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INTERSTRAIN MITOCHONDRIAL DNA POLYMORPHISM DETECTED IN *ACANTHAMOEBA* BY RESTRICTION ENDONUCLEASE ANALYSIS

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The genus *Acanthamoeba* includes pathogenic and nonpathogenic strains of amebas with unclear taxonomic and evolutionary relationships. To explore these relationships further, we have examined mitochondrial DNA fragment patterns obtained for 15 *Acanthamoeba* strains by use of five restriction endonucleases. The mitochondrial DNA molecules were circular, averaging 41.6 ± 1.5 kilobase pairs. Fragments resulting from endonuclease digestion of the DNA were separated by agarose gel electrophoresis. Ten distinct families of electrophoretic patterns (digestion phenotypes) were observed. Seven phenotypes were found for seven strains considered nonpathogenic or of unknown pathogenicity. Three phenotypes were associated with pathogenic strains. One of these phenotypes included a single pathogenic strain, a second included one pathogen and one strain of unknown pathogenicity, and the third included five pathogenic strains. The latter five were of widespread geographic origin and previously were assigned to two different species. The results suggest that extensive nucleotide sequence diversity occurs among strains from a single species of *Acanthamoeba*, but that subgroups of strains with similar sequences also occur. Thus, restriction enzyme analysis can identify clusters of strains and may be a useful approach to classification in the genus. Improvements in classification should help clarify relationships among pathogenic and non-pathogenic strains.

Key words: *Acanthamoeba*; Mitochondrial DNA; Molecular taxonomy; Genetic divergence.

INTRODUCTION

Classification of small free-living amebas of the genus *Acanthamoeba* has been a continuing problem. Approaches to this problem have relied on morphological [1–3], physiological [4], or molecular variation, including antigenic differences [5–8], variation in enzyme electrophoretic patterns [9–10], and hybridization of DNA [11]. Taxonomists

Abbreviations: ATA, aurintricarboxylic acid; BRL, Bethesda Research Laboratories; CL, criterion level; kbp, kilobase pairs; mtDNA, mitochondrial DNA; $\hat{p}$, genetic divergence (distance); S.D., standard deviation.

agree that distinctions among amebas are relatively clear at the genus level [12], but, as in other asexual organisms, the concept of the species is unclear. At present, it appears that a single species in the genus can comprise a mixture of pathogenic and nonpathogenic strains. Problems of identification and determination of pathogenicity are compounded by attenuation of virulence during laboratory culture [13]. Pathogenicity probably is opportunistic [14], and it is possible that all strains have a pathogenic potential, but this is uncertain and it is equally possible that pathogenic and nonpathogenic strains are equivalent to distinct species.

In this communication we explore relatedness among small free-living amebas utilizing a molecular approach based on electrophoresis of DNA fragments obtained by restriction enzyme digestion of mitochondrial DNA (mtDNA). This method has been used for the study of intra- and interspecific relationships in a variety of other organisms [15–19]. It is especially useful for examining affinities among closely related groups and has been applied to the study of three other protozoan genera, *Paramecium* [20], *Tetrahymena* [21], and *Trypanosoma* [22, 23]. We have tested the feasibility of using this approach for amebas by examining relationships among 15 strains from four species. Strains that have exhibited pathogenicity and others that have not were included in the study. Mitochondrial inheritance typically is a uniparental phenomenon in other organisms; in asexual *Acanthamoeba* the process is presumed to be clonal. Although we are interested in relationships between pathogenic and nonpathogenic strains, no relationship between the mitochondrion and pathogenicity has been assumed. mtDNA diversity has been used as an index of overall genetic relatedness as discussed in a preliminary report of this work [24].

MATERIALS AND METHODS

Acanthamoeba strains. The term *strain* has been used to designate an independent isolate from nature. *Lines*, which may or may not be *clones*, are subcultures that are phenotypically different or have been cultured independently for long periods. The following strains are the same as used previously [24]: *A. astronyxis* (Ray); *A. polyphaga* (Page 23, Jones [H]), *A. castellanii* (Castellani, Chang [M], Bos [H], Boyce [H], IMVS-Rus 22 [M], Ma [H], Neff, Lewin, Reich, Singh). [H] indicates isolation from a human infection and [M] indicates a strain isolated from the environment, but pathogenic to mice. Names in parentheses are strain designations used in our laboratory and in most cases indicate the person most closely associated with the isolation. Pathogenicity is documented in references cited elsewhere [24] except for IMVS-Rus 22 for which the evidence is unpublished (A.R. Stevens, personal communication). Two additional strains used in this study were obtained from A.R. Stevens: *A. polyphaga* (Nagington), also designated Elliott, isolated from a human eye infection in England [25]; and *A. griffini* (Griffin), isolated from seawater in Connecticut [26].

Cell growth and isolation of mitochondrial DNA. Ameba stocks were cultured as pre-

viously described [27]. For DNA preparations, cells were grown axenically in 2.5 liter low form flasks (Corning 4422) containing 1.5 liters of optimal growth medium (OGM) [28] agitated at 50 rev/min in a rotary shaker. About $2-5 \times 10^8$ midlog phase amebas were used per isolation (2 shaker flasks at 9×10^4 cells/ml). Cells were harvested and washed twice in wash buffer (660 mM sucrose, 50 mM KH_2PO_4, 25 mM EDTA, 200 mM aurintricarboxylic acid [ATA, a nuclease inhibitor], pH 6.8). The washed cell pellet was resuspended in 5–10 ml of lysis solution (500 mM NaCl, 300 mM EDTA, 1% digitonin, 2 mM ATA, pH 9). Cells were homogenized in the cold to 90% lysis using a Dounce homogenizer with a loose Size A pestle. Mitochondrial DNA isolation was then essentially as described for *Podospora anserina* [29] except for modifications at the CsCl gradient step. The lysed cell mixture was saturated with solid CsCl until the refractive index exceeded 1.4. Bisbenzimide (Hoechst No. 33258) was added to a final concentration of 0.025 mg ml^{-1} to enhance the separation of nuclear and mtDNA [30]. The refractive index of the solution was adjusted to 1.393 by the addition of DNA buffer (150 mM NaCl, 20 mM NaH_2PO_4, 6 mM EDTA, pH 7.2). The mixture was transferred to polyallomer tubes and centrifuged at 40 000 rpm for 48–60 h at 16°C in a Beckman Ti75 rotor. A good separation into nuclear and mitochondrial DNA bands, visualized with long wavelength UV, was obtained for each of the strains. Gradients were collected and fractions containing mtDNA (upper band) from several gradients were combined and recentrifuged at 38 000 rpm for 24 h. Bisbenzimide was extracted from the recovered band three times with CsCl-saturated isopropyl alcohol. The DNA-containing fractions were dialyzed once against DNA buffer supplemented with 1 M NaCl and twice against buffer alone. In a few cases, DNA was extracted directly from mitochondria purified as described elsewhere [31].

The method of DNA isolation worked well for all strains tested. Circular DNA was obtained at least for the six strains examined by electron microscopy. Degradation of mtDNA was observed when postlog cultures were used; diffuse bands of DNA were observed in the gradient rather than the sharp bands observed in younger cultures, lower molecular weight fragments against a smeared background were observed in electrophoretic gels, and the absence of circular molecules was noted by electron microscopy. Extensive mtDNA degradation begins during late log growth phase (L. King, unpublished observations); therefore, we isolated DNA from midlog phase cultures.

Restriction enzyme analysis. The endonucleases *Hind* III, *Sal* I, *Bcl* I, *Bgl* II, *Cla* I and *Xba* I were purchased from Bethesda Research Laboratories (BRL., Bethesda, MD) and *Eco* RI from BRL or Boehring Mannheim (Indianapolis, IN). Approximately 0.5–1 μg mtDNA was incubated overnight with 5 units of enzyme in a total volume of 50 μl using buffers specified by the enzyme supplier. The DNA fragments produced were ethanol precipitated, resuspended in 10–20 μl DNA buffer and 7.5 μl of a solution containing 50% (w/v) sucrose, 0.05% bromophenol blue and 0.1 M EDTA, and then loaded onto gels. Vertical slab gels of 1% agarose (gel electrophoresis grade; BRL) were used. Gels were run at 20 mA for 12–14 h in TEA buffer (40 mM Tris, pH 8.05, 20 mM sodium

acetate, 2 mM EDTA and 1.8 mM NaCl). Following electrophoresis, gels were stained with 1 μg ethidium bromide/ml for 20 min and destained in water for 30 min. Gels were visualized with a short wave UV transilluminator and photographed using an MP-4 Polaroid Land camera. Positive prints were obtained using Polaroid Type 667 film and negatives using Kodak Royal X-pan film.

Hind III and *Sal* I digests of lambda phage were included in each gel run as size standards. The sizes in kilobase pairs of *Hind* III fragments used for standard curves were 27.8 (fragments No. 1 and No. 4), 23.5 (No. 1), 9.7 (No. 2), 6.6 (No. 3), 4.3 (No. 4), 2.2 (No. 5), 2.1 (No. 6) and 0.59 (No. 7). Values were obtained directly from BRL; fragment No. 7 differs from the value published in the BRL 1981/82 catalogue. The *Sal* I fragment sizes were 33.2 (No. 1), 15.3 (No. 2) and 0.54 (No. 3). We plotted $\log_{10}$ of fragment size vs. distance migrated. The region from approx. 2 to approx. 15 kilobase pairs (kbp) was log-linear whereas the curve was nonlinear for the larger and smaller fragments. Curves were made to fit by eye.

Electron microscopy. The 'droplet' method [32] was used to spread DNA. A 40 μl drop containing 0.2 μg ml^{-1} DNA, 10 mM formaldehyde, 150 mM NH_4 acetate and 0.3 μg ml^{-1} cytochrome *c* at pH 7 was allowed to spread for 25 min. DNA was picked up on parlodion-coated grids and grids were stained in 2% uranyl acetate in 95% ethanol for 30 s, fixed for one min in 95% ethanol and rotary shadowed with platinum. The replicating form of phage φX174, which was used as an internal standard (BRL; 5386 bp), led to a 1.5% underestimation of the size of phage lambda (BRL; 49 kbp). Therefore, our electron microscopic size estimates for circular mtDNA molecules were increased by 1.5%.

Genetic divergence. Quantitative differences in mtDNA nucleotide sequences (genetic divergence) were estimated using the method of Upholt [33] as modified by Engels [34]. This approach estimates sequence differences from the proportion of polymorphic restriction sites, which can be determined directly from restriction site maps, or, as in our usage, indirectly from comparisons of fragment sizes. The estimated sequence difference between two mitochondrial genomes ($\hat{p}$) was calculated for each enzyme by,

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

where (k) is the number of polymorphic restriction sites in the two genomes; (j) is the number of base pairs per restriction site; and (m) is the sum of monomorphic and polymorphic restriction sites in the two genomes. Engels [34] provides a table of (k) values based on various combinations of (G), the number of monomorphic fragments (i.e. the same size in both genomes), and (F), the total number of fragments from both genomes. The total number of restriction sites (m) in a circular genome is calculated from

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

The various parameters are illustrated diagrammatically elsewhere [24]. Matrices of $\hat{p}$ values were subjected to cluster analysis by the UPGMA method [35], and used to construct the dendrograms. All calculations were performed using a computer program designed by and available from P.A. Fuerst, Department of Genetics, The Ohio State University.

RESULTS

DNA fragment patterns. Mitochondrial DNA isolated from several *Acanthamoeba* strains was digested with up to 17 different restriction enzymes to determine which enzymes would produce a relatively small number of fragments [18]. This permits shared fragments to be identified with less ambiguity than would occur if the number of fragments were larger [18]. *Bcl* I, *Bgl* II, *Cla* I, *Eco* RI, and *Xba* I, enzymes with 6-base recognition sequences containing two GC and four AT pairs were satisfactory. Fragment sizes obtained following digestion with these enzymes are given in Table I for the digestion pheno-

TABLE I

Fragment size estimates[a] for ten digestion phenotypes

		Digestion phenotype									
Endonuclease fragments		Castellani 1	Lewin 2	Neff[c] 3	Singh 4	Reich 5	Ray 6	Bos 7	Ma 8	Page 23 9	Griffin 10
Bcl I	a	17.0	11.4[b]	13.5	14.7	14.5	15.8	14.4	17.1	23.3	19.4
		1.0	*0.3*	*0.6*	*0.7*	*0.8*	*0.5*	*0.6*	*1.5*	*1.6*	*0.7*
	b	6.82	5.46	10.2	13.8	7.40	8.60	8.14	6.84	11.6	9.5
		0.05	*0.06*	*0.3*	*0.7*	*0.20*	*0.20*	*0.42*	*0.20*	*0.7*	*0.08*
	c	6.38	5.27	8.10	5.79	6.04	6.47	6.19	5.54	6.17	4.20
		0.15	*0.05*	*0.35*	*0.16*	*0.16*	*0.12*	*0.26*	*0.11*	*0.20*	*0.08*
	d	3.08	4.51	3.47	3.11	3.47	5.18	5.38	4.17	2.29	3.95
		0.11	*0.03*	*0.06*	*0.05*	*0.09*	*0.11*	*0.15*	*0.08*	*0.04*	*0.11*
	e	2.81	2.82	2.87[b]	2.86	3.10	4.75	5.01	3.06	0.85	2.69
		0.02	*0.11*	*0.06*	*0.03*	*0.08*	*0.10*	*0.14*	*0.08*	*0.05*	*0.25*
	f	2.48	1.55	0.73	1.08	2.91	1.98	2.79	2.44		
		0.05	*0.06*	*0*	*0.04*	*0.13*	*0.07*	*0.04*	*0.06*		
	g	1.80		0.39		1.60	1.17		1.72		
		0.10		*0*		*0.07*	*0.06*		*0.14*		
	h	1.15							1.11		
		0.06							*0.06*		

continued on p. 150.

Endonuclease fragments		Cast-ellani 1	Lewin 2	Neff[c] 3	Singh 4	Reich 5	Ray 6	Bos 7	Ma 8	Page 23 9	Griffin 10
Bgl II	a	14.9	14.5	14.7	7.10	15.2	30.8	15.1	10.0	11.2	11.7
		0.2	*0.5*	*0.6*	*0.29*	*0.8*	*1.6*	*0.5*	*0.5*	*0.3*	*0.8*
	b	9.29	12.8	9.35	5.62	11.4	14.4	8.66	6.50	10.0	7.67
		0.17	*0.5*	*0.31*	*0.19*	*0.5*	*0.4*	*0.19*	*0.18*	*0.5*	*0.34*
	c	6.80	3.62[b]	8.15	5.36	5.32		7.49	5.75	6.87	5.67
		0.18	*0.05*	*0.29*	*0.17*	*0.18*		*0.19*	*0.10*	*0.17*	*0.22*
	d	5.57	3.20	4.51	4.50	2.68		3.18	5.48[b]	6.44	5.19
		0.21	*0.06*	*0.12*	*0.10*	*0.03*		*0.05*	*0.13*	*0.18*	*0.21*
	e	4.26	2.56	3.51	4.00	1.62		2.45	4.19	3.48	4.83
		0.18	*0.09*	*0.09*	*0.07*	*0.06*		*0.03*	*0.11*	*0.06*	*0.14*
	f	3.04	2.49	1.40	2.88	1.60		2.12	2.88	2.22	4.75
		0.10	*0.05*	*0*	*0.10*	*0.03*		*0.04*	*0.08*	*0.10*	*0.16*
	g		1.16	0.73	2.86	0.52		1.19	2.34	2.08	
			0.02	*0*	*0.05*	*0.01*		*0.02*	*0.09*	*0.08*	
	h			0.55	2.51			1.31		1.39	
				0	*0.06*			*0.04*		*0.07*	
	i				2.48			0.80		0.72	
					0.08			*0.11*		*0.05*	
	j				1.52						
					0.09						
	k				0.88						
					0.03						
Cla I	a	9.46	16.0	9.46	17.3	13.9	37.2	14.9	17.2	13.1	16.6
		0.47	*1.3*	*0.26*	*1.3*	*0.5*	*2.6*	*1.0*	*1.3*	*1.4*	*1.4*
	b	8.50	9.52	7.39	7.29	12.4	6.77	6.99[b]	12.4	10.3	6.07
		0.32	*0.30*	*0.14*	*0.28*	*0.4*	*0.14*	*0.32*	*0.5*	*0.7*	*0.20*
	c	7.33	5.07	6.59	6.01	7.54		5.76	4.81	4.65	3.71
		0.28	*0.18*	*0.15*	*0.28*	*0.27*		*0.24*	*0.17*	*0.38*	*0.13*
	d	5.69	4.57	4.32[b]	4.61	4.07		4.36	2.88	3.18	3.55
		0.25	*0.15*	*0.21*	*0.21*	*0.09*		*0.13*	*0.12*	*0.12*	*0.12*

Endonuclease fragments		Castellani 1	Lewin 2	Neff[c] 3	Singh 4	Reich 5	Ray 6	Bos 7	Ma 8	Page 23 9	Griffin 10
Cla I (*contd.*)	e	4.76 *0.18*	2.85[b] *0.10*	4.11 *0.16*		2.46 *0.10*		2.03 *0.06*	1.96 *0.06*	3.14 *0.09*	3.42 *0.12*
	f	3.04 *0.09*	1.80 *0.05*	2.69 *0.12*					0.97 *0.09*	1.81 *0.05*	2.67 *0.07*
	g	2.58 *0.07*								1.35[b] *0.07*	1.81 *0.16*
	h									1.19 *0.07*	1.18 *0.05*
	i									0.99 *0.08*	
Eco RI	a	13.9 *0.8*	15.8 *0.7*	15.9 *0.6*	7.48 *0.19*	15.8 *0.6*	27.5 *0.5*	10.1[b] *0.4*	13.3 *0.4*	11.4 *0.4*	10.4 *0.7*
	b	12.5 *0.3*	6.94 *0.10*	11.6 *0.7*	6.26[b] *0.06*	5.48 *0.18*	12.9 *0.53*	9.54 *0.39*	12.6 *0.6*	10.4 *0.2*	8.19 *0.40*
	c	8.0 *0.20*	6.76 *0.05*	5.19 *0.20*	6.22 *0.09*	4.54 *0.11*	2.87 *0.06*	6.19 *0.32*	8.59 *0.17*	7.39 *0.24*	5.57 *0.16*
	d	4.81 *0.09*	5.94 *0.07*	3.28 *0.18*	5.32 *0.13*	3.67 *0.07*	1.70 *0.01*	3.75 *0.16*	4.77 *0.20*	6.85 *0.21*	3.76[b] *0.10*
	e	1.76 *0.05*	3.35 *0.14*	3.05 *0.17*	3.35 *0.07*	3.26 *0.11*		1.82 *0.15*	1.76 *0.08*	4.41 *0.13*	2.97 *0.06*
	f	1.45 *0.04*	2.90 *0.11*	1.81 *0.28*	1.85 *0.06*	2.04 *0.10*			1.44 *0.07*	2.92 *0.05*	2.77 *0.06*
	g		1.32 *0.05*	0.80 *0*	1.74 *0.08*	1.99 *0.08*					1.66 *0.05*
	h		1.27 *0.06*		0.92 *0.03*	1.89 *0.07*					
Xba I	a	25.4 *2.5*	18.5 *1.8*	11.5 *0.33*	10.6 *0.6*	8.04 *0.27*	18.1 *1.7*	10.1 *0.4*	12.4 *0.7*	11.5 *0.9*	14.8 *1.1*

continued on p. 152.

Endonuclease fragments		Cast-ellani 1	Lewin 2	Neff[c] 3	Singh 4	Reich 5	Ray 6	Bos 7	Ma 8	Page 23 9	Griffin 10
Xba I (*contd.*)	b	7.08	7.98	9.88	8.18	5.13	9.33	7.95	8.36	5.26	12.4
		0.24	*0.28*	*0.19*	*0.37*	*0.14*	*0.39*	*0.54*	*0.44*	*0.16*	*0.67*
	c	4.01	6.56	5.37	7.93	4.70	4.85[b]	7.79	3.93	4.63	6.68
		0.15	*0.28*	*0.14*	*0.36*	*0.11*	*0.17*	*0.56*	*0.15*	*0.31*	*0.13*
	d	2.75	4.49	3.17	4.92	4.21	2.45	4.80	2.99	3.28	3.89
		0.05	*0.13*	*0.12*	*0.14*	*0.11*	*0.07*	*0.16*	*0.08*	*0.10*	*0.57*
	e	1.35	2.04	2.01	4.34	4.05	1.19	4.25	2.71	3.21	1.70
		0.22	*0.07*	*0.03*	*0.15*	*0.09*	*0.03*	*0.16*	*0.09*	*0.10*	*0.24*
	f		1.68	1.66	2.57	3.12	0.92	2.51	2.57	2.63	
			0.05	*0.03*	*0.07*	*0.07*	*0.07*	*0.08*	*0.10*	*0.11*	
	g		1.32	1.58	1.36	2.08		1.33	1.72	2.47	
			0.04	*0.04*	*0.04*	*0.05*		*0.08*	*0.07*	*0.10*	
	h			0.95	0.90	1.90		0.93	1.42[b]	1.95	
				0.07	*0.03*	*0.05*		*0.11*	*0.07*	*0.05*	
	i			0.86	0.75	1.54		0.72	1.16	1.84	
				0.07	*0.02*	*0.03*		*0*	*0.07*	*0.06*	
	j			0.79		1.31			1.01	1.63	
				0.08		*0.04*			*0.05*	*0.07*	
	k					0.71			0.46	1.02	
						0.07			*0.05*	*0.06*	
	l									0.94	
										0.04	

[a] Average fragment sizes (kbp) are in first row of each fragment entry and standard deviations are given in italics. Size averages are for measurements from five gels.

[b] Relative fluorescent intensity of this band indicates the presence of two fragments.

[c] Similar values for *Eco* RI digest of Neff strain mtDNA have been published [42, 43].

types described below and in Table II. Variations in fragment sizes are readily visualized in diagrammatic representations of electrophoretic patterns (e.g. Fig. 1). Patterns were identical for mtDNA from whole cells or from purified mitochondria (not illustrated).

Fragments smaller than about 0.4 kbp were not detected by the assay conditions used

TABLE II

Digestion phenotypes and strains

Digestion phenotype (No. and name)	Strains with this phenotype	Species	Pathogenicity[a]
1 Castellani	Castellani	*A. castellanii*	
2 Lewin	Lewin	*A. castellanii*	
	Nagington	*A. polyphaga*	H
3 Neff	Neff	*A. castellanii*	
4 Singh	Singh	*A. castellanii*	
5 Reich	Reich	*A. castellanii*	
6 Ray	Ray	*A. astronyxis*	
7 Bos	Bos	*A. castellanii*	H
	Boyce	*A. castellanii*	H
	Chang	*A. castellanii*	M
	IMVS-Rus 22	*A. castellanii*	M
	Jones	*A. polyphaga*	H
8 Ma	Ma	*A. castellanii*	H
9 Page 23	Page 23	*A. polyphaga*	
10 Griffin	Griffin	*A. griffini*	

[a] H, isolated from human infection; M, pathogenic to mice.

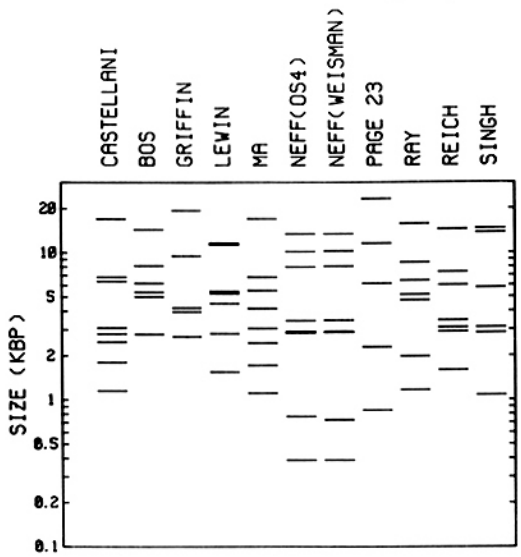

Fig. 1. Fragments of mtDNA obtained by *Bcl* I digestion. Data from Table I, except for the Neff strain. Only the OS4 line is represented in the table. Thicker lines indicate the presence of two fragments of this size. The fragment patterns illustrated accurately represent patterns observed on agarose gels within the range of about 2–15 kbp, but the correlation decreases outside this range (see Methods). Patterns for the Bos and Lewin strains are shared by other strains classified in the same digestion phenotypes (Table II).

in this study. Therefore, genome sizes obtained by summation of electrophoretic fragment sizes and by electron microscopic measurements of intact mtDNA molecules were compared to determine whether significant segments of the genome were missing from the fragment patterns (Table III). Good agreement between the two methods was obtained. The average size was 41.2 ± 1.4 kbp for six strains measured by electron microscopy and 41.4 ± 2.0 kbp for the same strains measured by gel electrophoresis. The overall average size for 15 strains measured by summation of electrophoretic fragments was 41.6 ± 1.5 kbp. The distributions of molecule sizes determined by electron microscopy indicated that a single homogeneous population of molecules was obtained from each strain (Fig. 2).

Ten distinct fragment size patterns were obtained with each of the five enzymes (Fig. 1, Table II) indicating a relatively high degree of nucleotide sequence diversity among the various isolates (see below). The collection of five fragment size patterns obtained for a single strain with the five enzymes is referred to as a digestion phenotype [15]; thus, 10 digestion phenotypes were identified. Seven of the 10 phenotypes observed were for strains classified as *A. castellanii*; the three remaining phenotypes were associated with: the Page 23 (*A. polyphaga*), Ray (*A. astronyxis*), and Griffin (*A. griffini*) strains respectively. For purposes of comparison, fragment size patterns were obtained with *Bcl* I, *Bgl* II, and *Eco* RI for two lines of the Neff strain, one from our laboratory (OS4) and one from the laboratory of R.A. Weisman (Wiesman). Only small differences in the patterns of the two lines were observed (e.g. Fig. 1). These differences were most likely

TABLE III

Genome sizes based on restriction endonuclease fragment sizes and electron microscopic measurements of whole mtDNA molecules[a]

Digestion phenotype	Restriction endonuclease					Average size	
	Bcl I	*Bgl* II	*Cla* I	*Eco* RI	*Xba* I	RE	EM[b]
Castellani	41.5	43.9	41.4	42.4	40.6	42.0 ± 1.3	41.2 ± 1.6 (34)
Bos	41.9	43.0	41.0	41.5	40.4	41.6 ± 1.0	–
Lewin	42.4	44.0	42.7	44.3	42.6	43.2 ± 0.9	42.9 ± 1.4 (30)
Ma	42.0	42.6	40.2	42.5	40.2	41.5 ± 1.2	–
Neff (OS4)	42.0	42.9	38.9	41.6	37.8	40.6 ± 2.2	41.0 ± 1.6 (48)
Reich	39.0	38.7	40.4	38.7	36.8	38.7 ± 1.3	39.5 ± 0.9 (28)
Singh	41.3	39.7	37.4	39.4	41.6	39.9 ± 1.7	39.9 ± 1.4 (27)
Page 23	44.2	44.4	41.1	43.4	44.2	43.5 ± 1.4	–
Ray	44.0	45.2	44.0	45.0	41.7	44.0 ± 1.4	42.9 ± 1.3 (36)
Griffin	39.7	39.8	39.0	39.1	39.5	39.4 ± 0.4	–

[a] Genome sizes in kilobase pairs. Averages for restriction endonuclease fragments (RE) and electron microscopic (EM) measurements include the standard deviation.

[b] Numbers of whole molecules measured are in parentheses. Corrected EM values used (see Methods).

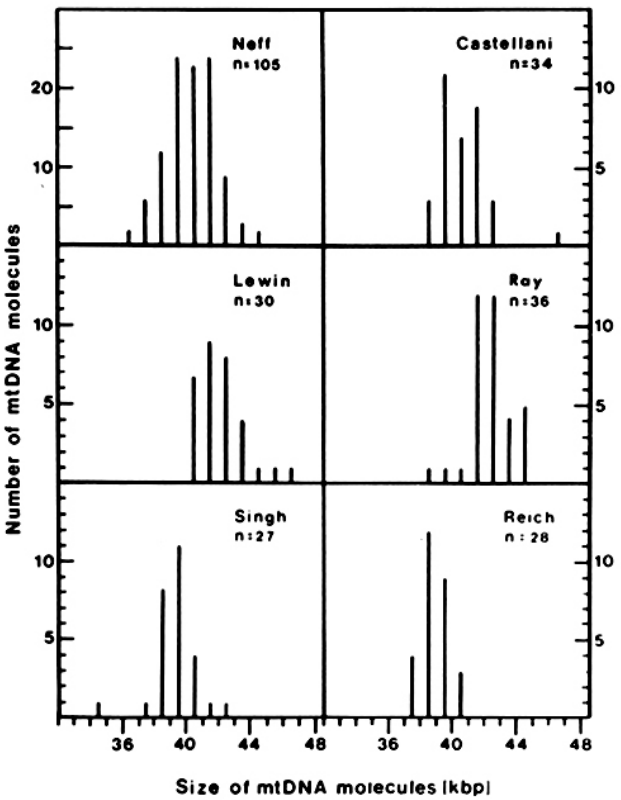

Fig. 2. Size distribution of mtDNA molecules measured by electron microscopy. The Neff strain included both OS4 and Weisman lines.

the result of averaging fragment sizes from different DNA digests when the two lines were run on different gels. Differences between the patterns were not evident when the two lines were run side-by-side on the same gel.

Three phenotypes were associated with pathogenic strains. Five of these strains, including Bos, Boyce, Chang, Jones and IMVS-Rus 22, belong to a single digestion phenotype. The identity of these patterns with the phenotype was unambiguous, as illustrated for four of the strains digested with *Bcl* I and *Cla* I (Fig. 3). The Jones strain previously has been identified as *A. polyphaga* [24], but the other four were classified as *A. castellanii* by E. Willaert (unpublished). The Nagington strain, which was isolated from a human eye infection and classified as *A. polyphaga*, has a digestion phenotype identical to the Lewin strain (*A. castellanii*), which is of unknown pathogenic potential. The Ma strain, recently isolated from a human eye infection, has a digestion phenotype that is quite similar to, although distinct from, the Castellani strain, which was isolated in 1930 from a yeast culture. The similarity between these two strains is evident in the *Eco* RI and *Bcl* I digests (Table I, Fig. 1), but also can be demonstrated by quantitative analysis of all five digests (see below).

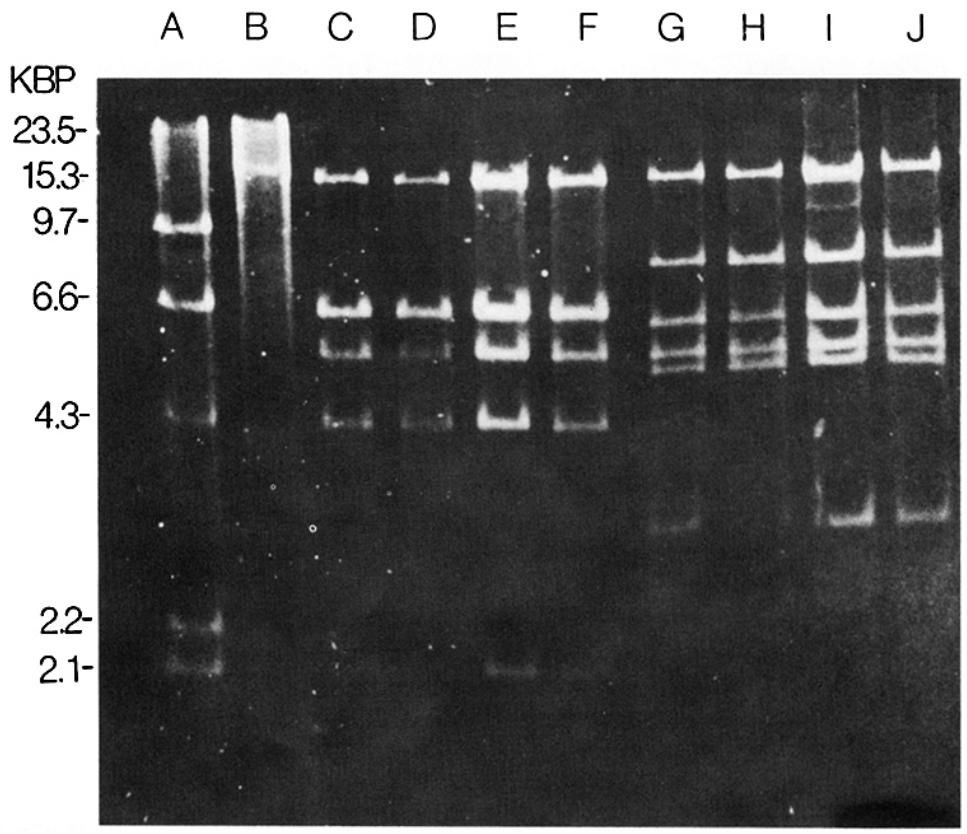

Fig. 3. Agarose gel electrophoretic patterns for *Bcl* I and *Cla* I digests of the Bos, Boyce, Chang and IMVS-Rus 22 strains. Lanes contain: A, *Hind* III digest of lambda phage; B, *Sal* I digest of lambda; C-F, *Cla* I digest of mtDNA from Chang, IMVS-Rus 22, Bos and Boyce strains, in sequence; G-J, *Bcl* I digests from Chang, IMVS-Rus 22, Bos and Boyce, in sequence.

Genetic divergence. In addition to determining the existence of qualitatively distinct digestion phenotypes, the data can be used to quantitate the amount of genetic differentiation between the strains. Genetic divergence among mtDNAs from the various strains was estimated by the method of Engels [34]. To use this method, the proportion of common fragments must be determined for each pairwise comparison of mtDNA digests. In our study, this proportion depends on the criteria used to determine whether two fragments measured on different gels are the same size. The most definitive criterion, whether the fragments have the same electrophoretic mobility when run side-by-side on the same gel, should be used when two very similar patterns are to be compared. For large scale surveys, a computer compilation of fragment size averages (e.g. Table I) may be the most convenient reference source. Where appropriate, computer generated predictions of relationships among strains can be rigorously checked by using side-by-side co-electrophoresis of digests.

Because there is measurement error associated with estimating fragment sizes, standard deviations were determined. A computer program determined size identity of fragments at four different criterion levels (0.5, 1, 1.5, and 2 standard deviation units). For example, at a criterion level of one S.D. ($CL_{1.0}$), two fragments were considered homologous when the size difference between them was less than or equal to one times the standard deviation of either fragment. The test is an empirical comparison, not a statistical one, since the CL values do not represent levels of statistically significant deviation in mobility. This empirical method was shown to have validity for protein electrophoresis in a study of human hemoglobin variants with known amino acid sequence differences [36].

Estimates of the proportion of fragments shared by two strains and the estimates of genetic divergence obtained from such data were determined at each of the four criterion levels. Data for $CL_{1.0}$ are given in Table IV. We tried to select a criterion level that revealed maximum diversity without introducing artificial fragment size polymorphism. $CL_{0.5}$ was too stringent because it indicated fragment size differences between the Weisman and OS4 lines of the Neff strain, whereas, no differences in fragment mobilities were observed when digests of the two strains were co-electrophoresed. These artificial differences disappeared at $CL_{1.0}$ and this criterion level proved most useful to describe the majority of work from our laboratory (Table IV). In a few cases, especially when the work of different technicians was compared, $CL_{1.5}$ was judged the most stringent test possible.

Dendrograms. Estimates of genetic divergence were used to construct dendrograms depicting average sequence differences among strains. Diagrams based on analysis at two different criterion levels differed only slightly in details of branching (Fig. 4). The Ray strain is clearly distinct from all others. The remaining strains seem to form a diffuse

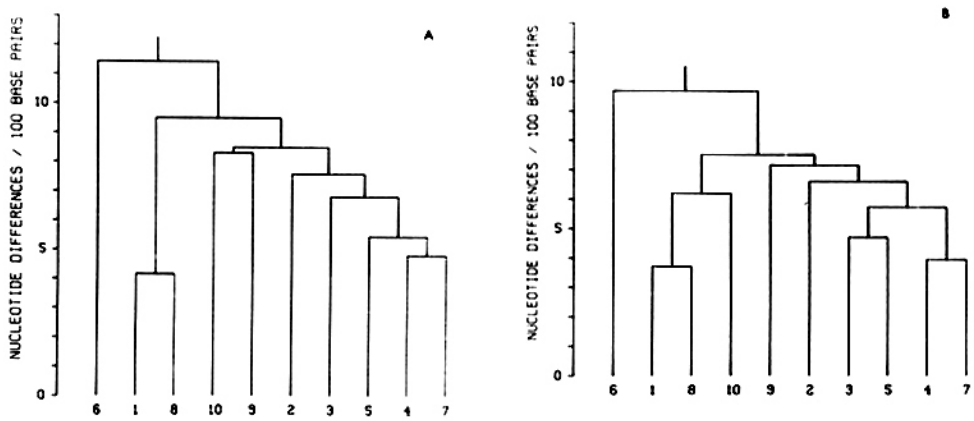

Fig. 4. Dendrograms representing average genetic distances among ten digestion phenotypes. Numbers at bottom of dendrogram represent phenotypes listed in Table II. Distances calculated using data in Table I and average $\hat{p}$ values for five enzymes. (A), analysis of $\hat{p}$ at $CL_{1.0}$; (B), analysis at $CL_{1.5}$.

TABLE IV

Fractions of common fragments and estimates of genetic divergence in pairwise comparisons of digestion phenotypes[a]

	1[b]	2	3	4	5	6	7	8	9	10
	Eco RI	0/14	1/13	1/15	0/14	1/10	1/12	5/12	0/12	1/14
	Bgl II	1/14	2/14	1/17	2/13	0/8	1/15	2/14	1/15	1/12
1[b]	*Bcl* I	1/15	1/16	1/14	2/15	2/15	2/14	6/16	0/13	1/13
	Cla I	1/13	3/14	2/12	1/12	0/9	1/13	1/13	1/17	0/15
	Xba I	1/12	0/15	1/14	2/16	1/12	1/14	3/17	0/19	1/10
			3/15	1/17	2/16	1/12	1/14	0/14	2/14	1/16
			1/16	2/19	1/15	1/10	2/17	0/16	0/17	0/14
2	9.80[c]		1/15	1/13	2/14	1/14	2/13	1/15	0/11	1/12
			1/13	1/11	0/11	0/8	0/12	1/12	2/16	1/14
			2/17	3/16	3/18	1/14	2/16	2/19	2/21	2/12
				3/16	3/15	1/11	1/13	1/13	2/13	2/15
				1/19	1/15	1/10	2/17	0/16	3/17	0/14
3	8.80	7.02		2/14	2/15	0/15	1/14	0/16	1/12	1/13
				1/12	2/12	0/9	1/13	0/13	1/17	1/15
				2/19	2/21	1/17	3/19	2/22	5/24	1/15
					3/17	1/13	2/15	1/15	1/15	1/17
					2/18	0/13	2/20	3/19	1/20	2/17
4	7.41	7.46	7.08		3/13	0/13	1/12	2/14	0/12	1/11
					1/10	0/7	2/11	2/11	1/15	2/13
					3/20	2/16	9/18	3/21	3/23	1/14
						0/12	2/14	0/14	0/14	2/16
						0/9	1/16	1/15	1/16	2/13
5	8.57	7.66	5.98	5.56		0/14	3/13	2/15	1/11	1/12
						0/7	1/4	1/11	1/15	0/13
						1/18	5/20	2/23	4/25	2/16
							1/10	2/10	1/10	1/12
							0/11	0/10	0/11	0/8
6	9.88	8.62	11.29	12.36	14.44		0/13	1/15	0/13	0/12
							1/8	0/8	0/12	0/10
							3/16	1/19	3/21	0/12
								1/12	1/12	2/14
								0/17	2/18	1/15
7	7.35	8.33	7.46	4.83	5.41	9.77		0/14	0/13	1/11
								0/12	1/16	0/14
								3/21	3/23	1/14

	1[b]	2	3	4	5	6	7	8	9	10
									0/12	0/14
									2/17	1/14
8	4.17	11.40	12.92	5.83	8.97	10.22	12.28		1/12	2/13
									3/16	2/14
									3/26	3/17
										2/14
										1/15
9	13.16	8.75	7.54	9.13	8.94	11.62	7.50	9.13		0/10
										2/18
										2/19
10	9.90	9.31	9.26	7.41	8.57	14.82	8.82	7.87	8.33	

[a] Ratios above the diagonal ([*G*/*F*]; see Methods) are provided for each of the 5 enzymes used in Table I. Values below the diagonal are estimates of genetic divergence ($\hat{p}$) based on the aggregate data from 5 enzymes analyzed at $CL_{1,0}$.

[b] Digestion phenotypes (Table II).

[c] $\hat{p} \times 100$.

cluster, but the taxonomic level of the grouping is unclear. One possibility is that the various phenotypes included represent a complex of sibling species (see Discussion). There is a suggestion of some structural hierarchy within the cluster, but the only definitive subgroups are the Lewin and Bos phenotypes which each contain more than one strain, and which are each represented by single endpoints in Fig. 4.

DISCUSSION

The discovery of seven different restriction digestion phenotypes for various isolates of *A. castellanii* (Table II) suggests considerable nucleotide sequence diversity within the species. The 4.2–12.9% estimated divergence among phenotypes within this 'species' (Table IV) is at least as great as the 3.6–8.2% divergence calculated from the data of Cummings for variation among sibling species of *Paramecium aurelia* [20]. (Our calculations combined the data of Cummings for five enzymes and assumed a 0% S.D. for fragment size.) It also is similar to the 2.6–9.7% variation in $\hat{p}$ calculated from the data of Goldbach et al. for phenosets (sibling species?) of *Tetrahymena pyriformis* [21]. (Calculations in this case were for strains with one or more fragments in common and assumed a 4% S.D. for average fragment size.) These two species complexes were selected for comparison because they include protozoans with mitochondrial genomes comparable in size to the genome of *Acanthamoeba*.

The most closely related *Acanthamoeba* strains are those that share the Lewin or Bos phenotypes. With the extensive variability in DNA fragment patterns found among strains of one species, *A. castellanii*, it seems reasonable to assume that strains which share the same phenotype also should belong to a single species. However, both the Lewin and Bos phenotypes are shared by two species, *A. castellanii* and *A. polyphaga.* This result may be due to difficulties in distinguishing these two species, or may be evidence for parallel or convergent evolution of mitochondrial genomes. The first explanation seems most probable.

In contrast to a previous suggestion which was based on less data [24], it now appears that the Page 23 phenotype (*A. polyphaga*) is as closely related to the seven phenotypes of *A. castellanii* (ave. $\hat{p}$ = 9.2 ± 1.2%) as the latter are to each other (ave. $\hat{p}$ = 8.0 ± 0.9%). The Griffin phenotype (*A. griffini*) is also related to the phenotypes of *A. castellanii* to about the same extent (ave. $\hat{p}$ = 8.7 ± 0.9%). The Ray phenotype is the most distinct, differing from all other phenotypes by an average $\hat{p}$ of 11.4 ± 2.1%. Inclusion of all strains, except Ray, in a single species complex would be consistent with the estimated genetic divergences. Judgement on the taxonomic value of $\hat{p}$ calculated from fragment size polymorphism should be reserved, however, until more is known about details of sequence organization in the mitochondrial genome of acanthamoebas and about the relationship of $\hat{p}$ to actual nucleotide sequence diversity.

We used DNA fragment size polymorphism to estimate nucleotide sequence polymorphism in the expectation that the latter would correlate with overall genetic relatedness of the various strains [24]. The method of Engels [34], like the original Upholt approach [33], assumes that the frequency and distribution of restriction sites in the mitochondrial genome are random; that the gain or loss of restriction sites during evolution occurs primarily by single nucleotide changes, at least for very closely related organisms; and that the probability of nucleotide change is the same for all nucleotide positions. There is evidence that the frequency and/or linear distribution of restriction sites for some, but not all, enzymes is nonrandom in human mtDNA [37]; that the probability of nucleotide changes becoming fixed during evolution is not equal for all nucleotide positions in rat [38] or primate [39] mtDNA; and that evolutionary change is not limited to single nucleotide substitutions in larger mitochondrial genomes where, for example, major insertions have occurred in the approx. 70 kbp yeast genome [40] and more extensive rearrangements have occurred in the approx. 500 kbp genome of corn [41]. It is probable, therefore, that our quantitative estimates of genetic divergence will require revision as information about mtDNA sequence organization in *Acanthamoeba* develops. Whether these revisions significantly alter our interpretation of overall genetic relatedness among strains remains to be seen.

One perplexing question has been whether all strains of *Acanthamoeba* have a pathogenic potential, or whether there are distinct pathogenic subgroups of strains. Attenuation of pathogenicity during laboratory culture [13] may make it difficult to determine whether a number of presently maintained lines were originally pathogenic. Our discovery that the five members of the Bos group share a single restriction digestion pheno-

type is consistent with the possibility that there are distinct pathogenic subgroups. It is clear, however, that pathogenicity is not associated with a single subgroup. The pathogenic Nagington and Ma strains have very different phenotypes, each distinct from the Bos phenotype and from two other pathogenic phenotypes recently identified by preliminary work in our laboratory. Since pathogenicity has not been tested in some of the strains that we have examined, further study of this character will be necessary before we can determine how many pathogenic subgroups might exist.

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