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A COMPARATIVE STUDY OF INTRASPECIFIC GENETIC HETEROGENEITY IN ACANTHAMOEBA POLYPHAGA (AMOEBIDA, ACANTHAMOEBIDAE), BY ISOENZYME ANALYSIS

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By

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ABSTRACT

A COMPARATIVE STUDY OF INTRASPECIFIC GENETIC HETEROGENEITY IN *ACANTHAMOEBA POLYPHAGA* (AMOEBIDA, ACANTHAMOEBIDAE), BY ISOENZYME ANALYSIS

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The intraspecific genetic heterogeneity of *Acanthamoeba polyphaga*, a free-living, asexual amoeba from natural soil populations, was examined by isoenzyme analysis. In this study, 15 isoenzymes were used to analyze random, clonal isolates of amoebae from soil at three different study sites. Isolates were obtained from a northern hardwood forest in Michigan in 1986 and 1987. The results showed that 1, there were no statistically significant differences in mean genetic distances between sites and between the two years; the mean genetic distances ranged from 0.42 to 0.50; 2, analysis of zymograms suggest that this species may be a diploid organism; 3, greater genetic heterogeneity exists in natural populations of this organism than those of sexual reproductive organisms, but similar to those of asexual organisms. The reason for this may be due to the absence of sexual reproduction or due to rare genetic recombination.

Dedicated to my wonderful parents,

my lovely wife and my daughter

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INTRODUCTION

Isoenzyme banding pattern analysis with electrophoresis has been widely used in the identification of species and to survey intra- or interspecies genetic diversity. Among amoebae, species and strains of Acanthamoeba and Naegleria have been studied.(7,11,17).

However, in most previous work the data were obtained from a limited number of axenic laboratory strains. Also considerable genetic selection may take place in establishing axenic cultures of amoebae. At this point, the genetic heterogeneity of the parent, natural population for each laboratory strain is unknown (11).

The intraspecific heterogeneity of Acanthamoeba polyphaga was examined by Jacobson & Band (11). In the present study, a comparative analysis of isoenzymes for two consecutive years was performed to determine if the intraspecific genetic heterogeneity of Acanthamoeba polyphaga remained the same or changed, when using natural populations. A. polyphaga is a free-living, asexual amoeba, found widely in soil and freshwater. The isolates were obtained from a northern hardwood forest at three different sites in 1986 and 1987. The population size of soil amoebae (number of amoebae per gram soil) for these two years at the study sites was smaller than previous years, possibly due to dry weather (13).

The results indicated that the genetic heterogeneity within A. polyphaga was high. The mean genetic distance calculated was 0.42 to 0.50. However, there were no statistically significant differences in average heterogeneity between the two years and between the sites.

MATERIAL AND METHODS

Sampling location and collection procedure. Random soil samples were collected from three 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 (11).

With the aid of a tube sampler (diameter : 2 cm.) both organic and mineral layers were collected and subsequently two inches from each horizon was brought back to the laboratory. Amoebae were isolated from the soil samples using a 96-multi-well plate, soil dilution technique and Escherichia coli (strain K-12) as the food organism (11). The above procedures were followed between the months of June to October for the two consecutive years of 1986 through 1987.

Microorganism identification and clonal isolation. Microorganisms were identified as Acanthamoeba polyphaga on the basis of cyst morphology (16). To each well identified as containing A. polyphaga, one drop of LS saline (50 mM NaCl, 4.6 mM $MgSO_4$, 0.36 mM $CaCl_2$)(2) 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) (LS-agar). 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 the lower edge of a slanted Petri dish containing LS-agar. Then amoebae were cultured at 23°C with E. coli (K-12) spread over the dish. The amoebae migrated from the lower edge towards the top of the Petri dish and distributed widely. A single amoeba was isolated from this plate. Then, the cyst shape was checked again and the same procedure was repeated one more time.

Strains. A total of 30 strains of A. polyphaga were randomly isolated and cloned from 30 soil samples of the three sites, 10 strains per site, for each year tested.

Culture methods and sample preparation for isoenzyme electrophoresis. E. coli (K-12) was cultured in 200 ml LB medium (NaCl 10 g, Bacto Tryptone 10 g, Bacto Yeast Extract 5 g, Difco Laboratories, Detroit, MI, U.S.A., H_2O to 1 Liter, pH 7.5) for 18 - 20 hr at 37°C. The bacteria were harvested by centrifugation (9000 g) and washed two times in LS saline, then suspended in 30 ml of LS saline and distributed in 10 ml volumes to three 250 ml plastic tissue culture flasks (Corning Science Products). Subsequently, clonal isolates of A. polyphaga were inoculated into these tissue culture flasks and incubated at 23°C. Once the peak of population growth was reached (about 36 - 72 hr), the amoebae from three flasks, representing 18-24 x 10^6 amoebae, were harvested by centrifugation, washed twice in LS saline to remove bacteria and pelleted. The amoebae were then

suspended in 200 ul of lysis buffer and frozen over liquid N_2 until electrophoresis. Lysis buffer consisted of 10 mM Tris, 1 mM EDTA, (pH 6.8) to which 0.05 mM NADP was added before use.

Gel preparation. A vertical discontinous polyacrylamide gel slab was used (12). Spacer gel was 6% (w/v) (pH 6.7) and resolving gel (pH 8.2) was used at different concentrations (Table I).

Gel electrophoresis. Samples were removed from liquid N_2, thawed at room temperature, suspended in 200 ul of sample solution (10 ml glycerol, 90 ml electrode buffer (12)). After centrifugation in a microcentrifuge (Fisher model 235B) for 30 sec, 50 ul of sample supernatant was injected into gel electrophoresis wells. Gel electrophoresis was run with cold water in an ice bath; running time was about 8 hr at 200 v with pH 8.2 electrode buffer. Control experiments with E. coli. (K-12) was also carried out on the same gel in order to differentiate between the bands of A. polyphaga and E. coli. A 50 ul sample of 250 mg (dryweight)/ml bacterial supernatant was loaded into a gel well. When a different bacteria species, Klebsiella pneummiae was used as the food source for A. polyphaga, the zymograms were the same.

Isoenzymes studied, staining recipes and staining time. Fifteen isoenzymes were examined (Table I). Staining was

done in darkness at 37°C; chemical reagents for staining were from Sigma Chemical Company.

Loci and allele assignment. Loci assigned were based on clustering patterns and numbered from anode to the cathode according to each isoenzyme banding pattern (17). The alleles at each locus (based on banding patterns in which one band represents a homozygote and multiple bands a heterozygote (10)), were also coded from the most anodic band to the most cathodic band. Homozygotes were designated by one number and heterozygotes by two numbers. 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 (10).

Null alleles. The absence of isoenzyme activity at each corresponding locus was defined as a "null allele", and also given a 0 number in order to be in agreement with the allele assignment for each locus.

The estimation of genetic distances. Calculations of genetic distance were based on Nei's standard formula (14) and done with the aid of a computer program (8, 9). Genetic distance is the genetic difference between populations as expressed by a function of gene frequences. Nei's standard genetic distance formula is a statistical method by which the average number of codon differences per locus can be estimated from gene frequency data. The frequencies of

alleles segregating at each locus is as follows : it will be equal to 1 for a homozygote with a given allele, the frequency of the other allele at the same locus then being 0. For a heterozygote, the two alternative alleles of a given locus both have a frequency 0.5. Hence the alleles frequencies have value 0, 0.5 and 1 (17) (see Appendix A for further detail).

RESULTS

Genotypes. The zymograph of the 30 isolates for each year showed monomorphic and polymorphic banding patterns. A total of 30 loci were assigned. Three out of 30 loci were monomorphic, AAP 2, PGM 2 and LTD 2 (for abbreviations see Table I). Tables II, IV, VI, VIII, X and XII showed the genotypes for each clonal isolate of different sites and different years based on allelic frequencies. These tables indicated varying degrees of diversity in genotypes among the polymorphic loci and there was not any two isolates which had the same patterns over the 30 loci. For example, between the isolates 4 and 5 (site A, 1987 at Table VIII), 27 (including three monomorphic loci) out of 30 loci showed the same genotypes, two loci (ME & HK) had one common allele at each locus and one locus (SOD 2) between the isolates had unlike alleles. Between the isolates 2 and 8 (site B, 1986 at Table IV), however, only eight out of 30 loci had the same genotypes, eight loci had a common allele in each locus and the other 14 loci had unlike alleles. The differences between any other paired-isolates in genotypes varied between these two extremes.

Control experiment. No isoenzyme activity of E. coli was detected on these gels, confirming that all the bands belonged to amoebae.

Repeated experiment. In later, repeated experiments for isolates with fresh cell extract were done and identical banding patterns were observed. The results indicated that the banding patterns for isolates were stable over a period of time.

Genetic variation. Genetic distances between clonal isolates from each site were calculated and given in Tables III, V, VII, IX, XI and XIII.. The values of genetic distance ranged from 0.067 to 0.839 over the three sites and the two years. The two extreme values were the allelic comparisons made between isolates 4 and 5 (site A, 1987) and isolates 2 and 8 (site B, 1986).

Statistics. Mean genetic distances for each site (Tables XIV & XV), one-way analysis of variance for three soil sites each year (Tables XIV & XV) and two-way analysis of variance for different sites and different years (Table XVI) were done respectively. The values of mean genetic distance ranged from 0.42 (site A, 1986) to 0.50 (site B, 1986). Yet, there were no statistically significant differences in the average genetic distances calculated between the sites and between the years (in one-way and two-way analysis of variance). Therefore, it was concluded that there was no change in genetic distances between the two consecutive years and there were no differences due to site effects.

DISCUSSION

The level of ploidy of A. polyphaga is unknown (4). However, the zymograms showed complex banding patterns at most of the loci. One possible explanation, in terms of the multiple banding patterns, is that the double-band pattern was not due to heterozygotes, but due to the mixing of two clonal isolates, this was unlikely. For example, isolate 2 (table VIII, 1987) showed double bands at the GDH 2 locus (genotype 1/2). One may think that this was the result of mixing of isolates 3 and 4 (genotypes 2 & 1), but this assumption could not be explained by the banding pattern in ICD and LDH for isolate 2 (genotypes 1/3 & 0). The genotypes of isolate 3 and 4 at these two loci were 1 & 1 and 0 & 1/2. There may exist two other explanations for the complex banding patterns such as gene duplication in a haploid organism or alternative splicing of a transcript. However, this is not my case because 25 out of 30 loci showed multiple bands. Therefore, the above two possibilities cannot explain the high frequencies of multiple banding paterns even if they are not rare events. The banding patterns for several enzymes suggested that A. polyphaga may be a diploid organism because the zymograms of several enzymes showed that the banding patterns were similar to those patterns commonly observed in diploid organisms. For instance, at LDH, PGM 1 and ME loci zymograms

exhibited three band-patterns, of which the middle band was stained more darkly than the two outside bands (the activities of the isozymes formed in heterozygotes are 1:2:1 for dimer, see Fig.1) and the three bands exhibited equidistance between bands. This is the typical dimeric heterozygous pattern, found in diploid organism (5,10 & 18). When the number of chromosomes in a species is difficult to count, zymograms of isoenzymes are used to estimate the ploidy level (4). Although this is a good method to be used for estimating ploidyism, it may not be as accurate, and therefore, it is not the most reliable method. My data didn't rule out the possibility of polyploidy in this species. In Amoeba proteus, it was reported that there may exist polyploid cells in its natural population, and they may play a role in genetic variability of those asexually growing organisms (1). Because there is no closer diploid species of this genus as a control to compare the zymograms and sometimes the banding patterns between diploid and polyploid organism for some enzymes may be the same (19), therefore, it is difficult to judge whether A. polyphaga is a polyploid species. More other lines of evidence are needed to prove it further. In zymograms, the complex banding patterns and high loci numbers for some enzymes may be due to the addition of genome, or by the gene duplication and then during the course of evolution they diverged.

Nei's genetic distance measure has been widely used to evaluate the genetic variation within species or between species. This method applies to any kind of organism without regard of ploidy or mating scheme. This is because the definition of I and D (see Appendix A) depends solely on gene frequencies rather than on others (14). The mean genetic distances I calculated of A. polyphaga ranged from 0.42 to 0.50. It was much greater than those of multicellular, sexual organisms within species (15). For example, within the Drosophila willistoni group, the genetic distance is 0.008 - 0.049. However, the value of genetic distance between the sibling species of D. willistoni group is 0.54 $\pm$ 0.05, non-sibling species of D.willistoni group is 1.1 $\pm$ 0.06 (15). For the unicelluar, free-living amoebae of the genus Naegleria, including three species, N. lovaniensis, N. fowleri and N. australiensis, the genetic distances within each species were obviously low (17) and similar to those of sexual organisms (15). The mean genetic distance for N. lovaniensis was 0.081, for N. fowleri 0.022 and for N. australiensis 0.023; six to twenty times lower than that of A. polyphaga. In contrast, the value of interspecific genetic distance was 1.222 between N. lovaniensis and N. fowleri, 3.733 between N. lovaniensis and N. australiensis and 3.273 between N. fowleri and N. australiensis. On the basis of the statistical analyses of their data and other evidence, Carlou & Pernin speculated

that genetic recombination existed in Naegleria (5). Based upon the above examples, the heterogeneity within A. polyphaga is similar to that which is derived from comparisons made between sibling species of the D. willistoni group, and much greater than that within each species of Naegleria. If A. polyphaga were a sexually reproducing organism, the value of genetic distance within this species could not possibly be so high. Here is another example. There was little genetic diversity when analysing isozyme banding patterns (14 out of 16 loci is the same) among sexual isolates in Dictyostelium discoideum, which are simple soil amoebae. In contrast, asexual isolates differed from sexual isolates at 13 of 16 loci. High heterogeneity existed between sexual isolates and asexual isolates (3). I think the high genetic heterogeneity of A. polyphaga may be due to the accumulation of mutations and the absence of sexual reproduction or very rare genetic recombination, thus inhibiting the gene flow. Interestingly, a chi-square test showed that, of the genotype frequencies, 14 out of 25 polymorphic loci in this species didn't fit the Hardy-Weinberg Equilibrium. Thus, if mating between isolates occurred, it was rare and not random. Similar high genetic heterogeneity within Trypanosoma cruzi, which is a unicelluar, diploid parasite, has been reported. The mean genetic distance of this species was 0.757 (18). Their statistical study indicated that large linkage

disequilibrium between alleles existed at paired loci, which reached maximum possible values for almost all of the locus pairs (20). The authors stated that this result was most consistent with the hypothesis that genetic recombination is absent or very rare in T. cruzi natural populations. There is no definitive evidence for sexual reproduction of A. polyphaga (4). These data don't eliminate the possibility of mating in this species, but more direct evidence is needed. An idea, which if achieved would yield such proof, would be to genetically mark isolates differently. For example, one could use two different genetic markers to mark two or more different strains; the different strains would then be mixed, cultured, and processed through electrophoresis. If it was found that in one cell the genetic markers of the two different strains existed, one could infer that mating occurred.

The genetic distances calculated by Jacobson (11) for the A. polyphaga ranged between 0.286 and 2.106 over three sites and mean genetic distance were 1.06 to 1.15, which were higher than my results. Two possible explanations for that is this. One is that fewer strain numbers were used and fewer loci were used as well. The data were based on 15 strains and 10 loci, but my data came from 30 strains and 30 loci. The result I calculated showed that the value of the mean genetic distance decreased by increasing the numbers of strains and loci used. According to this, the value of the

mean genetic distance within species may be related to the number of strains and loci that were used. To obtain more reliable results, a greater number of isolates were compared using many different isoenzymes. This idea was also supported by statistics, the larger number of strains you use, the shorter confidence interval of the mean, i.e. the more accurate mean internal.

The other explanation may be due to climate, which may be an important factor that affects the genetic heterogeneity within this species. The population sizes (number of amoebae per gram soil) of A. polyphaga in 1986 ($8-16\times10^3$ amoebae/per gram soil) and 1987 ($20-50\times10^3$ amoebae/per gram soil) were much smaller due to a drought (13) in the sampling areas than that in 1985 ($1.1-1.6\times10^6$ amoebae/per gram soil)(the isolates Jacobson (11) used were from 1985). During the wet season, there were more kinds of food for amoebae and less presure of genetic selection, therefore decreasing competition within and between the species, large population sizes and greater genetic diversity. On the other hand, a dry season provides less kinds of food, increasing competition and greater presure of genetic selection, small population sizes and less genetic diversity within species.

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TABLE 1. Isoenzymes studied, staining time, recipes and gel electrophoresis concentrations for each isoenzyme.

Isoenzyme	Resolving Gel(%)	Staining time(hr)[a]	Staining recipe[b]
Acetyl esterase (AE)	10.75	3	A
Arginine amino peptidase (AAP)	10	overnight	A
Butyryl esterase-2 (BE-2)	10.75	2.5	A
Glutamate dehydrogenase (GDH)	10.75	2.5	B
Hexokinase (HK)	10.75	2	B
B-hydroxybutyrate dehydrogenase (B-HBDH)	10.75	2	B
Isocitrate dehydrogenase (ICD)	10	2	B
Lactate dehydrogenase (LDH)	10.75	2.5	A
Leucine amino peptidase (LAP)	10	overnight	A
Malic enzyme (ME)	10	1.5	B
Phosphoglucomutase (PGM)	10	1	B
6-phosphogluconate dehydrogenase (6-PGD)	11.25	2.5	B
Propionyl esterase (PE)	10.75	3	B
Superoxide dismutase (SOD)	11.25	overnight	B
L-threonine dehydrogenase (LTD)	10.75	1.5	B

[a]Staining in the dark at 37°C.
[b]A, from Daggett (6); B, from Pernin (17).

TABLE II. Genotypes (site A, 1986)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	0	0	3	1/4	3	1/4	1/4	3	1/4	1/4
AE 2	1/2	2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
AE 3	1/3	1/2	1/3	1/2	1/3	1/3	1/3	1/3	1/3	1/2
AE 4	2/3	2	2	1/5	2	1/5	1/5	2	1/5	1/5
AAP 1	1	1/2	1/3	1/3	1/3	1/2	1/3	1/3	1/2	1/2
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	0	0	0	0	0	1/2	1/2	1/2	1/2	1/2
BE-2 2	1/2	1	1/2	1	1/2	1	1	1	1	1
BE-2 3	1/2	2	1/2	2	1/2	2	2	2	2	2
BE-2 4	2	1	1	1	1	1/2	1/2	1/2	1/2	1/2
BE-2 5	1	1	1	1	1	1/2	1/2	1/2	1/2	1/2
BE-2 6	1	1/2	1/2	1/2	1/2	1	1	1	1	1
GDH 1	0	1	1	1	1	1	1	0	0	1
GDH 2	1/2	1	1/2	2	2	1	1/2	1/2	2	1
HK	2	1	1	1	1	1	1	1	1	1
B-HBDH	2	0	2	1/2	2	0	2	2	0	0
ICD	1/3	2	2/3	1/3	1	2	2	2	2	3
LDH	1/2	1	1/2	0	1/2	1/2	1/2	0	1/2	1/2
LAP	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	2	2
ME	1/2	1	1	1	1	1	1/2	1/2	1/2	1/2
PGM 1	1/2	0	1/2	0	1/2	1/2	1/2	1/2	1/2	1/2
PGM 2	1	1	1	1	1	1	1	1	1	1
6-PGD	1/2	0	1	0	1/2	0	1/2	1/2	0	0
PE 1	2	2	2	2	1/4	1/4	1/4	2	1/4	1/4
PE 2	1/5	2/5	1/5	2/5	2/4	2/4	2/4	2/5	2/4	2/4
PE 3	0	0	0	0	1/3	1/3	1/3	0	1/3	1/3
SOD 1	3	1	2/3	2/3	2/3	1/3	1/3	2/3	1/3	1/3
SOD 2	1/2	1	1/2	1	1	2	1/2	1	1/2	1/2
LTD 1	2	1/2	1/2	1/2	2	1/2	1/2	1/2	1/2	1/2
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 reprsents a null allele.

TABLE III. Site A (1986). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.572	0.323	0.548	0.452	0.710	0.533	0.366	0.613	0.681
2		-	0.345	0.293	0.518	0.388	0.517	0.467	0.500	0.415
3			-	0.314	0.189	0.525	0.385	0.261	0.646	0.580
4				-	0.372	0.519	0.456	0.389	0.513	0.457
5					-	0.536	0.326	0.400	0.530	0.550
6						-	0.141	0.516	0.150	0.109
7							-	0.310	0.194	0.210
8								-	0.399	0.571
9									-	0.173
10										-

[a]Null alleles included.

TABLE IV. Genotypes (site B, 1986)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	3	0	3	3	1/4	1/4	1/4	1/4	3	1/4
AE 2	1/2	1/2	1/2	1/2	0	0	0	0	1/2	0
AE 3	2/3	1/3	1/3	1/3	0	0	0	0	1/3	0
AE 4	2	2	2/3	2	1/5	1/5	3/5	1/5	2	1/5
AAP 1	1	1/3	1/3	1/3	1/2	1/3	1/2	1/2	1/2	1/3
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	0	1/2	2	0	1/2	0	1/2	1/2	1/2	1/2
BE-2 2	1/2	1/2	1	1/2	1	1/2	1	1	1	1
BE-2 3	1/2	1/2	1/2	2	2	1/2	2	2	2	2
BE-2 4	1	1	1	1/2	1/2	1/2	1/2	1/2	1/2	1/2
BE-2 5	1	1	1	1/2	1/2	1	1/2	1/2	1/2	2
BE-2 6	1	1	1	1/2	2	2	1	1	1	1
GDH 1	1	0	0	1	1	1	1	1	1	1
GDH 2	1	2	2	1	1	1/2	1	1	1	2
HK	2	2	2	1	1	2	1	1	1	1
B-HBDH	1	2	2	0	0	0	0	0	0	1
ICD	2	0	3	1	2	2	2	2	1	2/3
LDH	0	1/2	0	0	1/2	0	1/2	1/2	1/2	1/2
LAP	1/2	1/2	1/2	2	1/2	1/2	1/2	2	2	2
ME	1	1/2	1	1	1/2	1/2	1	2	1	1/2
PGM 1	0	1/2	1/2	0	1/2	1	1/2	1/2	1/2	1/2
PGM 2	1	1	1	1	1	1	1	1	1	1
6-PGD	1	1	2	0	1	1/2	1	0	1	0
PE 1	2	2	2	1/4	1/4	1/4	1/4	1/4	2	1/4
PE 2	1/5	1/5	1/5	4	2/4	2	2/4	2/4	1/5	4
PE 3	0	0	0	1/3	1/3	1/3	1/3	1/3	0	1/3
SOD 1	1/3	2/3	3	3	1/3	2/3	1/3	1/3	1/3	1/3
SOD 2	2	1	2	1	2	1	2	2	2	1
LTD 1	2	1/2	2	2	2	1/2	1/2	1/2	1/2	1/2
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 represents a null allele.

TABLE V. Site B (1986). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.502	0.381	0.527	0.596	0.539	0.521	0.690	0.350	0.756
2		-	0.304	0.758	0.828	0.637	0.728	0.839	0.496	0.608
3			-	0.644	0.804	0.689	0.689	0.816	0.463	0.768
4				-	0.538	0.447	0.538	0.512	0.453	0.384
5					-	0.336	0.087	0.139	0.328	0.322
6						-	0.423	0.454	0.749	0.533
7							-	0.125	0.239	0.276
8								-	0.340	0.243
9									-	0.479
10										-

[a]Null alleles included.

TABLE VI. Genotypes (site C, 1986)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	2	3	3	0	3	1/4	1/4	1/4	1/4	1/4
AE 2	0	1/2	1/2	1/2	1/2	1/2	2	1/2	1/2	1/2
AE 3	1/3	1/3	1/3	1/3	1/3	1/2	1/2	1/2	1/2	1/2
AE 4	2/4	2	2	2	2	1/5	1/5	1/5	1/5	1/5
AAP 1	1/3	1	1/3	1/3	1/2	1/2	1/3	1/3	1/2	1/3
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	0	0	2	1/2	2	1/2	1/2	1/2	1/2	1/2
BE-2 2	1	1/2	1/2	1	1/2	1	1	1	1	1
BE-2 3	1/2	2	1/2	1/2	1/2	2	2	2	2	2
BE-2 4	2	1	1	1	1	1/2	1/2	1/2	1/2	1/2
BE-2 5	0	1	1	1	1	1/2	1/2	1/2	1/2	1/2
BE-2 6	1/2	1	1/2	1/2	1/2	1	1	1	1	1
GDH 1	0	1	1	1	0	1	0	1	1	0
GDH 2	1/2	1	1	1/2	2	1/2	1	1	1	1/2
HK	2	2	1	1	2	1	1	2	1	1
B-HBDH	2	2	2	2	1/2	2	2	0	0	0
ICD	3	1/3	1/2	1/2	2/3	2	2	2	2	2
LDH	1/2	0	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
LAP	1/2	1	1/2	1/2	1	1/2	1/2	1/2	1/2	2
ME	1/2	1	1	1	1	1/2	1	1/2	1	1/2
PGM 1	1/2	1/2	1/2	1/2	1/2	1/2	1/2	0	1/2	1/2
6-PGD	1/2	2	1	1	1	1/2	1/2	0	0	0
PE 1	2/3	2	2	2	2	1/4	1/4	1/4	1/4	1/4
PE 2	3/5	2/5	1/5	1/5	1/5	4	2/4	4	4	4
PE 3	2/3	0	0	0	0	1/3	1/3	1/3	1/3	1/3
SOD 1	1/3	1/3	3	2/3	2/3	2/3	1/3	1/3	1/3	1/3
SOD 2	1/2	1/2	1	1	1	1	1/2	1/2	2	1
LTD 1	2	2	1/2	2	2	2	2	2	2	2
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 represents a null allele.

TABLE VII. Site C (1986). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.511	0.670	0.594	0.520	0.635	0.519	0.705	0.791	0.658
2		-	0.287	0.348	0.339	0.555	0.529	0.598	0.609	0.816
3			-	0.104	0.226	0.404	0.433	0.638	0.548	0.596
4				-	0.243	0.326	0.406	0.606	0.518	0.526
5					-	0.547	0.559	0.721	0.781	0.654
6						-	0.142	0.241	0.179	0.182
7							-	0.284	0.191	0.166
8								-	0.122	0.178
9									-	0.148
10										-

[a]Null alleles included.

TABLE VIII. Genotypes (site A, 1987)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	2/3	1	2/3	2/3	2/3	2/3	2/3	3	2/3	2
AE 2	1/2	1/2	1/2	2	2	2	2	1/2	1/2	1/2
AE 3	1	1	1	0	0	2	2	2	1	1/2
AE 4	1	1	1	1/2	1/2	1/2	1/2	1/2	1	1
AAP 1	1/3	1	1/3	1/2	1/2	1/2	1/3	1/2	1/3	1
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	0	0	2	1/2	1/2	1/2	0	0	1/2	1/2
BE-2 2	1/2	2	2	1/2	1/2	2	2	1/2	2	2
BE-2 3	1/2	1/2	1/2	2	2	1	2	1/2	1/2	2
BE-2 4	2	0	2	1/2	1/2	1/2	1	1/2	2	2
BE-2 5	1	1	1	1/2	1/2	1/2	0	1/2	1	1
BE-2 6	1	1	1	1	1	1/2	1	2	1	1
GDH 1	0	0	1	1	1	1	1	1	1	0
GDH 2	1	1/2	2	1	1	1	1/2	1	1/2	1/2
HK	2	2	2	1	1/2	1	2	2	2	2
B-HBDH	1	2	2	0	0	0	0	0	2	1/2
ICD	1	1/3	1	1	1	3	1/3	1	1	1
LDH	0	0	0	1/2	1/2	1/2	1/2	1	0	0
LAP	1/2	2	1/2	2	2	2	1/2	1/2	1/2	1/2
ME	1	1	1	1	1/2	1	1/2	1	1	1
PGM 1	0	1	0	1/2	1/2	1/2	1	1/2	0	0
PGM 2	1	1	1	1	1	1	1	1	1	1
6-PGD	0	1/2	1/2	1	1	1	0	1	0	1
PE 1	1/2	1	1/2	3	3	1/2	1/2	1/2	1/2	2
PE 2	1/3	1/3	1/3	2	2	2	2	2/3	1/3	1/3
PE 3	1/2	1/2	1/2	2/3	2/3	2/3	2/3	2/3	1/2	1/2
SOD 1	2	2	2/3	1/3	1/3	1/3	1/3	1/3	2	2/3
SOD 2	2	2	1/2	0	2	1/3	1/3	1/3	2	1/2
LTD 1	1/2	1/2	1/2	2	2	1/2	1/2	1/2	1/2	1/2
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzymes. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 represents a null allele.

TABLE IX. Site A (1987). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.281	0.266	0.770	0.661	0.832	0.640	0.578	0.147	0.197
2		-	0.315	0.813	0.702	0.735	0.661	0.659	0.298	0.326
3			-	0.641	0.579	0.628	0.619	0.556	0.078	0.151
4				-	0.067	0.262	0.444	0.372	0.672	0.592
5					-	0.299	0.389	0.350	0.570	0.533
6						-	0.345	0.246	0.682	0.618
7							-	0.303	0.590	0.630
8								-	0.607	0.548
9									-	0.187
10										-

[a]Null alleles included.

TABLE X. Genotypes (site B, 1987)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	1/2	2/3	2/3	3	1/3	2/3	2/3	2/3	1/2	1/2
AE 2	1/2	2	2	2	1/2	2	1/2	1/2	1/2	1/2
AE 3	1	2	1	0	2	2	1/2	1	1	1
AE 4	1	1/2	1/2	1/2	1	1/2	0	1/2	1	1
AAP 1	1	1/2	1/2	1	1/3	1/2	1	1/3	1	1/3
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	2	2	1/2	1/2	2	1/2	2	1	1/2	1/2
BE-2 2	2	2	1/2	1/2	1/2	1/2	1/2	1/2	2	2
BE-2 3	1/2	2	1	1	1/2	1	1/2	2	1/2	1/2
BE-2 4	0	1/2	1/2	1/2	0	1/2	1	1	1	1
BE-2 5	1	1/2	0	1/2	1	1/2	1	0	1	1
BE-2 6	1	1	1	1	1	1	1	1	1	1
GDH 1	0	0	1	1	1	1	1	1	0	1
GDH 2	2	1	1	1	1/2	1	1/2	1/2	1/2	1/2
HK	2	1/2	2	1/2	2	1/2	2	1/2	2	2
B-HBDH	2	1/2	0	0	2	2	2	0	2	2
ICD	2	1	1	1	1/3	1	1	0	1	0
LDH	0	1	1/2	1/2	0	1/2	1	1	0	0
LAP	1/2	0	2	1/2	1/2	1/2	1/2	0	1/2	1/2
ME	1	1/2	1	1	1	1	1	1/2	1	1
PGM 1	0	1/2	1/2	0	0	1/2	0	1/2	0	1
PGM 2	1	1	1	1	1	1	1	1	1	1
6-PGD	1	0	0	1/2	2	1	0	2	0	0
PE 1	1/2	1/2	1/2	2	1/2	2	1/2	1/2	1/2	1/2
PE 2	1/3	2	2	2	1/3	2	1/3	3	1/3	1/3
PE 3	1/3	1/2	1/2	2/3	1/3	2/3	1/2	2/3	1/2	1/2
SOD 1	2/3	1/3	1/3	1/3	2/3	1/3	2	1/3	2/3	2/3
SOD 2	2	1/3	1/3	1/3	2	3	2	1/3	2	2
LTD 1	1/2	2	2	1/2	1/2	1/2	1/2	1/2	0	1/2
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 represents a null allele.

TABLE XI. Site B (1987). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.692	0.747	0.671	0.225	0.639	0.374	0.818	0.205	0.291
2		-	0.352	0.457	0.629	0.314	0.461	0.537	0.507	0.579
3			-	0.248	0.640	0.265	0.438	0.405	0.518	0.444
4				-	0.474	0.185	0.444	0.500	0.581	0.640
5					-	0.463	0.273	0.682	0.313	0.291
6						-	0.433	0.586	0.589	0.511
7							-	0.618	0.236	0.270
8								-	0.714	0.457
9									-	0.186
10										-

[a] Null alleles included.

TABLE XII. Genotypes (site C, 1987)[a]

	Clonal isolates									
Loci	1	2	3	4	5	6	7	8	9	10
AE 1	3	2/3	2/3	2/3	1/3	2/3	2/3	2/3	1/2	2/3
AE 2	1/2	2	2	2	1/2	1/2	2	2	1/2	2
AE 3	1	1	1	0	1	1	1	1/2	1	2
AE 4	1	1	1/2	1/2	1	1	1/2	1	1	1
AAP 1	1	1	1/2	1/2	1/3	1/3	1/3	1/3	1/1	1
AAP 2	1	1	1	1	1	1	1	1	1	1
BE-2 1	2	0	1/2	1/2	0	0	1	0	2	1/2
BE-2 2	2	2	1/2	1/2	2	2	1/2	1/2	0	1/2
BE-2 3	1/2	2	1	2	1/2	1/2	1/2	2	1/2	2
BE-2 4	2	1	1/2	1/2	1	0	1	2	0	2
BE-2 5	1	1	0	1/2	1	1	0	1	1	1
BE-2 6	1	1	1	1	1	1	1	1	1	1
GDH 1	1	0	0	1	1	1	1	1	1	1
GDH 2	1/2	1	1	1	1	1/2	1/2	1/2	1/2	1/2
HK	2	2	1	1/2	2	2	1/2	2	2	2
B-HBDH	2	0	0	0	2	0	2	1	2	1/2
ICD	1	0	1	1/3	1	2	3	1	2	1
LDH	0	0	1/2	1/2	0	0	0	1	0	0
LAP	1/2	1/2	2	2	1/2	1/2	1/2	1/2	1/2	1/2
ME	1	1	1	1/2	1	1	1/2	1	1	1
PGM 1	1	0	1/2	1/2	1	1	1/2	0	1	0
PGM 2	1	1	1	1	1	1	1	1	1	1
6-PGD	1	0	0	1	0	0	2	0	0	0
PE 1	1/2	1/2	1/2	3	1/2	2	2	1	1/2	1/2
PE 2	1/3	1/3	2	2	1/3	1/3	1/3	3	1/3	3
PE 3	1/3	1/3	2/3	2/3	1/3	1/3	2/3	2/3	1/3	2/3
SOD 1	2	2/3	1/3	1/3	2/3	2/3	1/3	2/3	2/3	2/3
SOD 2	2	1/2	1/3	0	2	1/2	1/3	1/2	2	1/2
LTD 1	1/2	1/2	1/2	2	1/2	1/2	1/2	1/2	1/2	0
LTD 2	1	1	1	1	1	1	1	1	1	1

[a] Abbreviations represent the loci coding for the various enzymes, the numbers representing multiple forms of the enzyme. Alleles are numbered from the most anode to the most cathod. Homozygotes are designated by one and heterozygotes by two numbers; 0 represents a null allele.

TABLE XIII. Site C (1987). Nei's genetic distances.[a]

	Clonal isolates									
	1	2	3	4	5	6	7	8	9	10
1	-	0.416	0.621	0.571	0.178	0.298	0.487	0.380	0.243	0.294
2		-	0.475	0.629	0.241	0.243	0.522	0.322	0.431	0.351
3			-	0.394	0.543	0.570	0.434	0.562	0.693	0.620
4				-	0.650	0.660	0.534	0.570	0.773	0.589
5					-	0.184	0.398	0.316	0.210	0.345
6						-	0.456	0.379	0.174	0.411
7							-	0.638	0.491	0.520
8								-	0.459	0.158
9									-	0.395
10										-

[a] Null alleles included.

TABLE XIV. Statistics for genetic distance (1986) [a]

Site	Mean genetic distance ± SD
A	0.4213 ± 0.151
B	0.5084 ± 0.199
C	0.4522 ± 0.208

One-way ANOVA : (for site A, B and C)

	d.f.	M.S.
Among	2	0.0729
Within	132	0.0340
	F = 2.144 (NS)	

[a]Abbreviations : SD, standard deviation; ANOVA, analysis of variance; d.f., degree of freedom; M.S., mean square; F, F-test; NS, not significant at the 0.05 level.

TABLE XV. Statistics for genentic distance (1987) [a]

Site	Mean genetic distance ± SD
A	0.4764 ± 0.209
B	0.4644 ± 0.168
C	0.4406 ± 0.157

One-way ANOVA : (for site A, B and C)

	d.f.	M.S.
Among	2	0.0151
Within	132	0.0323

F = 0.4675 (NS)

[a]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.

TABLE XVI. Two-way ANOVA [a]

Source of variation	df	M.S.	F
Site	2	MS1=0.0452	MS1/MS4=1.3373 NS
Year	1	MS2=1.9818E-06	MS2/MS4=1 E-04 NS
Site x Year	2	MS3=0.0573	MS3/MS4=1.6953 NS
Experiment error	264	MS4=0.0338	

[a] Abbreviations: ANOVA, analysis of variance; df, degree of freedom; M.S., mean square; F, F-test; NS, not significant at the 0.05 level.

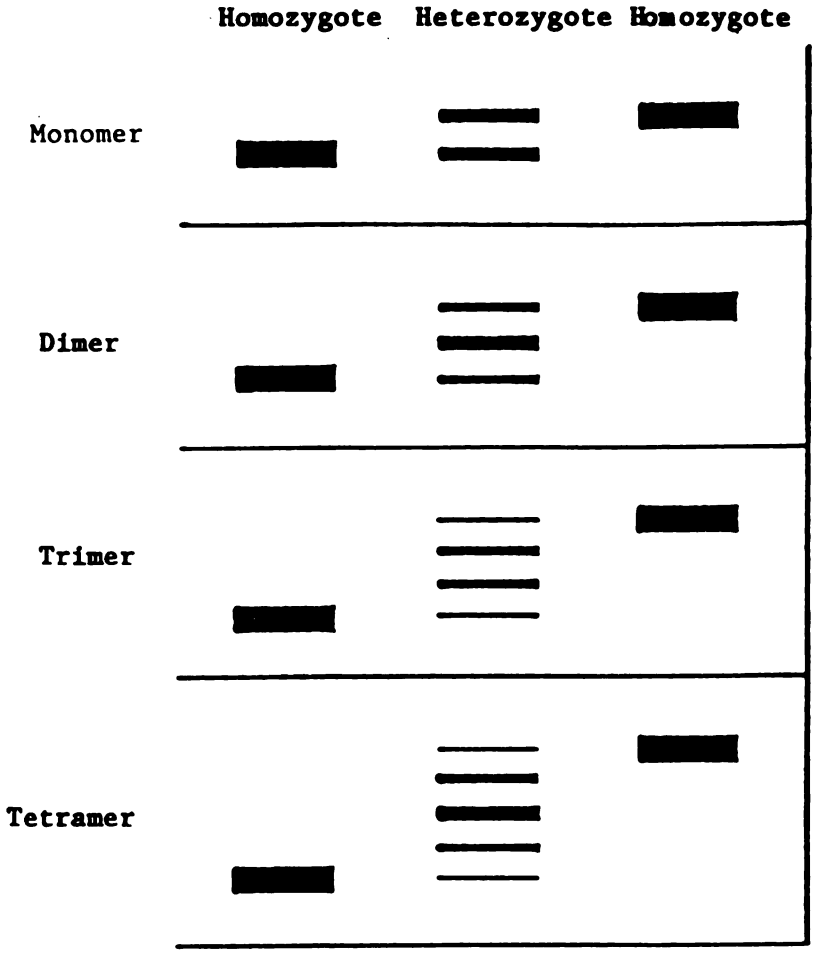

Fig.1. Diagram of characteristic isozyme patterns expected in heterozygotes in the case of enzymes which are monomers, dimers, tricmers or tetramers. The activities of the isozymes formed in heterozygotes are 1:1 for monomers, 1:2:1 for dimers, 1:3:3:1 for trimers, 1:4:6:4:1 for tetramers and so on.

(from ref. 10)

APPENDIX

The Nei's standard genetic distance between two populations X and Y is : $D = -\text{Log}_e I$, where I is the noramlized identity of genes between X and Y with repect to all loci, $I = Jxy / JxJy$. Let x_i and y_i be the frequencies of the i^{th} alleles in X and Y, respectively. The probability of identity of two randomly chosen genes is $j_x = \sum x_i^2$ in population X, while it is $j_y = \sum y_i^2$ in population Y. The probability of identity of two genes, chosen at random, one from each of the populations, is $j_{xy} = \sum x_i y_i$; then designate Jx, Jy and Jxy the arithmetric means if j_x, j_y and j_{xy} over all loci, including monomorphic ones, respectively. If we have the gene frequencies from both populations, the genetic distance between these two populations can be calculated. If populations X and Y have the same genefrequencies, then I = 1, D = 0; if X and Y don't have the same gene frequencies, then I = 0, $D = \infty$.