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GENETIC HETEROGENEITY IN A NATURAL POPULATION OF ACANTHAMOEBA POLYPHAGA FROM SOIL, AN ISOENZYME ANALYSIS

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By

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ABSTRACT

GENETIC HETEROGENEITY IN A NATURAL POPULATION OF ACANTHAMOEBA POLYPHAGA FROM SOIL, AN ISOENZYME ANALYSIS

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Acanthamoeba polyphaga, a free-living, bacterial feeder found in freshwater and soil, reproduces asexually and is morphologicaly distinguishable from other acanthamoebae. Isoenzyme analyses were done on 15 random, clonal isolates from soil. Electrophoretic patterns indicated that enzyme bands occurred in clusters consistent with that of a diploid organism. The data indicates that natural populations of A. polyphaga have a greater genetic diversity than laboratory isolates of other amoebae, resembling the heterogeneity observed for natural populations of bacteria.

Dedicated to my mom and dad,

Darryl and Jerrold

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INTRODUCTION

Free-living, asexual amoebae (e.g. Acanthamoeba, Naegleria) are widespread in soil and freshwater. Some species are also pathogenic to man (13, 26). Isoenzyme analyses have been used to clarify the taxonomy of axenic, laboratory isolates obtained from a variety of sources and indicate a low genetic heterogeneity for those studied (23). This is in contrast with isoenzyme analyses of natural populations of bacteria in which considerable genetic heterogeneity has been reported (12, 15, 16, 24).

In the present study, isoenzyme electrophoresis was performed on clonal isolates to determine genetic heterogeneity in natural populations of A. polyphaga from soil. The genetic heterogeneity observed was greater than results obtained for axenic, laboratory cultures, yet consistent with the heterogeneity reported for bacterial populations (12, 15, 16, 24).

I question the use of isoenzyme analyses for strain and species classification of laboratory cultures of small amoebae. By using random isolates from various sources, the genetic heterogeneity of the parent, natural population for each laboratory isolate is being ignored. Further, considerable genetic selection takes place in establishing axenic cultures of amoebae (20, 27).

Results from this study were presented at the American Society for Micrbiology Convention, Washington, D.C., March 23-28, 1986.

MATERIALS AND METHODS

Sampling location and collection procedure. Random soil samples were collected from 3 sites (A, B, C) in Dickinson County, Michigan. Sites A and C were located in Felch township at lat. 46° 07' N, long. 87° 54' W and lat. 46° 03' N, long. 87° 55' W respectively, while site B was located in Norway township at lat. 45° 56' N, long. 87° 56' W. Each site consisted of a 10 by 20 meter area in a northern hardwood forest.

With the aid of a tube sampler (diameter:2 cm) both organic and mineral layers were collected and subsequently 2 inches from each horizon was brought back to the laboratory. Amoebae were enriched from the soil samples using a 96 multi-well plate, soil dilution technique and Escherichia coli (strain K-12) as the food organism (8).

Microorganism identification and clonal isolation. Microorganisms were identified as Acanthamoeba polyphaga on the basis of cyst morphology (22). To each well identified as containing A. polyphaga, one drop of LS saline (50 mM NaCl, 4.6 mM $MgSO_4$, 0.36 mM $CaCl_2$) (3) was added; then the amoebae were withdrawn with a pipet and placed in a Petri dish containing LS saline and agar (1.5% w/v). To obtain single amoebae for clonal isolates, a piece of agar which contained the smallest number of A. polyphaga was cut out and placed on another Petri dish containing LS saline and agar (1.5% w/v) (LS-agar). At this point, had a single A. polyphaga been isolated, it was cultured with E. coli (strain K-12) at 23°C on LS agar. If however, an isolate had not been achieved, amoebae were subcultured to

the bottom of a fresh Petri dish containing LS-agar, with E. coli spread over the dish, which was slanted during incubation. The slant allowed the amoebae to migrate towards the top and distribute widely over the plate which eased the isolation of individual organisms.

Culture methods. E. coli was cultured in 200 ml Tryptone broth(0.5 g NaCl, 1.0 g Tryptone, Difco Laboratories, Detroit, Michigan U.S.A. and H_2O to 100 ml) for 18 h at 37°C. The organisms were harvested and washed 2 times in LS saline, suspended in 30 ml of LS saline and distributed in 10 ml volumes to 250 ml plastic tissue culture flasks (Corning Science Products). Subsequently, clonal isolates of A. polyphaga were inoculated into the tissue culture flasks containing E. coli and incubated at 23°C. Once the peak of population growth had been reached (approx. 48-72 h), the amoeba cultures were harvested by centrifugation, washed once in LS saline and pelleted. Culture pellets were then frozen and stored over liquid nitrogen.

Gel Electrophoresis. A vertical discontinuous polyacrylamide gel was used (14): (resolving gel (7.5%), resolving gel buffer (pH 8.9) and spacer gel (5%), spacer gel buffer (pH 6.7). Samples were removed from liquid nitrogen, thawed and suspended in 1 ml sample solution (10 ml, 5.98 g Trizma base/100 ml, pH 6.7, 80 ml H_2O, 10 ml glycerol). After centrifugation in a microcentrifuge (Fisher Model 235B) for 1 min, 50 µl of sample supernatent (approx. 2-4 x 10^4 cells) was loaded into each gel electrophoresis well. Electrophoresis was run for approximately 4 h at 300 V with pH 8.3 electrode buffer (14). Gels were stained for acetyl esterase (7), propionyl esterase (7) and

tetrazolium oxidase (2). All reagents used in the enzyme staining procedures were obtained from Sigma Chemical Company. For comparison, A. polyphaga, American Type Culture Collection strain #30487, was used in this study.

Data analysis procedures. Loci were coded by number from the most anodic banding cluster, designated number 1 and so on to the most cathodic cluster (23). Allele number was assigned based on isoenzyme patterns expected for heterozygotes in the case of enzymes which are monomers, dimers, trimers or tetramers (10). The calculation of genetic distances was based on Nei's formula (17) and was done with the aid of a computer program (9) and a correction to that program (11).

RESULTS

Loci identification. Isoenzyme analyses indicated a clustering of the electrophoretic bands for acetyl esterase (AE), propionyl esterase (PE) and tetrazolium oxidase (TO). Based on these clustering patterns, loci were assigned by number from the most anodic to the most cathodic for each of the enzymes. The number of loci observed for each enzyme varied by soil site (Tables 1, 3 and 5). Three loci were observed for PE from sites A and C, while four were observed from site B. Four loci were observed for AE from sites A and B; three from site C. Three loci were observed for TO from site A and B; four from site C.

Allele assignment. All loci were assigned allele numbers in accordance with the isoenzyme patterns expected in heterozygotes. Alleles were assigned with numbers increasing from the most anodic electrophoretic band to the most cathodic. Heterozygous monomers consisted of two electrophoretic bands, dimers three bands, trimers four bands and so on, with the homozygote represented by the two outer bands of the respective heterozygote.

Data given below showed that all of the clonal isolates differed from one another, although isolate B5 was identical to the banding pattern of A. polyphaga strain #30487 from the American Type Culture Collection. Replicate electrophoretic gel runs showed identical banding patterns.

Null alleles. Absence of enzyme activity was noted for each of the enzymes studied from the three sites. The alleles responsible for

this loss of activity ("null alleles") were assigned a number in relation to the corresponding allele assignments for each locus.

Genotypes. Genotypes were described based on the allelic information for each clonal isolate (Tables 1, 3 and 5). The homozygotes had been designated by one allele and the heterozygotes by both alleles. A range of variation existed in the genotypes between the isolates at a given site. Between isolates A1 and A2 (Site A), six of ten loci showed the same alleles in identical frequencies, two showed one allele the same yet in different frequencies and the remaining two loci had unlike alleles. Between isolates C1 and C3 (Site C), only two of ten loci had genotypes with the same alleles in identical frequencies. The remaining eight loci had different alleles. Differences in the genotypes between the other isolates varied between these two extremes.

Genetic variation. Genetic similarities ("identities") were determined and subsequently used to calculate genetic distances between clonal isolates from each site (Tables 2, 4 and 6). Values ranged from 0.122 to 0.751 over the three sites. These two values corresponded to the allelic comparisons made between isolates C1 and C3 and isolates A1 and A2 respectively.

Genetic distances ranged from 0.286 to 2.106 corresponding to comparisons between A1 and A2 and C1 and C3 respectively. The greater the proportion of loci with the same alleles, with identical frequencies between the two isolates, the greater the genetic similarity and subsequently, the smaller the genetic distance.

Statistics. The data indicated large differences between clonal isolates at each of the soil sites. Yet there is no statistically significant difference in mean genetic distances between the three sites (Table 7).

DISCUSSION

Isoenzyme electrophoresis is a technique for the study of genetic variation within a species (25). Electrophoretic banding patterns provide data in the form of allelic variants for specific genes. This information can be readily converted to allelic frequencies which are subsequently applied to the chosen formula for genetic variation.

A measure of heterozygosity (18) would be determined if one wished to calculate genetic variation at each enzyme locus. A measure of genetic distance (17) would be calculated to determine genetic variation between isolates over all loci.

In previous studies, a measure of heterozygosity was used to express genetic variation when each individual electrophoretic band was the product of a separate genetic locus as seen in haploid systems (12). On the other hand, genetic distance was calculated when a clustering of the electrophoretic bands suggested a diploid structure for the genome (23). Calculation of genetic distances was similarly used in this study to interpret the data. However, genetic distance calculations are not dependent on ploidy or mating scheme (17).

By calculating the values for genetic distance it was evident that genetic heterogeneity did exist for the 15 clonal isolates at each of the 3 soil sites. Since random isolates taken from 3 different soil sites did not differ in terms of mean genetic distance, the values obtained were not unique to a particular site. The data for these 15 clonal isolates represented a single species, A. polyphaga, rather than a mixture of Acanthamoeba species. This amoeba

species can be readily identified by a unique cyst morphology (22). The cyst protoplast tends to be angular so that a mixture of square and triangular protoplasts, as well as other shapes, are seen in a population of cysts. The variation that existed among the isoenzyme patterns of the 15 clonal isolates also provided evidence that a single species was studied. If the clonal isolates had represented more than 1 species, a few repetitive banding patterns between isolates of the same species would have been observed. The only similarity that was found in the isoenzyme patterns was that isolate B5 was identical to the banding pattern of A. polyphaga strain #30487 from the American Type Culture Collection.

The genetic heterogeneity observed for the 15 A. polyphaga isolates was high. Genetic distances were calculated to be between 0.286 and 2.106 over the 3 soil sites. These values were higher than those calculated by Pernin (23) for axenic laboratory stocks of Naegleria. The species N. lovaniensis, considered to be the most genetically variable of the species studied, had genetic distances ranging from 0.11 to 0.146. These low values for genetic distance may be due in part to axenic selection which has been observed in the cellular slime mold Dictyostelium (20).

The values of genetic distance for N. lovaniensis (23) which ranged from 0.11 to 0.146 were determined by comparison between strain 6, an isolate from a thermally polluted water source in the U.S.A., and strain 8, an isolate from a hydrotherapeutic swimming pool in Belgium; and between strain 1, an isolate from a thermally polluted water source in France, and strain 7, an isolate from a

hydrotherapeutic swimming pool in Belgium, respectively. It would be useful to determine the amount of genetic heterogeneity within a population of a Naegleria species from a single source and with clones that had not been selected for axenic growth. An isoenzyme study of axenic, laboratory isolates of Acanthamoeba (6) also would have benefited for similar reasons.

The results of this study indicate that there is greater genetic diversity in this asexually reproducing species than in sexually reproducing diploid animals (1). In the absence of normal genetic recombination, the dispersal of new genotypic elements within the population may be inhibited which would prevent new species from forming and would consequently lead to greater heterogeneity within the original species.

The basis for this heterogeneity may be some type of recombination such as defective DNA repair proposed in fungi (21). Perhaps genetic variation could be explained by the presence of plasmids, found in wild-type strains of the cellular slime mold Dictyostelium discoideum (19) or by the presence of transposons, also reported in D. discoideum (4,5).

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TABLE 1. GENOTYPES (Site A)**

	CLONAL ISOLATES				
LOCI	A1	A2	A3	A4	A5
PE 1	1	1	2	1/3	2
PE 2	1/3	2	2	1/3	1/2
PE 3	1	2/3	4*	1/2	2
AE 1	2*	2*	2*	1	2*
AE 2	1/2	2	3*	1	3*
AE 3	2*	2*	2*	1	2*
AE 4	1	1	1	1/2	2
TO 1	1/2	1/2	2	2/3	3
TO 2	1/2	2	2	1/2	3*
TO 3	2*	2*	2*	1	1

*Null alleles.

**Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anodic to the most cathodic. Homozygotes are designated by one allele and heterozygotes by both alleles.

TABLE 2. Site A. NEI'S GENETIC DISTANCES(Above the diagonal)/GENETIC IDENTITY(Below the diagonal)*

(A Site)

	CLONAL ISOLATES				
	A1	A2	A3	A4	A5
A1	----	0.286	0.685	0.834	1.820
A2	0.751	----	0.434	1.378	1.417
A3	0.504	0.648	----	1.719	0.916
A4	0.434	0.252	0.179	----	1.087
A5	0.162	0.243	0.400	0.337	----

*Null alleles included.

TABLE 3. GENOTYPES (Site B)**

	CLONAL ISOLATES				
LOCI	B1	B2	B3	B4	B5
PE 1	1/3	1/2	2	2	2
PE 2	1/3	4*	2	1/2	1
PE 3	2/3	4/6	7*	3/5	1/5
PE 4	1	3	4*	2	2
AE 1	3*	1/2	3*	1/2	3*
AE 2	1	2*	2*	2*	1
AE 3	2*	2*	2*	2*	1
AE 4	1/4	2	1	3	3
TO 1	1/2	3	5*	3/4	2/3
TO 2	2	2/3	2	2	1/2
TO 3	2*	1	2*	1	2*

*Null alleles.

**Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anodic to the most cathodic. Homozygotes are designated by one allele and heterozygotes by both alleles.

TABLE 4. Site B. NEI'S GENETIC DISTANCES(Above the diagonal)/GENETIC IDENTITY(Below the diagonal)*

	(B Site)				
	CLONAL ISOLATES				
	B1	B2	B3	B4	B5
B1	----	1.609	0.906	1.642	0.899
B2	0.200	----	1.498	0.636	2.001
B3	0.404	0.224	----	0.938	1.305
B4	0.194	0.529	0.391	----	0.857
B5	0.407	0.135	0.271	0.424	----

*Null alleles included.

TABLE 5. GENOTYPES (Site C)**

	CLONAL ISOLATES				
LOCI	C1	C2	C3	C4	C5
PE 1	1	2	3*	1	1
PE 2	1	1/2	1	1	1
PE 3	2	1/2	1	3*	2
AE 1	1	2/3	4*	4*	1
AE 2	2/4	1/3	3	1/3	3
AE 3	1	2	1	2	2
TO 1	2	1	1	3*	2
TO 2	2/3	1/2	4*	4*	2/3
TO 3	1/3	1/2	2	1/2	1/3
TO 4	1	2*	2*	2*	2*

*Null alleles.

**Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anodic to the most cathodic. Homozygotes are designated by one allele and heterozygotes by both alleles.

TABLE 6. Site C. NEI'S GENETIC DISTANCES(Above the diagonal)/GENETIC IDENTITY(Below the diagonal)*

(C Site)

	CLONAL ISOLATES				
	C1	C2	C3	C4	C5
C1	----	1.638	2.106	1.824	0.377
C2	0.194	----	0.896	0.819	0.837
C3	0.122	0.408	----	0.752	1.445
C4	0.161	0.441	0.471	----	0.758
C5	0.686	0.433	0.236	0.469	----

*Null alleles included.

TABLE 7. STATISTICS FOR GENETIC DISTANCE*

SITE	MEAN GENETIC DISTANCE ± SD
A	1.0576 ± .521
B	1.2291 ± .444
C	1.1452 ± .565

One-way ANOVA: (For sites A, B & C)

	D.F.	M.S.
Between	2	.0735
Within	27	.2628

F= .2798 (NS)

*Abbreviations: SD, standard deviation; ANOVA, analysis of variance; D.F., degrees of freedom; M.S., mean square; F, F-test; NS, not significant at the 0.05 level.