperature. Detection of bound biotinylated probes was performed using the Blugene Nonradioactive Nucleic Acid Detection System (BRL) as described by the manufacturer.

Genetic divergence. An estimate of interstrain genetic divergence was quantitated using the method of Engels (1981) as previously applied to Acanthamoeba (Bogler et al., 1983). The proportion of mismatched bases between two genomes was indirectly calculated using summed "k" (number of dimorphic sites) and "m" (total number of monomorphic and dimorphic restriction sites) values determined by various observed fractions of common, homologous

fragments, for all selected enzymes by:

$$\hat{p} = k / (2jm - jk)$$

where (j) is the number of base pairs per restriction site. A table "k" values based on various combinations of "G" (number of monomorphic (identically sized) fragments in both genomes) and "F" (total number of monomorphic and dimorphic (different-sized) fragments) is provided by Engels (1981). Assuming equiprobable permutations of monomorphic and dimorphic sites, the maximum likelihood estimator, which is independent of average base composition and expected fragment length, is used to estimate "k" from "F" and "G" (Engels, 1981). For a circular genome, "m" is calculated by the expression:

$$m = (F + k) / 2.$$

Matrices of pairwise genetic distances ($\hat{p}$ values) were analyzed and clustered by the UPGMA method (Hedrick, 1985; Sneath and Sokol, 1973) in order to construct dendrograms. The UPGMA cluster method assumes a constant evolutionary rate over time for all lineages.

RESULTS

Restriction fragment analysis.

(a) CsCl isolated, purified mitochondrial DNA. Six of the eleven hexanucleotide recognizing restriction endonucleases tested for fragment pattern appropriateness were found to be satisfactory for this analysis. Bcl I, Bgl II, Cla I, Eco RV, Hin dIII, and Sst I, each produced a simple, distinct fragment pattern when used to digest the mitochondrial DNA from the vahlkampfiid amoeba strains. An average of approximately six fragments was produced from each restriction enzyme digestion ($\bar{x} = 5.5 \pm 2.6$ fragments/digest; variance = 6.5 fragments). Production of a small number of restriction fragments allows the comparison of patterns to be performed with less ambiguity (Lansman et al., 1981). Fragments smaller than ~0.4 kbp were not detected by the assay conditions used in this study. Fragment sizes produced upon digestion with restriction enzymes are given in Table II. Fragment patterns were identical for mtDNA from whole cells or from enriched mitochondrial fractions. Strains Nf66 and MB-41 of Naegleria fowleri gave nearly identical fragment size patterns for Bcl I, Bgl II, Eco RV, and Hin dIII digestions, but differences between the patterns for the Hin dIII digestion became apparent when the two strains were run side-by-side on the same gel (Fig. 1). The 6088 strain of

a

TABLE II. Restriction endonuclease fragment size estimates

Vahlkampfiid Strains								
EGs	1518/1a	30298	MB-41	Nf66	TS	6088	0400	
<u>Bcl</u> I digestion								
a	17.0	14.0 <u>0</u>	19.7	36.0	34.3	20.6	43.8	16.3
	<u>+0.9</u>	1.0	1.3	9.2	5.9	0.0	2.0	0.7
b	13.4	10.9	16.9	15.2	15.1	10.6	15.1	12.2
	<u>+0.5</u>	1.0	1.1	1.0	0.6	0.1	0.2	0.4
c	8.48	6.21	13.0	3.25	3.16	9.21	3.16	7.22
	<u>+0.16</u>	0.34	0.6	0.11	0.10	0.08	0.06	0.10
d	6.11	4.25	3.22	1.21	1.16	7.77		6.99
	<u>+0.09</u>	0.21	0.12	0.03	0.02	0.13		0.04
e	4.38	3.21	2.58			3.47		3.49
	<u>+0.02</u>	0.16	0.10			0.05		0.09
f	3.12	2.55	2.29			3.14		3.20
	<u>+0.01</u>	0.13	0.17			0.06		0.09
g		1.62						2.58
		0.10						0.04
h								1.93
								0.03

Table II (cont'd.)

<u>Bgl</u> II digestion								
a	16.2	17.3	21.2	16.2	17.1	15.9	16.1	13.9
	<u>+0.2</u>	0.7	1.6	1.7	1.1	0.9	0.6	1.0
b	8.17	8.71	14.8	6.62	6.66	7.49	6.65	8.62
	<u>+0.05</u>	0.07	0.7	0.25	0.16	0.22	0.13	0.30
c	7.48	7.22	10.2	6.09	6.14	5.66	6.16	7.83
	<u>+0.03</u>	0.06	0.6	0.23	0.15	0.14	0.19	0.30
d	6.21	5.76 <u>0</u>	7.77	4.88	4.88	4.55	4.84	5.75
	<u>+0.07</u>	0.10	0.17	0.20	0.15	0.12	0.13	0.22
e	3.91	4.54		4.72	4.73	3.54	4.67	5.71
	<u>+0.09</u>	0.07		0.19	0.17	0.18	0.16	0.22
f	3.55	3.82		4.57	4.59	2.96	4.59	4.94
	<u>+0.05</u>	0.03		0.20	0.17	0.15	0.14	0.20
g	3.36	1.29		4.37	4.42	2.47	4.38	3.94
	<u>+0.06</u>	0.07		0.19	0.15	0.05	0.14	0.18
h	2.23	0.95		1.41	1.79	2.03	1.37	
	<u>+0.02</u>	0.14		0.05	0.03	0.04	0.04	
i	0.83							
	<u>+0.04</u>							
j	0.49							
	<u>+0.06</u>							

Table II (cont'd.)

<u>Cla</u> I digestion							
a	29.0	24.5	27.9	23.7	24.6	29.1	26.7 <u>D</u> 12.3
	<u>+1.7</u>	0.5	2.1	1.4	0.5	0.7	0.3 0.6
b	7.04	14.6	15.7	15.3	16.2	14.2	8.90 8.08
	<u>+0.10</u>	0.4	1.0	1.0	0.1	0.7	0.12 0.24
c	6.01	12.5	8.02	8.66	7.08		3.69 7.67
	<u>+0.14</u>	0.1	0.20	0.23	0.09		0.07 0.26
d	4.39	2.40		6.71	3.68		6.23
	<u>+0.11</u>	0.05		0.16	0.10		0.19
e	0.73	1.35			2.43		4.88
	<u>+0.01</u>	0.05			0.06		0.17
f							4.75
							0.14
g							4.61
							0.12
h							4.27
							0.13

Table II (cont'd.)

<u>Eco</u> RV digestion								
a	26.8	25.2	20.4	35.1	34.7	36.9	43.7	14.0
	<u>+3.4</u>	2.9	1.4	6.5	5.6	0.8	2.3	0.4
b	12.4	15.5	9.24	19.4	19.2	8.16	18.0	10.8 <u>0</u>
	<u>+0.8</u>	1.4	0.33	0.5	0.3	0.03	0.5	0.2
c	9.29	11.1	5.34					8.5
	<u>+0.32</u>	0.1	0.08					0.08
d	7.27	6.33	4.78 <u>0</u>					5.29
	<u>+0.19</u>	0.25	0.08					0.03
e	2.35	2.50	4.15					2.48 <u>0</u>
	<u>+0.09</u>	0.13	0.01					0.03
f			1.96					
			0.07					

Table II (cont'd.)

<u>Hin</u> dIII digestion								
a	14.6	18.9	10.8	16.6	16.0	24.1	15.1	32.6
	<u>+0.5</u>	2.6	0.1	0.4	0.2	0.0	0.2	0.6
b	9.93	10.4 <u>D</u>	6.79	11.7	11.4	13.8	11.1	11.7
	<u>+0.12</u>	0.6	0.73	0.3	0.2	0.1	0.3	0.2
c	9.10	5.01	5.63 <u>D</u>	8.78	8.92	11.8	8.68	10.6
	<u>+0.14</u>	0.19	0.04	0.21	0.14	0.0	0.09	0.2
d	5.62	3.11	4.80	4.50	4.28	3.79	4.25	4.64
	<u>+0.05</u>	0.05	0.25	0.17	0.12	0.03	0.00	0.02
e	3.08	2.42	3.92	4.33	4.06	1.79	4.05	1.97
	<u>+0.03</u>	0.07	0.05	0.19	0.11	0.02	0.00	0.03
f	2.70	1.38	3.53	3.64	3.57	1.35	3.56	1.47
	<u>+0.04</u>	0.09	0.04	0.15	0.09	0.02	0.04	0.01
g	2.29 <u>D</u>	1.19	2.38	2.80	2.75	1.07	3.26	
	<u>+0.04</u>	0.01	0.35	0.11	0.09	0.02	0.04	
h	1.58		1.97	1.74	1.44		1.47	
	<u>+0.06</u>		0.01	0.47	0.03		0.07	
i	1.35						1.09	
	<u>+0.05</u>						0.04	
j	0.90							
	<u>+0.01</u>							

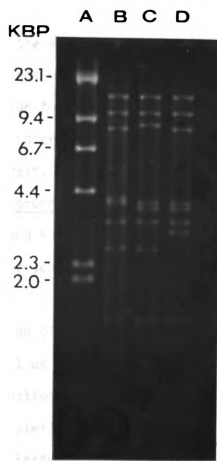
Table II (cont'd.)

<u>Sst</u> I digestion							
a	20.5	23.8	29.6 <u>D</u>	30.0 <u>D</u>	29.0 <u>D</u>	29.3 <u>D</u>	27.7 <u>D</u> 31.0 <u>D</u>
	<u>+0.9</u>	1.0	0.3	1.0	0.3	0.6	0.0 1.2
b	15.2	20.2			2.49		2.52 2.35
	<u>+0.6</u>	0.9			0.07		0.05 0.06
c	12.7	14.1					
	<u>+0.4</u>	0.7					
d	1.68	2.58					
	<u>+0.05</u>	0.05					

a
Average fragment sizes (kbp) are in the first row of each fragment entry and standard deviations are in the second row. Averages are for measurements made from three gel replicates. The symbol D indicates the presence of two different fragments of the same size as determined by the relative fluorescent intensity of the band.

Figure 1. Agarose gel electrophoretic patterns for Hin dIII mitochondrial DNA digests of MB-41, Nf66, and 6088 strains of *N. fowleri*. Lane A contains Hin dIII digest of wild type Lambda phage DNA. Lanes B, C, and D contain Hin dIII digest of mtDNA from MB-41, Nf66, and 6088 strains, respectively.

1





Naegleria fowleri also had a digestion pattern that was similar yet quite distinct from the other N. fowleri strains.

Genome sizes determined by summation of fragment sizes are shown in Table III. The average standard deviation found between identical enzyme digestions of the eight vahlkampfiid amoeba strains (± 4.6) is equivalent to the average standard deviation found between different enzyme digestions of the same strain (± 4.8). Therefore, the mtDNA length variation found between the strains cannot be readily attributed to insertion/deletion events as the presented data might initially suggest. Average mitochondrial DNA sizes for the two strains of N. gruberi and the three strains of N. fowleri were 54.6 ± 3.3 kbp and 55.6 ± 2.2 kbp, respectively. The overall average genome size for seven strains of Naegleria used in this study was 54.8 ± 2.7 kbp.

Examination of uncleaved Naegleria mtDNA band mobility in the presence of $0.1 \mu\text{g ml}^{-1}$ free ethidium bromide indicated a circular genome conformation, since the migration rate was faster than that of linear DNA of similar size.

(b) Enriched mitochondrial DNA fraction. Mitochondrial DNA enrichment from each vahlkampfiid amoeba strain was digested to completion with the restriction endonucleases, Bcl I, Hin dIII, Eco RV, Bgl II, Cla I, and Sst I. A schematic diagram of the different, electrophoretically resolved, restriction fragment patterns is shown in Fig. 2. An average of approximately three additional fragments was produced from each restriction enzyme digestion ($\bar{x} = 3.4 \pm 1.7$

Table III. Genome sizes based on summation of restriction endonuclease
^a
 fragment sizes.

Strain ^b	Restriction endonuclease						Average size
	<u>Bcl</u> I	<u>Hin</u> dIII	<u>Eco</u> RV	<u>Bgl</u> II	<u>Cla</u> I	<u>Sst</u> I	
EGs	52.5	53.4	58.1	52.4	47.2	50.1	52.3 $\pm$ 3.6
1518/1a	56.7	52.8	60.6	55.4	55.4	60.7	56.9 $\pm$ 3.1
ATCC30298	57.7	45.5	50.7	54.0	51.6	59.2	53.1 $\pm$ 5.0
MB-41	55.7	54.1	54.5	48.9	54.4	60.0	54.6 $\pm$ 3.6
Nf66	53.7	52.4	53.9	50.3	54.0	60.5	54.1 $\pm$ 3.4
TS	54.8	57.7	45.1	44.6	43.3	58.6	50.7 $\pm$ 7.1
6088	62.1	52.6	61.7	48.8	66.0	57.9	58.2 $\pm$ 6.5
0400	53.9	63.0	54.4	50.7	52.8	64.4	56.5 $\pm$ 5.7

^a
 Genome size in kilobase pairs. Averages include standard deviation.

^b
 See Table I for species details.

Figure 2. Schematic diagram of digestion fragments of enriched mtDNA from eight strains of Naegleria and one strain of Vahlkampfia by restriction endonucleases Bcl I, Hin dIII, Eco RV, Bgl II, Cla I, and Sst I. Hin dIII digest of Lambda phage DNA was used for size markers. M = mitochondrial DNA restriction fragment.

2

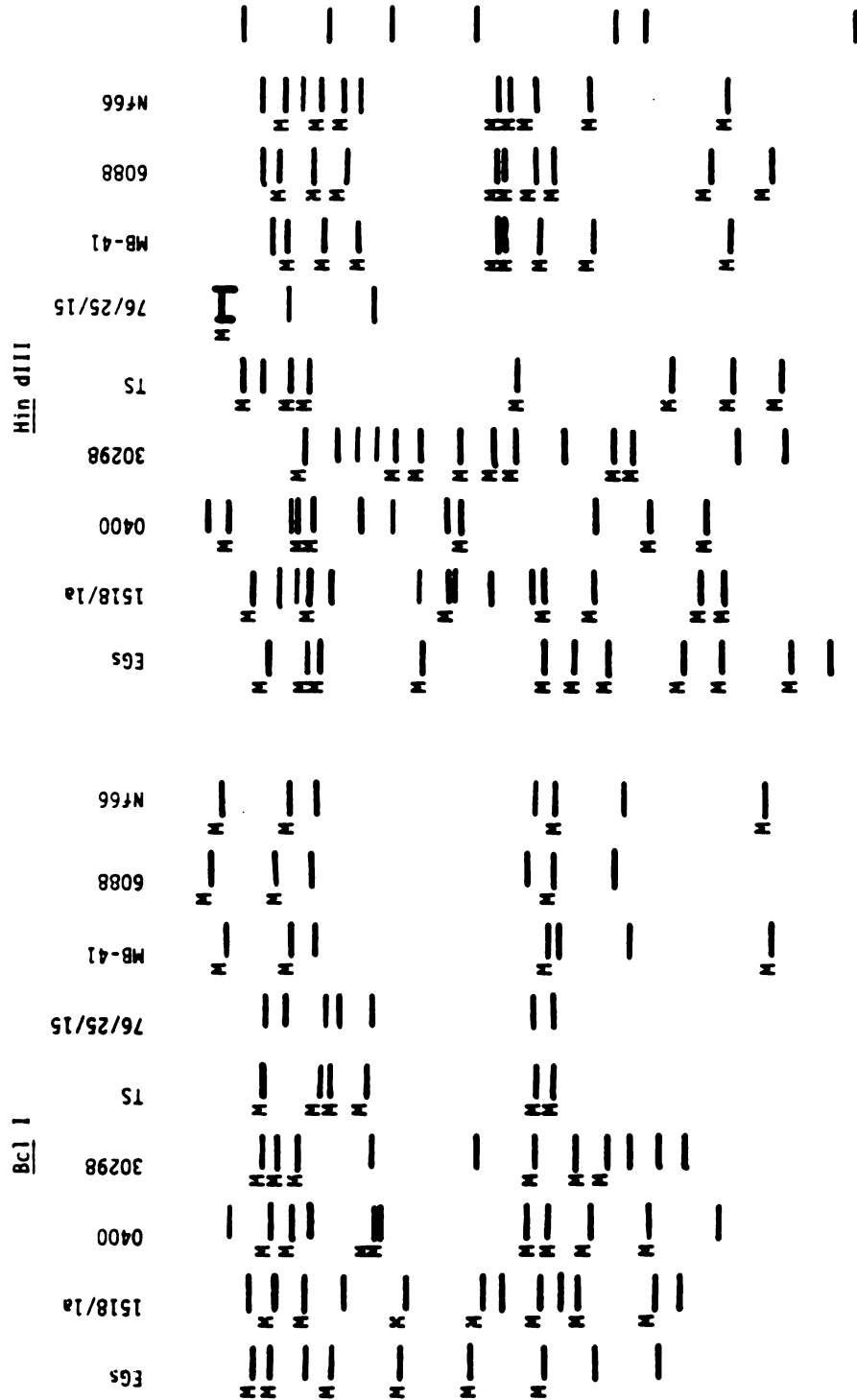


Figure 2 (cont'd.)

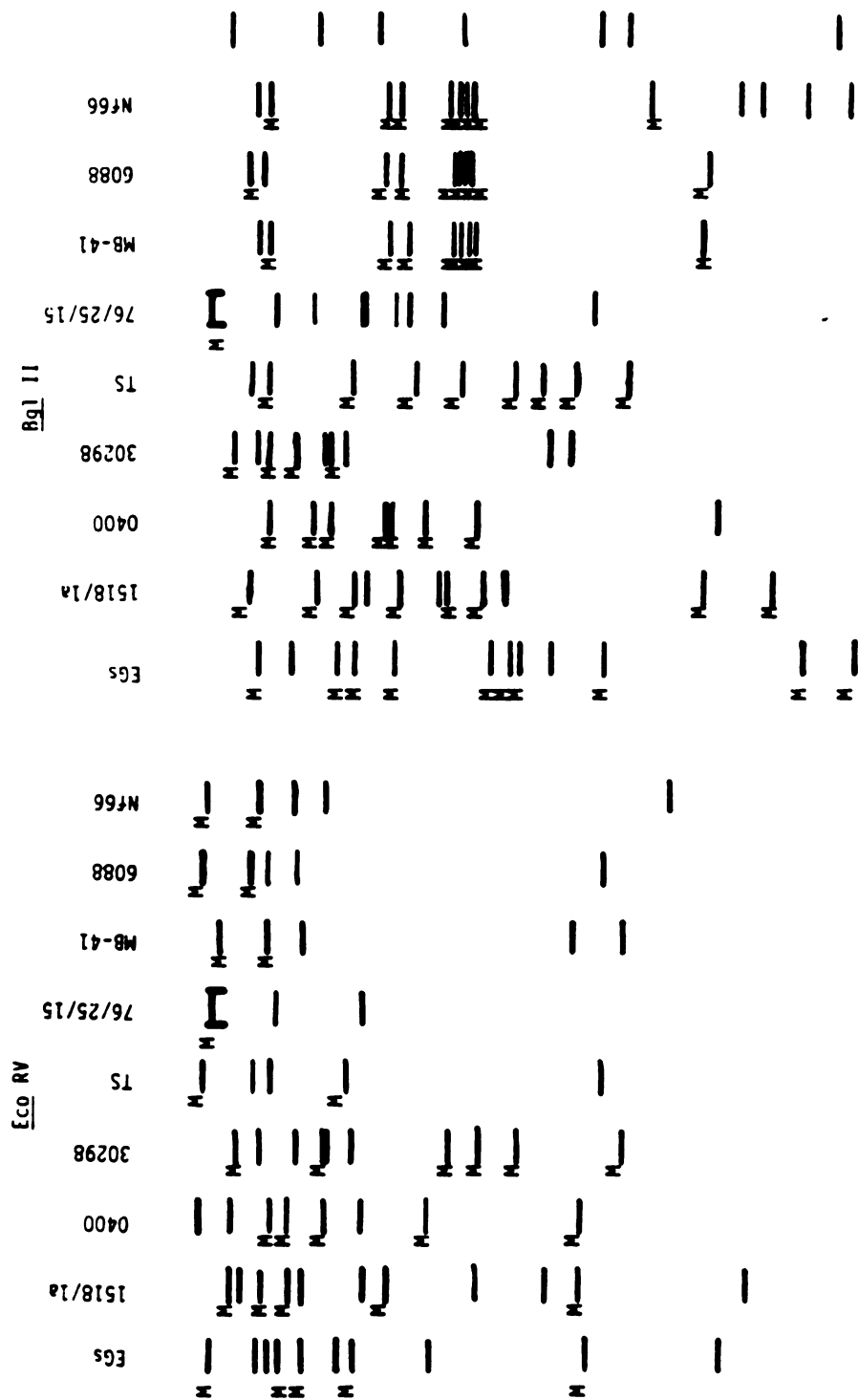
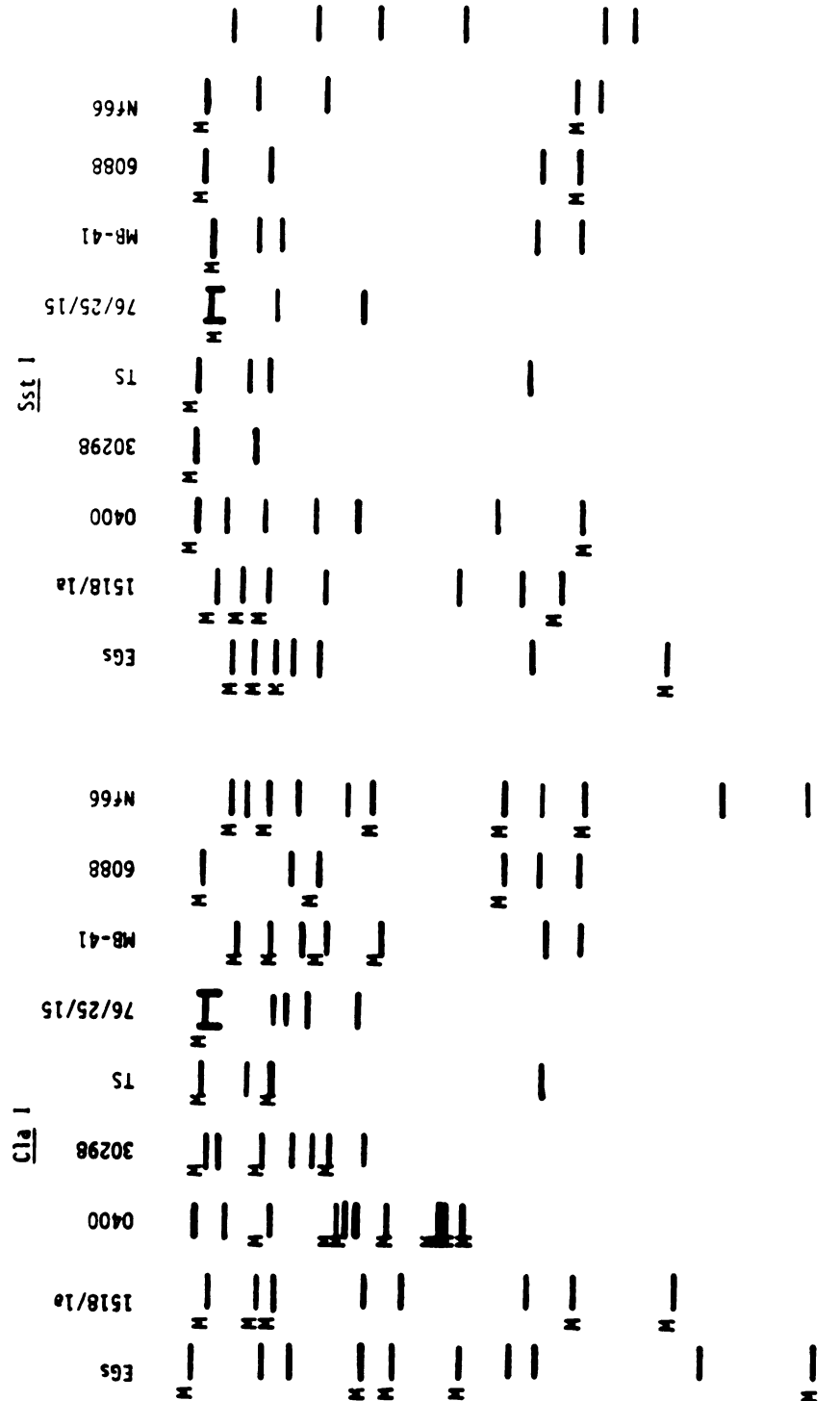


Figure 2 (cont'd.)



additional fragments/digest; variance = 2.8 fragments) when compared to the restriction fragment patterns from CsCl purified mtDNA, suggesting that an additional extrachromosomal element (i.e. plasmid) may be present in this enriched mtDNA fraction. Strains MB-41, 6088, and Nf66 of Naegleria fowleri produced highly similar fragment size patterns for Bcl I, Bgl II, and Hin dIII digestions which differed by the presence of 1 to 4 additional bands (Fig. 2). Naegleria lovaniensis, strains TS and 76/25/15, gave highly similar fragment patterns for the Bcl I digestion only, since the mtDNA from strain 76/25/15 was uncleaved (undigested) by the other five restriction enzymes. Readily observable differences in fragment patterns between Naegleria gruberi, strains EGs and 1518/1a, N. jadini, strain 0400, and Vahlkampfia lobospinosa, strain ATCC30298 were exhibited for all six restriction enzyme digestions. Stability of these DNA gel patterns was observed for stock cultures stored at 15-30° C (25-37° C for high-temperature tolerant strains).

Putative plasmid DNA. Table IV gives fragment size estimates for the extrachromosomal, non-mitochondrial DNA. The sizes of these additional bands were determined by electrophoresis of the mtDNA enriched digests in lanes adjacent to lambda phage DNA fragments of known length. The close similarity between the three strains of N. fowleri is further supported by the presence of comparable extrachromosomal Bcl I, Cla I, and Hin dIII fragment sizes.

Electrophoresis of undigested, enriched mtDNA fractions directly extracted from eight Naegleria strains demonstrated five

TABLE IV. Extrachromosomal (non-mitochondrial DNA) restriction
endonuclease fragment size estimates^a

Vahlkampffid Strains									
EGs	1518/1a	0400	30298	TS	76/25/15	MB-41	6088	Nf66	
<u>Bcl</u> I digestion									
a	9.95	18.1 ^b	29.3 ^c	7.45	15.0 ^b	14.3 ^b	10.4	10.3	10.4
b	2.47	8.25	10.3	4.53			3.12	3.50	3.45
c	1.83	3.82	1.36	2.11			2.21	2.28	2.25
d		2.87		1.84 ^e					
e		1.60		1.63					
<u>Bgl</u> II digestion									
a	10.4	7.05	13.4 ^b	15.3 ^b	15.0 ^b	14.3 ^b	17.7 ^b	16.2 ^b	15.7 ^b
b	2.90	4.95	6.35 ^e	8.85		10.3 ^e		13.5 ^e	1.23 ^e
c		3.58	1.31 ^e	7.90 ^e		7.63 ^e			1.09 ^e
d				2.90		7.58 ^f			0.82 ^e
e				2.63		6.55 ^e			0.45 ^e
f						6.09			
g						5.14			
h						2.47			

Table IV (cont'd.)

<u>Cla</u> I digestion									
a	12.6 ^e	7.00	31.0 ^c	26.7 ^d	15.0 ^b	14.3 ^b	10.5	10.7	16.8 ^b
b	10.0 ^e	5.85	23.1 ^d	10.7 ^f	12.2	12.4 ^e	3.10	3.10	10.8
c	6.90	3.17	7.24 ^f	9.25	3.09	10.3 ^e	2.57	2.58	7.70 ^e
d	3.47			6.95		7.58 ^f			3.10
e	3.05								1.34
f	1.38 ^e								0.84

<u>Eco</u> RV digestion									
a	14.4 ^b	18.5 ^b	31.0 ^c	15.3 ^b	15.0 ^b	14.3 ^b	10.8	16.2 ^b	15.4
b	12.8 ^e	10.0 ^e	23.0 ^d	10.8 ^f	12.8	7.58 ^f	2.77	13.1	11.4 ^e
c	8.10	7.25	13.4 ^b	8.85	2.29		2.17	10.7 ^e	9.20 ^e
d	5.15	4.14	7.25 ^f	7.65				2.28	1.73
e	1.29 ^e	2.91							
f		1.07							

Table IV (cont'd.)

<u>Hin dIII digestion</u>									
a	13.5	12.9 ^f	33.0 ^c	8.85	15.0 ^b	14.3 ^b	17.3 ^b	16.2 ^b	16.6 ^b
b	0.71	11.1 ^e	12.2 ^b	7.90 ^e		7.58 ^f			11.9 ^e
c		8.95	7.70 ^f	7.14 ^e					8.00 ^e
d		5.85 ^e	6.55	2.87					
e		4.83 ^e	5.01	1.26					
f		4.09	2.45	1.02					
g		3.33							

<u>Sst I digestion</u>									
a	10.3 ^e	8.65	23.1 ^d	15.3 ^b	15.0 ^b	14.3 ^b	18.0 ^b	12.7	15.9 ^b
b	8.75	4.48	13.7 ^b		12.4	7.58 ^f	13.2	3.10	9.20 ^e
c	3.19	3.60	9.20 ^e		3.28		3.30 ^e		2.44 ^e
d	1.68		7.20 ^f				2.64		
e			3.78 ^e						

-
- a Fragment sizes (kbp) are for measurements from a single gel.
- b Presumptive conformation form III (linear DNA).
- c Unidentified DNA component.
- d Presumptive conformation form II (open circular DNA).
- e Partially digested fragment as determined by size and/or intensity.
- f Presumptive conformation form I (supercoiled DNA)

common variable-sized components and one common non-variable-sized component (Fig. 3). Band mobility of components 3 and 5 did not vary relative to linear DNA markers with the presence of increasing quantities of ethidium bromide (up to 0.4 ug ml^{-1}), suggesting that these two components had linear conformations. The main band of non-variable-sized component 3 was found to have an average size of $54.3 \pm 1.4 \text{ kbp}$ for the eight strains of Naegleria indicating that this was the linear conformation of the mtDNA genome. Component 5 varied in size among strains from 12.5 to 16.4 kbp (Table V) and was believed to be conformation form III (linear) of the extrachromosomal, non-mitochondrial DNA since migration related faster (component 6: presumptive conformation form I (supercoiled)) and slower (component 4: presumptive conformation form II (open circular)) bands were observed in each strain. Circular components 1 and 2 are believed to be related forms of the extrachromosomal DNA since their mobility in gels is correlated with the migration of the linear component.

The presumptive circular plasmid genome size was determined by summation of completely digested fragment sizes or from linear DNA conformations (Table V). The average standard deviation found between identical enzyme digestions of the nine vahlkampfiid amoeba strains (± 1.3) was more than twice the average standard deviation found between different enzyme digestions of the same strain (± 0.6), indicating that the presumptive plasmid size variation found between

Figure 3. Schematic diagram of electrophoretically migrated components from undigested, enriched mtDNA fractions extracted from the eight strains of Naegleria. Hin dIII digest of lambda phage DNA was used for fragment size markers.

3



Table V. Presumptive plasmid genome sizes based on summation of restriction endonuclease fragment sizes or linearized DNA^a compared to undigested, linear conformation.

Strain	Restriction endonuclease						Average size	Undigested
	<u>Bcl</u> I	<u>Hin</u> dIII	<u>Eco</u> RV	<u>Bgl</u> II	<u>Cla</u> I	<u>Sst</u> I		
EGs	14.3	14.2	13.3	13.3	13.4	13.6	13.7 ± 0.5	14.0
1518/1a	16.5	16.4	15.4	15.6	16.0	16.7	16.1 ± 0.5	16.4
0400	11.7	14.0	13.4	13.4	----	13.7	13.2 ± 0.9	12.5
TS	15.0	15.0	15.1	15.0	15.3	15.7	15.2 ± 0.3	15.4
76/25/15	14.3	14.3	14.3	13.7	14.3	14.3	14.2 ± 0.2	13.7
MB-41	15.7	17.3	15.7	17.7	16.2	15.8	16.4 ± 0.9	15.5
6088	16.1	16.2	15.4	16.2	16.4	15.8	16.0 ± 0.4	15.5
Nf66	16.1	16.6	17.1	15.7	16.1	15.9	16.3 ± 0.5	15.2
ATCC30298	15.7	14.0	16.5	14.4	16.2	15.3	15.4 ± 1.0	----

^a Genome size in kilobase pairs. Averages include standard deviation.

^b Presumptive plasmid genome size based on summation of completely digested restriction endonuclease fragment sizes unless indicated otherwise.

^c Presumptive plasmid genome size based on linear conformation.

^c Gel lacks completely digested fragments and linear DNA conformation for size estimate.

strains may be attributed to insertion/deletion events. Average presumptive plasmid genome sizes determined primarily by summation of fragment sizes ranged from 13.2 to 16.4 kbp while plasmid sizes determined solely from undigested linear conformations ranged from 12.5 to 16.4 kbp. Since there is significant variability between the strains in the number and size of the restriction fragments produced, substantial nucleotide sequence variation exists.

Thermal denaturation temperature (T_m) determination. The T_m values for nuclear DNA (from N. fowleri, strain MB-41) and mitochondrial DNA (from N. gruberi, strain EGs) in 0.1 X SSC were estimated at 64.6° C and 50.8° C, respectively.

DNA-DNA hybridization studies. At T_m - 25° C, biotin-labeled plasmid probes hybridized with restriction enzyme digested, positive control DNA (i.e. hybridized with itself), but failed to form thermally stable cross-hybrids with the mtDNA from Naegleria. At low stringent conditions (T_m - 45° C), the biotin-labeled mtDNA probe (Nf66) produced stable, but faint hybrids with several Hin dIII mtDNA restriction fragments from all species of Naegleria (i.e. N. fowleri (strains Nf66, MB-41, and 6088), N. gruberi (strain EGs), N. lovaniensis (strain TS), and N. jadini (strain 0400)).

Genetic divergence. Utilizing the method of Engels (1981), as applied previously (Bogler et al., 1983) to strains of Acanthamoeba, to estimate the genetic divergence between the various valkhampioid strains, fractions of common, homologous fragments were determined for each pairwise examination as shown in Tables VI and VII. Standard

Table VI. Proportions of homologous fragments and estimates of interspecific genetic divergence in pairwise comparisons of Bgl II, Cla I, Eco RV, Hin dIII, and Sst I digestion fragment patterns.

	EGs	Nf66	TS	0400
EGs	<u>Bgl</u> II	1/18	5/18	2/17
	<u>Cla</u> I	2/10	2/7	0/13
	<u>Eco</u> RV	0/7	0/7	0/12
	<u>Hin</u> dIII	0/19	2/18	0/17
	<u>Sst</u> I	0/7	0/6	0/7
Nf66			2/16	2/15
			0/7	0/13
	12.30		1/4	0/9
			0/15	0/14
			2/5	0/6
TS				2/15
				0/10
	7.14	9.93		0/9
				1/13
				0/5
0400	14.14	13.74	11.54	

^a

Fractions, [G/F], where G equals the number of monomorphic fragments in both genomes, and F equals the total number of fragments from both

Table VI (cont'd.)

genomes, are provided for five of the six restriction endonucleases used in Table II. Comparisons of fragment sizes produced from Bcl I digestions were not made since instability in enzyme activity was observable with some DNA substrates (i.e. from TS and O400). Values to the left of the [G/F] comparisons are estimates of genetic divergence, $\hat{p} \times 100$, based on aggregate data from five enzymes. Both analyses were performed at a fragment identity level of one-half the standard deviation.

Table VII. Proportions of homologous fragments and estimates of intraspecific and interspecific genetic divergence in pairwise comparisons of Bcl I, Bgl II, Cla I, Eco RV, Hin dIII, and Sst I digestion fragment patterns .

EGs		1518/1a	30298	MB-41	Nf66	6088
EGs	<u>Bcl</u> I	1/14	2/12	0/10	1/10	0/9
	<u>Bgl</u> II	0/19	0/14	2/18	1/18	3/18
	<u>Cla</u> I	0/10	0/8	0/9	2/10	0/9
	<u>Eco</u> RV	1/10	2/12	0/7	0/7	0/7
	<u>Hind</u> III	1/19	5/20	1/19	0/19	0/20
	<u>Sst</u> I	2/8	0/6	0/6	0/7	0/7
1518/1a			4/14	2/12	2/12	1/11
			0/13	2/17	3/17	1/17
	10.83		0/8	0/9	3/10	0/9
			0/12	0/7	0/7	0/7
			1/17	0/16	0/16	0/17
			0/6	0/6	0/7	0/7
30298				2/10	1/10	1/9
				0/12	0/12	0/12
	9.26	11.90		2/7	1/8	0/7
				0/9	0/9	0/9
				1/17	1/17	0/18
				2/4	0/5	0/5

Table VII (cont'd.)

						4/8	2/7
						12/16	14/16
MB-41	12.32	12.19	9.32				
						0/9	0/8
						3/4	0/4
						4/16	2/17
						0/5	0/5
						4/7	
						12/16	
Nf66	11.50	9.90	11.20	5.17	2/9		
						0/4	
						5/17	
						1/6	
6088	13.81	13.24	15.00	7.31	3.39		

^a

Same as in Table VI except values based on fragment patterns produced from Bcl I, Bgl II, Cla I, Eco RV, Hin dIII, and Sst I digestions.

deviations were determined for each fragment size determination (Table II) and used to set limits for size identity comparisons. Fragment size comparisons and genetic divergence analyses in Tables VI and VII were performed at a fragment identity limit of one-half the standard deviation. At a fragment size identity limit of one standard deviation, two fragments are considered homologous when the size difference between them is less than or equal to one times the standard deviation of either fragment (Bogler et al., 1983). When fragments were compared at this size identity limit for identical patterns of Bcl I or Eco RV digests and highly similar patterns of Hin dIII digest, few fragments were found to be non-homologous between strains Nf66 and MB-41 of N. fowleri. Twenty out of 28 fragments compared were considered homologous. When the estimate of genetic divergence between the two strains was determined at this identity level for each of the three enzyme digests, no genetic difference was concluded ($\hat{p} = 0\%$). However, since differences in Hin dIII restriction fragment mobility between these two N. fowleri strains are striking in side-by-side, single gel comparisons (Fig. 1), a more stringent identity level must be implemented in order to detect this nucleotide sequence difference in the $\hat{p}$ value estimate. The identity level limit of one-half the standard deviation proved to be appropriate for this purpose and fragment identity data and genetic divergence estimates at this stringency appear in Tables VI and VII.

Dendrograms. Dendrograms based on fragment identities at a size stringency of one-half the standard deviation are shown in Fig. 4 and 5.

Figure 4. Dendrogram representing estimated genetic divergence between strains from four *Naegleria* species: *N. jadini* (0400), *N. fowleri* (Nf66), *N. lovaniensis* (TS), and *N. gruberi* (EGs), using UPGMA cluster analysis. Percent nucleotide divergence calculated using $\hat{p}$ values in Table VI.

4

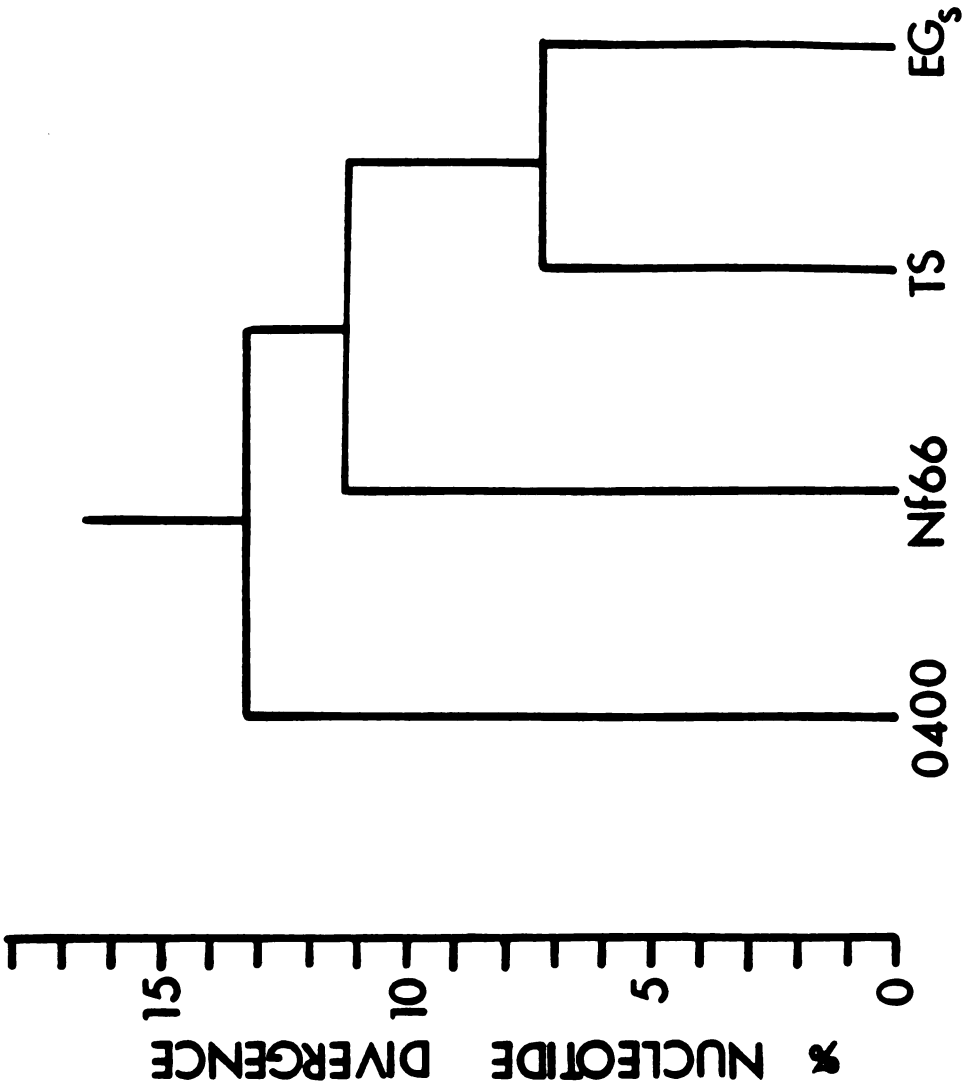
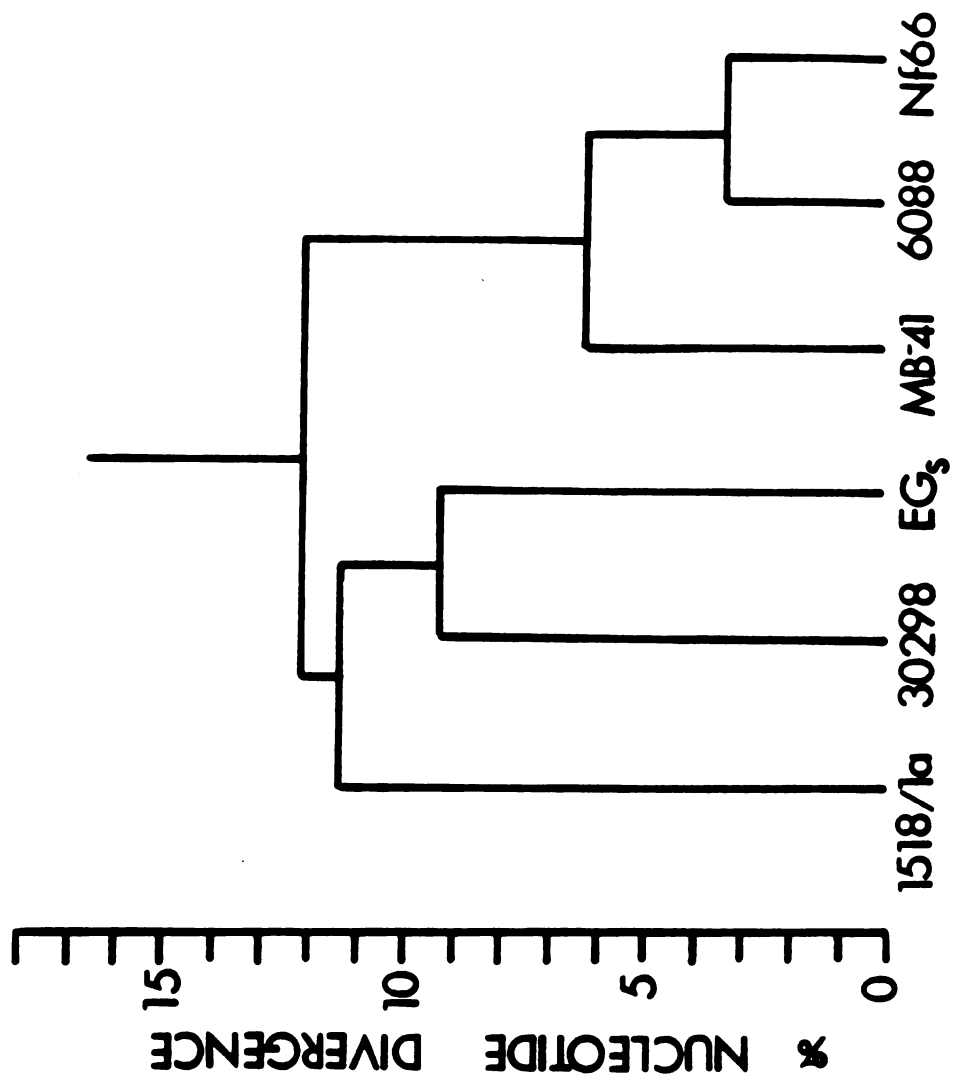


Figure 5. Dendrogram representing estimated genetic divergence between strains 1518/1a and EGs of N. gruberi, strain ATCC30298 of Vahlkampfia lobospinosa, and strains MB-41, 6088, Nf66, of N. fowleri using UPGMA cluster analysis. Percent nucleotide divergence calculated using $\hat{p}$ values in Table VII.

5



Naegleria jadini, strain 0400, is quite distinct from the compared strains of N. gruberi (EGs), N. fowleri (Nf66), and N. lovaniensis (TS) (Fig. 4) as demonstrated by 13.1% average sequence differences as estimated by $\hat{p}$. Naegleria fowleri, strain Nf66, is as divergent from N. gruberi, strain EGs, with sequence mismatches estimated at 12.3% (Table VI) and average sequence differences of 11.1% (Fig. 4) with the EGs-TS cluster. This relationship is contrasted with the two clusters, ((Nf66 6088) MB-41) x ((EGs ATCC30298) 1518/1a), depicted in Fig. 5, where interstrain sequence differences within the same Naegleria species is no more than 7.3% for N. fowleri and as large as 10.8% for N. gruberi (Table VII). Estimated overall sequence divergence between the two Naegleria clusters is approximately 12.1% thereby indicating that N. fowleri is indeed clearly distinct from N. gruberi.

DISCUSSION

Restriction fragment analysis.

(a) Enriched mitochondrial DNA fraction. Species and strains of Naegleria can be differentiated based on the restriction endonuclease digestion patterns of enriched fractions of mtDNA. Enriched mitochondrial DNA fractions from N. fowleri (strains MB-41, Nf66, and 6088) gave highly similar restriction fragment patterns for Bcl I, Bgl II, and Hin dIII digestions with higher levels of variation seen for other enzymes (see Fig. 2). With Bcl I, the major difference between the strains was the absence of the smallest mtDNA band in the successfully drug-treated strain 6088. The most prominent difference with Bgl II was the absence in the american strains (MB-41 and 6088) of the four smallest non-mitochondrial bands. With Hin dIII, strain Nf66 differed from the american strains by the presence of two faint non-mitochondrial bands while strain 6088 varied from the two pathogenic N. fowleri strains by the presence of an additional small mtDNA band. Thus, the restriction fragment patterns provide evidence for a high similarity and suggest a relatively recent origin for these three strains of N. fowleri. This extremely close similarity detected between N. fowleri strains which has been shown to exist using isoenzyme electrophoresis of acid phosphatase, propionyl esterase, and leucine aminopeptidase (Nerad & Daggett, 1979) and immunoelectrophoresis

(Visversvara & Healy, 1975) was not observed among strains from N. gruberi (EGs and 1518/1a) or N. lovaniensis (TS and 76/25/15). However, some similarities in the fragment size patterns could be detected. For instance, the two strains of N. lovaniensis exhibited highly similar patterns for the Bcl I digestion only. Strain TS could be easily distinguished from strain 76/25/15 using the remaining five restriction enzyme digestions. De Jonckheere (1987) could not differentiate between strains of N. lovaniensis using restriction enzyme fragment patterns of repetitive whole-cell DNA suggesting that many genetic differences were undetected by this gross technique.

Several band identities could be observed between strains of N. fowleri and strains of N. lovaniensis indicating a relationship, but the digestion fragment patterns from the two species were distinct and easily distinguishable in support of their current taxonomic standing. A close similarity of N. fowleri and N. lovaniensis has been indicated in serological results (Stevens et al., 1980; Dubray et al., 1987; Marciano-Cabral et al., 1987), but significantly large differences between the two species have been shown to exist using protein profiles (Marciano-Cabral et al., 1987), isoenzyme banding (Daggett & Nerad, 1983; Nerad & Daggett, 1979), and restriction enzyme analysis of whole-cell DNA (De Jonckheere, 1987).

Few similarities in digestion fragment pattern between strains of Naegleria gruberi, Naegleria jadini, and Vahlkampfia lobospinosa could be detected, thereby supporting the present classification scheme.

Strains EGs and 1518/1a of N. gruberi show distinctly different band patterns supporting the conclusion based on whole-cell DNA patterns by De Jonckheere (1987) that the species is comprised of an assortment of strains that have not yet been assigned to other species. Additional evidence for this has been derived from isoenzyme analysis (Daggett & Nerad, 1983; Nerad & Daggett, 1979).

A close relationship between the three strains of N. fowleri is detected when the extrachromosomal, non-mitochondrial DNA fragment sizes are contrasted for Bcl I, Cla I, and Hin dIII enzyme digestions (Table IV). For example, when Bcl I digests are compared, three identical fragments are produced from each strain with approximate size ranges of 10.3-10.4, 3.1-3.5, and 2.2-2.3 kbp. For Cla I, three to four fragments are produced with size ranges of 10.5-10.8, 3.1, and 2.6 or 1.3 and 0.8 kbp. With Hin dIII, one large fragment with an approximate size range of 16.2-17.3 kbp is produced.

Electrophoresis of undigested, enriched mtDNA fractions extracted from each of the eight Naegleria strains exhibited band migration patterns (see Fig. 3) highly similar to that found for the undigested, enriched extrachromosomal fraction from N. gruberi, strain NEG-M (ATCC30224), in which a circular, self-replicating extrachromosomal element carrying the organism's rRNA genes had been identified (Clark & Cross, 1987). The main large, linear component (#3) had an average size of 54.3 ± 1.4 kbp for the eight Naegleria strains which is identical to the average mitochondrial DNA genome size of 54.8 ± 2.7 kbp determined by fragment size summation, indicating

that this component is mtDNA instead of contaminating chromosomal DNA as presumed by Clark & Cross (1987). The smaller linear component (#5) varied in size from 12.5 to 16.4 kbp (Table V), with the size estimate from strain EGs of Naegleria gruberi consistent with the total size estimate from restriction digestion of the fractionation-enriched ribosomal DNA (rDNA) plasmid from derived clonal isolate strain NEG-M (both 14 kbp). Presumptive supercoiled (component #6) and open circular (component #4) conformations are present in all Naegleria strains indicating that the extrachromosomal element is circular in conformation. Previous electron microscopic studies of N. gruberi, strain EGs, have shown the existence of capsidless virus-like particles (VLP) with nuclear developmental origins (Schuster & Dunnebacke, 1974). Copy number estimates of VLPs per nucleus were similar to that found for the nuclear rDNA plasmid isolated from strain NEG-M. Clusters of unbound, intranuclear particles have also been observed in strains NF66 and MB-41 of N. fowleri (Schuster & Dunnebacke, 1977).

Restriction fragment sizes of the extrachromosomal element from N. gruberi, strain EGs were found to be similar to those of the derived subclone strain NEG-M for Bgl II, Hin dIII, and Sst I. For Bgl II digestions, two fragments were produced in strain EGs (10.4 and 2.9 kbp) compared to the three fragments in strain NEG (11.0, 2.6 and 0.4 kbp) (Clark and Cross, 1987). With Hin dIII, two extrachromosomal fragments were separated from both strains (13.5 and 0.7 kbp from

EGs and 13.4 and 0.6 kbp from NEG-M). When Sst I digestions were analyzed, three completely digested fragments (8.8, 3.2, and 1.7 kbp) from strain EGs were compared to the four fragments (8.6, 3.0, 1.5, and 0.9 kbp) from strain NEG-M.

Average size estimates of the presumptive fractionation-enriched plasmid were based on restriction fragment summations and ranged from 13.2 to 16.4 kbp (Table V). Close agreement was obtained between the size estimates determined from the digestion sums and the undigested linear conformations for all Naegleria strains. Identifiable plasmid size and sequence variation between species supports the current classification scheme and suggests another possible extrachromosomal source of genetic deviation. For instance, the three strains of N. fowleri have quite similar plasmid sizes and digestion patterns lending additional support to the idea that they are closely-related strains.

Thermal denaturation temperature (T_m) determination. The T_m value for nuclear DNA from Naegleria fowleri, strain MB-41, compares favorably with previously published % GC data on vahlkampfiid amoebae. For example, the main nuclear DNA component of N. gruberi, strain NEG was found to be 34% GC (Schuster, 1979). Nuclear DNA from N. fowleri (MB-41) melted with an approximate T_m of 64.6° C in 0.1 X SSC which indicates a GC content of approximately 34%. The GC content of the mitochondrial DNA component from N. gruberi strain, NB-1, was calculated from CsCl gradient density measurements and found

to be 23% (Fulton, 1970). The T_m of mitochondrial DNA from N. gruberi, strain EGs, was approximately 50.8°C in 0.1 X SSC, thereby suggesting a GC content significantly less than the technique's lower measureable limit of 25%. This A + T-rich nature of Naegleria mtDNA is further substantiated by its ability to consistently form component bands of lower buoyant density in all bisbenzimidide - CsCl gradients.

DNA-DNA hybridization studies. A weak cross-hybridization at nonstringent conditions ($T_m - 45^{\circ}\text{C}$) between mtDNAs isolated from different Naegleria species suggests a significant amount of nucleotide sequence divergence. Annealings at $T_m - 50^{\circ}\text{C}$ permit the detection of regions of homology with as much as 33% base mismatch (Howley et al., 1979). Regions of detectable homology between Naegleria mtDNAs possessed 67% or greater base matching. The existence of large mtDNA sequence divergences among these closely-related amoebae may suggest an ancient separation of the various species/strains from the ancestral Naegleria root.

Intraspecific diversity. The estimated intraspecific genetic divergence, as measured by $\hat{p}$, ranges from 3.4 to 7.3% nucleotide substitutions (average $\hat{p} = 5.3 \pm 2.0\%$) for paired strains of N. fowleri. This level of variation is consistent with $\hat{p}$ values determined among phenotypically distinct "strains" of protozoan species such as Acanthamoeba castellanii (4.2 - 12.9%), Paramecium aurelia (3.6 - 8.2%), and Tetrahymena pyriformis (2.6 - 9.7%) (Bogler et al., 1983). Pathogenic strain Nf66 and successfully drug-treated strain 6088

clearly represent the most closely-related isolates within the N. fowleri cluster (Fig. 5) with a genetic variation of only 3.4%, whereas pathogenic strain MB-41 is distinctly different from the other two with a mean divergence of 6.2%. The intraspecific genetic divergence estimated between strains EGs and 1518/1a of N. gruberi ranked much higher than N. fowleri at 10.8% (Table VII). This level of divergence exceeds the intraspecies phenotypic range found for the similarly-sized mitochondrial genome of Tetrahymena pyriformis. The $\hat{p}$ value found between these two strains of N. gruberi is similar to genetic divergence levels found between separate species of Acanthamoeba ($\hat{p}$ range: 7.4-14.8%) (Bogler et al., 1983). However, it should be noted that since only small numbers of strains were sampled, estimates of intraspecific genetic divergence may not completely reflect the actual variation range.

Interspecific diversity. With the exception of N. lovaniensis, strain TS, the genetic variation found between strains belonging to different Naegleria species is comparatively high with ranges from 12.3 - 14.1% nucleotide substitutions (Table VI). Naegleria gruberi, N. fowleri, and N. jadini are distinctively different from one another, as measured by $\hat{p}$, to be justifiably placed in separate taxonomic complexes. Naegleria lovaniensis is less divergent from each of these three species of Naegleria with N. gruberi possessing the greatest estimated nucleotide sequence homology with a $\hat{p}$ value (=7.1%) in the range commonly found among strains of the same species.

Naegleria lovaniensis, strain TS, a Vero cell culture isolate, shares many morphological and biological characteristics with isolates of N. gruberi. Among these similarities are cyst thickened ring pores, ovoid-shaped mitochondria, extreme sensitivity to Concanavalin A agglutination, and lack of pathogenicity to experimental animals (Stevens et al., 1980). Similarities of N. lovaniensis with N. fowleri strains include its ability to grow at 45° C and the possession of a low, but highly variable, number of cyst pores. Results of previous antigenic studies (De Jonckheere, 1977; Stevens et al., 1980) clearly establish an antigenic divergence of N. lovaniensis from N. gruberi, N. fowleri, and N. jadini. One of the environmental nonpathogenic isolates (strain Aq/9/1/45D) studied shares about one-half of its antigenic structure with N. fowleri and about one-third of its antigenic components with N. jadini and N. gruberi. However, N. lovaniensis strain TS, was shown to be less divergent from N. gruberi, strain BG-6, than strain Aq/9/1/45D using the indirect immunofluorescent antibody technique (Stevens et al., 1980). These results in addition to the estimated level of mtDNA nucleotide sequence homology suggest that N. lovaniensis, strain TS, is definitely distinct from strains of N. gruberi and N. fowleri, but with genetic distances from N. gruberi, strain EGs, characteristic of that found between distant strains of the same species.

At present, it is assumed that the genus Vahlkampfia is a taxonomic convenience for strains of Naegleria that cannot be

transformed to flagellates in vitro or are incapable of transformation through mutation. Estimated genetic divergence between ATCC30298 strain of V. lobospinosa and strains EGs and 1518/1a of N. gruberi ($\hat{p}$ = 9.3% and 11.9%, respectively) suggest an intermediate relationship between the two groups with variation levels similar to that found between strains of closely-related, separate species. A comparison of $\hat{p}$ values places Vahlkampfia lobospinosa closer to strain EGs than to Wilmer's strain 1518/1a of N. gruberi (Fig. 5). This lack of a significantly large genetic distance to morphologically identical N. gruberi, strain EGs, suggests that V. lobospinosa should be classified at least as a species of Naegleria. The genetic divergence was estimated to be much larger between morphologically distinct strain 1518/1a and strains of N. gruberi, N. fowleri, and Vahlkampfia lobospinosa ($\hat{p}$ range: 9.9 - 13.2%) (Table VII), indicating that strains 1518/1a should be classified as a separate species of Naegleria instead of as a distinct strain of N. gruberi. Results from previous isoenzyme electrophoresis studies (Daggett and Nerad, 1983; Nerad and Daggett, 1979) lend support to strains EGs and 1518/1a being classified as a closely-related, separate species based on the similar, yet highly distinctive zymogram patterns.

Dendrograms. Dendrograms constructed from matrices of genetic divergence were used to position each vahlkampfiid amoeba isolate based on its estimated genetic variation from other isolates. The UPGMA clustering technique produced the tree topology as shown in

Fig. 4 and 5. Ordering within the N. fowleri cluster (Fig. 5) suggests that pathogenic strain Nf66 and drug-cured strain 6088 are more closely-related to one another than either is to the pathogenic strain MB-41. Strain 6088, isolated from cerebrospinal fluid from the first non-fatal case of primary amoebic meningo-encephalitis in the United States (Center for Disease Control, 1978), exhibits close interstrain genetic homology to pathogenic strain Nf66 with an estimated divergence of 3.4%. Pathogenic strain MB-41 appears to be genetically distinct from either strain 6088 or Nf66 with a mean nucleotide mismatch of 6.2%. However, estimates of genetic divergence reach no greater than 7.3% nucleotide differences between the three isolates suggesting that each should be considered a unique strain within the species N. fowleri. Identical isoenzyme patterns between these three human isolates (Daggett and Nerad, 1983) lends additional support to their strain status within the N. fowleri species complex.

Strains of N. fowleri are genetically separated from strains of N. gruberi as suggested by these two species being split into two distinct lineages with a mean nucleotide divergence of 12.1% (Fig. 5). Naegleria jadini diverges in nucleotide sequence from N. gruberi and N. fowleri to about the same extent ($\hat{p}$ = 14.1% and 13.7%, respectively) since there are few homologous restriction fragments detected using the five enzymes (Table VI). This indicates that N. jadini is at least as genetically different from N. gruberi and N. fowleri as each is from one another. Support for this substantial

level of genetic divergence can be found in morphological (Carosi et al., 1976), antigenic (Carosi et al., 1976; Stevens et al., 1980), and isoenzyme (Daggett and Nerad, 1983) differences unique to N. jadini. In addition, physiological differences observed during the course of this study, such as inability of growth in a hemin supplemented axenic nutrient medium and lack of similar temperature tolerance as shown to exist for the other Naegleria species, tended to illustrate the distinctness of this species. Naegleria lovaniensis is much less genetically divergent from N. gruberi (proportion of polymorphic nucleotides ($\hat{p}$) = 7.1%) than N. fowleri ($\hat{p}$ = 9.9%), since nearly twice as many fragments were detected having identical mobilities, indicating that N. lovaniensis could be considered a distant strain of N. gruberi instead of a separate species. Therefore, three of the four species used in this study appear to be distinctly different, as estimated by $\hat{p}$, in support of their current taxonomic standing within the genus Naegleria. Dendrograms constructed by Pernin et al. (1985) from matrices of Nei's average genetic distances for isoenzyme analysis of four species of Naegleria suggest two distinct evolutionary groups with N. lovaniensis and N. fowleri forming one cluster on one side of the ancestral Naegleria root with N. gruberi forming the second branch on the other side of the root. Considering the fact that infinite genetic distances were found between N. gruberi and the two species N. fowleri and N. lovaniensis as a result of no common alleles found for the enzymes screened, this

dendrogram is inconclusive regarding the true distance relationships comparing N. gruberi with the proposed N. fowleri - N. lovaniensis cluster. This is not the situation in our cluster analysis of estimated genetic divergence comparing N. gruberi, N. fowleri, N. lovniensis, and N. jadini since some genetic homology can be observed in the fragment identities, thereby producing significant $\hat{p}$ values.

As discussed in a previous mtDNA restriction fragment study (Bogler et al., 1983), inappropriate assumptions regarding mtDNA sequence organization and evolution (such as random restriction sites and restriction site changes due to random single nucleotide changes) may cause significant error in the quantitative estimates of $\hat{p}$. Recent evidence suggesting the presence of genetic recombination in N. lovaniensis (Cariou and Pernin, 1987) may be cause enough to change our conceptions of mtDNA evolution in this group since structural polymorphisms would then be possible through mitochondrial recombination as shown to exist in some taxa of protists and fungi (Birley and Croft, 1986). However, data from the present study provides no support for genetic recombination since highly similar mtDNA clones are found in distant geographic regions. As more knowledge becomes available regarding the exact organization and pattern of evolution in mtDNA from Naegleria, corrections can be made to obtain more significant estimates of genetic polymorphism.

Conclusions. The results of the restriction enzyme analysis

(Engels, 1981) of mitochondrial genomes from several strains of Naegleria support the current classification scheme for three of the four previously described species. Naegleria lovaniensis, strain TS, was found to be genetically distinct from strains of N. fowleri and N. jadini, but possessed significant nucleotide homology with N. gruberi, strain EGs, with an estimate of polymorphic nucleotides similar to that found between distant strains belonging to the same species. Pathogenic strain MB-41 of N. fowleri exhibited distinct genetic divergence from highly homologous, pathogenic strain Nf66 and drug-susceptible strain 6088. Morphologically distinct strains EGs and 1518/1a of N. gruberi appear to have significantly large estimated nucleotide divergence to be reclassified as separate species. Vahlkampfia lobospinosa, a species of small amoeba which cannot be transformed into flagellates, appears to be closely related to Naegleria gruberi, strain EGs, and should be classified at least as a species of Naegleria.

Restriction enzyme analysis of mtDNA structural polymorphism is a useful taxonomic tool for vahlkampfiid amoebae which also provides insight into their possible phylogenetic relationships. The ability to detect and quantify the genetic variation between strains of Naegleria makes this molecular approach extremely useful as a diagnostic aid for the identification and differentiation of potentially pathogenic isolates. This is the only technique which can differentiate between strains from either high-temperature tolerant species N. lovaniensis or

N. fowleri. The small estimated genetic divergence found between the typestrain of the N. fowleri species, Nf66, and the drug-cured strain 6088 suggests that the two isolates are not genetically distant which supports the idea that other strains of N. fowleri may also be drug-susceptible. Vahlkampfia lobospinosa appears to be closely-related to morphologically indistinct strains of N. gruberi suggesting that Vahlkampfia is a possible mutant form of Naegleria which is unable to transform itself.

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