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Genetic Studies of Cave and Spring Populations of the Freshwater Amphipod Crustacean *Gammarus Minus* Say

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GENETIC STUDIES OF CAVE AND SPRING POPULATIONS OF THE FRESHWATER AMPHIPOD CRUSTACEAN GAMMARUS MINUS SAY

by

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ABSTRACT

The amphipod Gammarus minus has three morphotypes in its Appalachian range : (1) a spring form with well developed eyes, (2) an intermediate cave form with slightly reduced eyes, and (3) an extreme cave form with greatly reduced eyes. This study compares the genetic structure of populations in two karst areas and analyzes the genetics of populations of the three morphotypes.

Genetic data were collected from populations in the Ward's Cove, Virginia and Greenbrier Valley, West Virginia karst areas using zone electrophoresis zymograms. One hundred percent of the Ward's Cove populations are polymorphic at both MDH-1 and PEP 1 loci with alleles and allelic frequencies nearly identical. Polymorphism is low in most of the Greenbrier Valley populations. The allele frequencies are also nearly identical.

The well integrated subterranean drainage systems of both the Greenbrier Valley and Ward's Cove karst areas favor intermittent gene flow among populations. Gene flow and random drift are probably responsible for the low levels of genetic variability. The genetic homogeneity seen in populations in certain drainage basins within these two karst areas is probably due primarily to gene flow.

No consistent genetic differences were found between the three morphotypes. A preliminary study of the chromosomal make-up also indicates no differences (2N= 53-54). This indicates that photoreceptor regression has not been accompanied by a major reorganization of the genome. Also, based on these data there is nothing to warrant the division of the species into separate taxa.

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INTRODUCTION

Cavernicoles have long been a curiousity to the evolutionary biologist because of their often bizarre adaptations to the cave environment. Knowledge of evolutionary processes involved in loss or degeneration of eyes and loss of pigmentation, increase in size and complexity of tactile sensory organs and other changes in cave organisms is still limited. Barr (1968) reviewed 12 hypotheses proposed to explain regressive evolution in cavernicoles. For other reviews see Vandel (1964), Poulson (1965), Greenwood (1967), and Poulson and White (1969). As Avise and Selander (1972) point out, little is known of the genic character of population genetics of cave organisms. What little is known concerns primarily vertebrates (see Kosswig, 1965; Sadoglu, 1956; Sadoglu, 1957; Sadoglu, 1967; Wilkins, 1971). An understanding of the gene pool is essential, however, in developing a cohesive theory of their evolution. The question arises how has a cave existence affected the genetic variability of troglobites (obligatory cavernicoles) compared to epigean (surface-dwelling) forms.

According to Barr (1968) many troglobites have evolved from troglophilic (facultative cavernicoles) ancestors which entered caves before the end of the Pleistocene. The cave populations became isolated concomitant with local extinction of the surface populations due to the effects of climatic changes brought on by glaciation. In order to compare accurately genetic variability, the troglobite and epigean populations should be of the same species, but under the strict definition of a troglobite this can never be the case. Since many troglobites have evolved from troglophilic ancestors, one way to determine the degree of change in genetic variability that might occur in the evolution of troglobites would be to study a troglophilic organism. The present study examines the population genetics of a morphologically variable troglophilic amphipod, having both surface and cave-form populations. Considering that the cave environment is relatively stable and uniform temporally and spatially, one might expect, according to both the gene flow and niche width hypotheses, that cave organisms would have low levels of genetic variability.

Also, genetic drift and inbreeding would produce low variability. But as Avise and Selander (1972) point out, assuming few organisms are able to adapt to cave life, the relatively depauperate nature of cave faunas should allow troglobites to expand their niches and utilize resources that under normal circumstances would be utilized by two or more species (ecological release). High levels of genetic variability would be expected under these circumstances.

The gammarid amphipod Gammarus minus Say is a common crustacean found in springs, small spring fed streams, and cave streams. This species is most often encountered in springs located in limestone areas of the eastern and middle-eastern United States. Say (1818) gave the first description of the species, and Shoemaker (1940), Hubricht (1943), Bousfield (1958), Minckley and Cole (1963), and Holsinger and Culver (1970) have also treated the species taxonomically. Holsinger and Culver (1970) divided G. minus into three morphological groups that correspond to habitat. The three morphotypes are: a brown spring form with well developed eyes and short antennae, Form III; a bluish intermediate cave form with slightly reduced eyes and long antennae, Form II; and a large, bluish-bodied cave form with degenerate eyes and long antennae, Form I. (See figures 1-3) Form I is restricted to mostly large cave systems in two disjunct areas: the Great Savannah karst of Greenbrier County, West Virginia and the Ward's Cove karst of Tazewell County, Virginia, about 75 miles to the southwest. Form II amphipods occur generally in small, semi-isolated caves over the Appalachian range of the species. Form III amphipods occur over the entire range of the species, being present from Pennsylvania southwestward to Georgia and west to the Ozarks.

Nothing is known about genetic or morphogenetic processes in the eye development of G. minus. Eye mutants are known, however, in Gammarus chevreuxi (Ford and Huxley, 1927); Wolsky and Huxley (1934) studied eye morphogenesis in this species.

Electrophoretic and histochemical staining techniques were chosen as the research tool primarily because these methods can provide considerable genetic information based upon protein variation. These techniques are quite useful when examining the population genetics of an organism like Gammarus minus

FIGURE 1 FORM III- Body and Eye Morphology

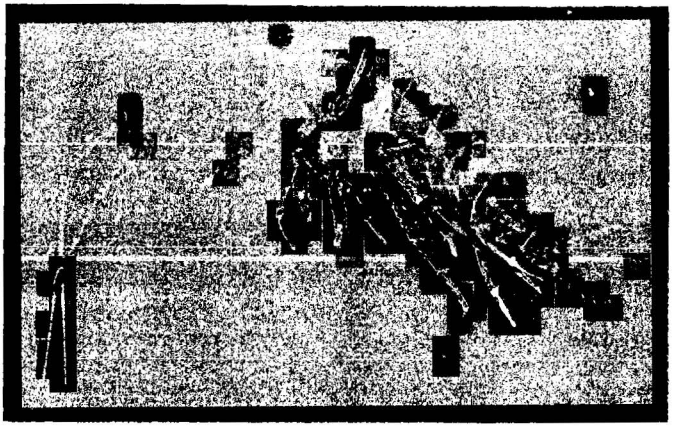


FIGURE 2 FORM II- Body and Eye Morphology

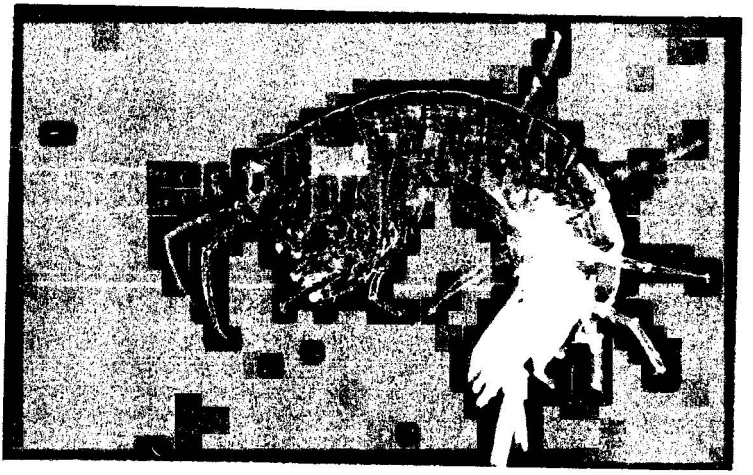


FIGURE 3 FORM I- Body and Eye Morphology

which is difficult to breed in the laboratory. This is especially true when compared to classical Mendelian experimentation. Electrophoretic and histochemical staining techniques have been used by other workers for detection of genetic variants at individual gene loci for over a decade, and especially since the work of Shaw (1965), Hubby and Lewontin (1966), and Lewontin and Hubby (1966). Using electrophoretic analysis, one can establish: (1) the number of loci associated with a specific protein system in a population and how many alleles are present at a locus, (2) the frequency of specific alleles in a population, and (3) whether the allelic frequency is in Hardy-Weinberg equilibrium.

The genetic bases of the allozymic polymorphisms demonstrated on the gels were not checked directly by progeny studies. This was not possible because of the length of the life cycle of G. minus and the difficulty of maintaining it alive in the laboratory. Genetic interpretation of observed variation in simple Mendelian terms is supported, however, by the fact that most of the proteins exhibit banding patterns similar to those described in other invertebrates and vertebrates in which crosses have been performed (see Wright, 1963; Hubby, 1963; Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; Yarbrough and Kojima, 1967; and Gaines and Krebs, 1971). As with other organisms difficult or impossible to cross under laboratory conditions, the interpretation of the genotypes is based on electrophoretic banding patterns (Gooch and Schopf, 1970; Selander, et al., 1970).

The objectives of this study were to determine: (1) the degree of genetic differentiation between the three morphotypes of G. minus within a geographic area; (2) The effects of geology and hydrology on possible gene dispersal patterns by comparing the allelic frequencies at polymorphic loci in populations throughout a large geographic area and also between two discontinuous limestone areas; and (3) whether cave form populations are less variable genetically than surface populations.

METHODS AND MATERIALS

Collections of the three morphotypes were made during the winter of 1970-71 and 1971-72, and the summer of 1973. Six distinct geographic areas separated by partial barriers to migration were sampled. From northeast to southwest, they are: (1) northern Pocahontas County, West Virginia (abbreviated NPC)--two collections, two form III; (2) southern Pocahontas County, West Virginia (SPC)-- three collections, two form II and one form III; (3) Great Savannah karst area, Greenbrier County, West Virginia (GSK)--12 collections, five form I, four form II, and three form III; (4) the area south of the Greenbrier River in Greenbrier and Monroe counties (SGR)-- four collections, one form I, one form II, and two form III; (5) Ordovician limestone belt, Monroe County, West Virginia (OLB)--three collections, three form III; (6) Ward's Cove karst area, Tazewell County, Virginia (WCK)--three collections, two form I, and one form III.

Collections were made at various points along cave streams and in springs using a Surber sampler. Generally, in all populations, larger, more conspicuous adult males were sampled more frequently than females. This was assumed to have no effect on the sample since comparisons by Gooch (1970) and Gooch (unpublished) of electrophoretic banding patterns in males and females of G. minus yielded no qualitative differences in allele frequencies of Malate dehydrogenase locus.

Vertical polyacrylamide and horizontal starch electrophoresis and histochemical staining were utilized in characterizing gene loci from six protein systems in individuals of G. minus, representing nine cave populations (six form I and three form II) and four surface populations (form III). Protein systems studied were: general protein (GP), "leucine" aminopeptidase (LAP) peptidase (PEP), esterases (EST), tetrazolium oxidase (TO), malate dehydrogenase (MDH), and phosphoglucose isomerase (PGI). At the polymorphic loci Malate dehydrogenase and Peptidase, 27 populations representing 15 cave populations (eight form I and seven form II) and 12 surface populations (form III) were sampled. The amphipods were immediately processed or frozen whole and stored at -60°C until electrophoresis could be carried out. Those processed

immediately were kept in plastic containers of spring or cave water at 10 ± 2°C. Those not processed immediately were removed from water and placed in air tight plastic bags, then sealed and stored at -60°C. Periods of storage were generally less than three weeks. This freezing procedure caused no obvious decrease in enzyme activity in this study. Most proteins do not denature in intact organs when stored in this fashion (Selander, et al., 1971). Extracts for electrophoresis were prepared by triturating the whole amphipod with a glass rod in a 1 ml polystyrene centrifuge tube in 100 ul cold 0.1 M tris-glycine buffer, pH 8.5 or distilled water (a 25 per cent sucrose solution was also used when utilizing polyacrylamide gel). The homogenate was centrifuged at 7,000 rpms for 15 minutes, care being taken not to warm the homogenate.

Polyacrylamide Electrophoresis technique

After centrifugation, aliquots of 15 ul of supernatant were pipetted into slots in 6 per cent polyacrylamide gel (apparatus of E.C. Corp., Philadelphia). Gels were run vertically for three hours at 75-125 mA and 350-400 v at 5°C. Polyacrylamide staining procedures follow Gooch and Schopf (1970) and Gooch, et al. (1972).

Starch Gel Electrophoresis techniques

Samples of supernatant were absorbed on 6 x 6 mm pieces of Whatman No. 3 filter paper, blotted to remove excess liquid, and inserted into a slit cut in gel. Electrostarch (12.5%), Lots 88 and 171 (Otto Hiller, Madison, Wisconsin), was prepared and poured in 9 x 190 x 210 mm lucite molds. The gel was cooled during electrophoresis by a tray of ice supported above the gel-mold by a glass plate. The gel was cut into 3 mm slices following electrophoresis and incubated in appropriate staining solutions. Electrophoresis procedures follow Selander, et al. (1971), primarily using continuous tris citrate buffer I, adjusted to 7.0. Gel staining followed Shaw and Prasad (1970). All protein systems scored migrated anodially.

The number of gene loci and the number of alleles per locus were determined from the pattern of enzymatically active bands using the criteria outlined in Gooch and Schopf (1970). A discrete protein zone is representative of a single locus if variation within it appears independent of other zones on the gel. Gene loci are numbered sequentially to correspond to these zones, from most cathodic to most anodic. Each allele at a locus produces a single enzyme band of characteristic mobility. They are also designated sequentially, the bands of highest mobility serving as the standard. Thus GP-$2^{1.00}$ is the reference allele for the general protein locus whose protein band system migrates second from the most cathodic system. This nomenclature has the added advantage that new alleles can be added easily; a newly-discovered high mobility allele might become GP-$2^{1.15}$.

Cytogenetic Techniques

Populations of each of the three morphotypes were sampled periodically between January, 1973 and October, 1974 using a Surber sampler. Form I collections were from Organ Cave and Fallen Rock Cave, while Form II collections came from Higginbotham Cave No. 1. Form III came from Taylor's Spring (the insurgence of Bone-Norman Cave) and a spring along the Greenbrier River which is the resurgence of General Davis Cave. Individuals were transported back to the laboratory in large plastic containers of spring or cave water and transferred to glass containers immediately upon return. They were maintained at 10°C until examined.

Embryos were removed from the brood pouches of ovigerous females. The embryos were transferred to distilled water for about 30 minutes, stained in aceto orcein for 15-30 minutes, teased apart and squashed. Cells examined were primarily from embryos developed up to and including the blastula stage because of larger cell sizes. Chromosome numbers for each morphotype were determined and compared using both phase contrast and bright field microscopy.

RESULTS

Electrophoretic Results

By electrophoresis of seven protein systems, 13 loci are identifiable. Eight are fixed for the same allele in all populations regardless of morphotypes and geographic area and five are variable. The genetic construction of one of the seven proteins, although variable, cannot be evaluated and, therefore, its importance in the overall scheme cannot be determined. The body of the paper, then, refers to the genetics of only six proteins. Allelic frequencies for all 13 populations surveyed for these six protein systems are presented in Table 1. Following are the descriptions of the proteins analyzed.

Malate Dehydrogenase - NAD-dependent malate dehydrogenase is the product of the MDH-1 locus. This locus is segregating for five alleles--MDH-$1^{0.75}$, MDH-$1^{0.87}$, MDH-$1^{0.94}$, MDH-$1^{1.00}$, and MDH-$1^{1.09}$. Homozygotes at the MDH-1 locus appear as one darkly-stained band on the electrophoresis gels. Heterozygotes have two dark bands with an intermediate light band. Presence of this intermediate band denotes MDH polypeptides probably associated in dimers. The MDH-1 locus is polymorphic. Additional populations (27 populations total) were surveyed at this locus in order to gain a better insight into the allelic distributions in various geographic areas. These data are presented in Table 2. Overall, the predominant allele is MDH-$1^{0.87}$. It is fixed in NPC, SPC, all but one population in GSK (General Davis Cave), and is fixed, or nearly so, in most SGR populations. MDH-$1^{0.75}$ is the predominant allele only in OLB. The dominant allele in WCK area is MDH-$1^{0.94}$, while both MDH-$1^{1.00}$ and MDH-$1^{1.09}$ are encountered, but in low frequency. MDH-$1^{1.00}$ is in high frequency only in Burnside Branch Cave (SGR).

Peptidase - Three loci were identified, one of which is segregating for 4 alleles, PEP-$1^{0.74}$, PEP-$1^{0.78}$, PEP-$1^{1.00}$, and PEP-$1^{1.09}$. PEP-2 and PEP-3 each have one allele which is fixed in all populations. Homozygotes at PEP-1 appear as one dark band. Heterozygotes have two dark bands and an

Table 1 Allele frequencies at seven protein loci in Gammarus minus

Protein locus and allele	FORM I						FORM II			FORM III			
	Organ Cave	The Hole	McClung's Cave	Ludington Cave Spring	Benedict's Cave	Fallen Rock Cave	Martha's Cave	Higginbotham's Cave	Coffman Cave	Lobelia Spring	Route 219 Spring	Fort Spring	Zenith Spring
General Protein-1													
GP-1$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
General Protein-2													
GP-2$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
General Protein-3													
GP-3$^{0.94}$	1.00		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
GP-3$^{1.00}$		1.00											
General Protein-4													
GP-4$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leucine Aminopeptidase-1													
LAP-1$^{0.98}$											1.00		
LAP-1$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00		1.00	1.00
Leucine Aminopeptidase-2													
LAP-2$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Peptidase-1													
PEP-1$^{0.74}$						0.97					0.04	0.17	
PEP-1$^{0.78}$	0.11	1.00	0.96	1.00	1.00	0.03	0.06	1.00	1.00		0.96	0.83	1.00
PEP-1$^{1.00}$	0.89		0.04				0.94			1.00			
PEP-1$^{1.09}$													
Peptidase-2													
PEP-2$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Peptidase-3													
PEP-3$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Esterase													
Est-1$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tetrazolium Oxidase													
TO-1$^{1.00}$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Malate Dehydrogenase													
MDH-1$^{0.75}$													0.80
MDH-1$^{0.87}$	1.00	1.00	1.00	1.00	1.00		1.00	1.00	1.00	1.00	1.00	1.00	0.20
MDH-1$^{0.94}$						0.87							
MDH-1$^{1.00}$						0.01							
MDH-1$^{1.09}$						0.12							
Phosphoglucose Isomerase													
*PGI-1													

* The PGI-1 locus is widely polymorphic, but bands are unresolved for some localities

Table 2 Allele frequencies at Malate dehydrogenase (MDH-1) and Peptidase (PEP-1) loci at sampled localities grouped by area

Locality	Morphotype	Sample size	MALATE DEHYDROGENASE-1 LOCUS					Sample size	PEPTIDASE-1 LOCUS			
			$MDH\text{-}1^{0.75}$	$MDH\text{-}1^{0.87}$	$MDH\text{-}1^{0.94}$	$MDH\text{-}1^{1.00}$	$MDH\text{-}1^{1.09}$		$PEP\text{-}1^{0.74}$	$PEP\text{-}1^{0.78}$	$PEP\text{-}1^{1.00}$	$PEP\text{-}1^{1.09}$
Northern Pocahontas County												
1 Linwood Spring	III	5	0	1.00	0	0	0	5	0	1.00	0	0
2 Tub Spring	III	5	0	1.00	0	0	0	6	0	1.00	0	0
Southern Pocahontas County												
3 Martha's Cave	II	8	0	1.00	0	0	0	8	0	0.06	0.94	0
4 Lobelia Spring	III	8	0	1.00	0	0	0	8	0	0	1.00	0
5 Marten's Cave	II	5	0	1.00	0	0	0	5	0	0	1.00	0
Great Savannah Karst												
6 Bone-Norman Cave	II	7	0	1.00	0	0	0	7	0	0.14	0.86	0
7 Route 219 Spring	III	12	0	1.00	0	0	0	12	0.04	0.96	0	0
8 Buckeye Creek Cave	II	7	0	1.00	0	0	0	7	0	1.00	0	0
9 Spring Creek Spring	III	5	0	1.00	0	0	0	5	0	0.60	0.40	0
10 The Hole	I	14	0	1.00	0	0	0	6	0	1.00	0	0
11 Higginbotham's Cave	II	18	0	1.00	0	0	0	10	0	1.00	0	0
12 Coffman Cave	II	10	0	1.00	0	0	0	10	0	1.00	0	0
13 Ludington Cave Spring	I	7	0	1.00	0	0	0	7	0	1.00	0	0
14 McClung's Cave	I	22	0	1.00	0	0	0	14	0	0.96	0.04	0
15 Benedict's Cave	I	23	0	1.00	0	0	0	12	0	1.00	0	0
16 Fort Spring	III	10	0	1.00	0	0	0	3	0.17	0.83	0	0
17 General Davis Cave	I	8	0	0.75	0.25	0	0	8	0.50	0.50	0	0
South, Greenbrier River												
18 Organ Cave	I	34	0	1.00	0	0	0	28	0	0.11	0.89	0
19 Dixon's Spring	III	7	0.07	0.93	0	0	0	7	0	1.00	0	0
20 Spring South of Second Creek	III	7	0	1.00	0	0	0	7	0	1.00	0	0
21 Burnside Branch Cave	II	10	0	0	0.10	0.90	0	10	1.00	0	0	0
Ordovician Limestone Belt												
22 Zenith Spring	III	5	0.80	0.20	0	0	0	5	0	1.00	0	0
23 Patton's Cave Spring	III	7	1.00	0	0	0	0	7	0	0	0.57	0.43
24 Kitchen Creek Spring	III	7	0.21	0.79	0	0	0	7	0	0.86	0.14	0
Ward's Cove Karst												
25 Fallen Rock Cave	I	34	0	0	0.87	0.01	0.12	34	0.97	0.03	0	0
26 Hugh Young Cave	I	11	0	0	0.86	0.09	0.05	11	0.86	0.14	0	0
27 Maiden Spring	III	37	0	0	0.92	0.08	0	39	0.94	0.06	0	0

intermediate lighter stained band, indicating peptidase polypeptides may also associate as dimers. PEP-$I^{0.74}$ is the predominant allele only in WCK populations and Burnside Branch Cave of SGR. Overall, PEP-$I^{0.78}$ is the dominant allele and is fixed or common in most populations of NPC, GSK, SGR and OLB. PEP-$I^{1.00}$ is predominant only in SPC, though it is of high frequency in Organ Cave in SGR and common in OLB. Only Patton's Cave Spring (OLB) population contain PEP-$I^{1.09}$. Peptidase like malate dehydrogenase was surveyed at a total of 27 populations because of its polymorphic nature. See Table 2 for allelic frequencies.

Esterase - The esterase system contains only one useable locus, even though the esterase system contains numerous banding zones and considerable interpopulation and intrapopulation variation occurs. This is similar to the results obtained by Gooch (1970). EST-1 locus has one allele EST-$I^{1.00}$ which is fixed in all populations sampled. Further genetic interpretation of this protein system awaits breeding experiments so progeny can be scored and compared with parental electrophoretic patterns.

Leucine Aminopeptidase - LAP banding shows two loci are present, LAP-1 and LAP-2. LAP-2 has one allele which is fixed in all populations; LAP-1 has two alleles, LAP-$I^{0.98}$ and LAP-$I^{1.00}$. Route 219 Spring (Form III) is monomorphic for LAP-$I^{0.98}$, whereas all other populations sampled are monomorphic for LAP-$I^{1.00}$.

Tetrazolium Oxidase - A one-protein band system occurs on photocatalized tetrazolium-stained gels. Gels stained for this protein become darkly pigmented while bands appear light or colorless. The single locus is monomorphic for one allele TO-$I^{1.00}$ which is fixed in all populations sampled.

General Protein - A number of faint and poorly defined bands occur in gels stained for non-specific, general protein. There are four loci: GP-1, GP-2. GP-3 and GP-4. Two alleles have been identified at GP-3 locus, GP-$3^{0.94}$ and GP-$3^{1.00}$. Form I population from The Hole is fixed for allele GP-$3^{1.00}$; all other populations are fixed for GP-$3^{0.94}$.

Phosphoglucose Isomerase - One phophoglucose isomerase locus is found in gels stained for this protein. The phosphoglucose isomerase locus PGI-1 is segregating at most localities, but bands are too closely spaced and poorly resolved in a variety of buffers for accurate genetic comparisons. The polymorphic nature of this locus would raise estimates of genetic variability, but its importance and interpretation also await breeding comparison studies.

Allele and genotype frequencies were surveyed for MDH-1 and PEP-1 loci at 27 populations over six geographic areas. Genotype frequencies were ascertainable directly from banding patterns, and from these allelic frequencies (see Tables 3 and 4 for genotype distributions and Table 2 for allelic frequencies). While MDH-1$^{0.87}$ is practically fixed in NPC, SPC and GSK, it varies in frequency from 0 to 1.00 in SGR, from 0 to 0.79 in OLB, and is completely absent in WCK. Peptidase, however, is more variable. PEP-1$^{0.78}$ is fixed in NPC populations but nearly absent in SPC. In GSK populations PEP-1$^{0.78}$ varies from 0.14 to 1.00, but in the majority of these populations the frequency is fixed or nearly so. In SGR and OLB populations PEP-1$^{0.78}$ varies from 0 to 1.00. The frequency range is much narrower in WCK populations--0.03 to 0.14.

One of the problems in population genetics has been accurately quantifying genetic differences between two populations (Lewontin, 1967). The use of indices of genotypic identity improves the determination of the degree of genetic differentiation. Hedrick (1971) and Nei (1972) use genotypic frequencies to measure genetic differentiation. Application of the index of probability of genotypic identity (Hedrick, 1971) to Gammarus minus data is presented for MDH-1 and PEP-1 in Tables 5 and 6, respectively. The index is determined by the following formula:

$$I_{x \cdot y} = \frac{\sum_{j=1}^{n} P_{j \cdot x} \; P_{j \cdot y}}{\frac{1}{2} \left(\sum_{j=1}^{n} P^2_{j \cdot x} + n \, P^2_{j \cdot y} \right)}$$

Table 3 Genotype distribution for Malate Dehydrogenase locus in Gammarus minus

Locality	Sample size	Genotype distribution								
		0.75/0.75	0.75/0.87	0.87/0.87	0.87/0.94	0.94/0.94	0.94/1.00	0.94/1.09	1.00/1.00	1.09/1.09
Northern Pocahontas County										
Linwood Spring	5	0	0	5	0	0	0	0	0	0
Tub Spring	5	0	0	5	0	0	0	0	0	0
Southern Pocahontas County										
Martha's Cave	8	0	0	8	0	0	0	0	0	0
Lobelia Spring	8	0	0	8	0	0	0	0	0	0
Marten's Cave	5	0	0	5	0	0	0	0	0	0
Great Savannah Karst										
Bone-Norman Cave	7	0	0	7	0	0	0	0	0	0
Route 219 Spring	12	0	0	12	0	0	0	0	0	0
Buckeye Creek Cave	7	0	0	7	0	0	0	0	0	0
Spring Creek Spring	5	0	0	5	0	0	0	0	0	0
The Hole	14	0	0	14	0	0	0	0	0	0
Higginbotham's Cave	18	0	0	18	0	0	0	0	0	0
Coffman's Cave	10	0	0	10	0	0	0	0	0	0
Ludington Cave Spring	7	0	0	7	0	0	0	0	0	0
McClung's Cave	22	0	0	22	0	0	0	0	0	0
Benedict's Cave	23	0	0	23	0	0	0	0	0	0
Fort Spring	10	0	0	10	0	0	0	0	0	0
General Davis Cave	8	0	0	6	1	1	0	0	0	0
South, Greenbrier River										
Organ Cave	34	0	0	34	0	0	0	0	0	0
Dixon's Spring	7	0	1	6	0	0	0	0	0	0
Spring South of Second Creek	7	0	0	7	0	0	0	0	0	0
Burnside Branch Cave	10	0	0	0	2	8	0	0	0	0
Ordovician Limestone Belt										
Zenith Spring	5	3	2	0	0	0	0	0	0	0
Patton's Cave Spring	7	7	0	0	0	0	0	0	0	0
Kitchen Creek Spring	7	0	3	4	0	0	0	0	0	0
Ward's Cove Karst										
Fallen Rock Cave	34	0	0	0	0	26	1	6	0	1
Hugh Young Cave	11	0	0	0	0	8	2	1	0	0
Maiden Spring	37	0	0	0	0	33	2	0	2	0

Table 4 Genotype distribution for Peptidase locus in Gammarus minus

Locality	Sample size	0.74/0.74	0.74/0.78	0.78/0.78	0.78/1.00	1.00/1.00	1.00/1.09	1.09/1.09
Northern Pocahontas County								
Linwood Spring	5	0	0	5	0	0	0	0
Tub Spring	6	0	0	6	0	0	0	0
Southern Pocahontas County								
Martha's Cave	8	0	0	0	1	7	0	0
Lobelia Spring	8	0	0	0	0	8	0	0
Marten's Cave	5	0	0	0	0	5	0	0
Great Savannah Karst								
Bone-Norman Cave	7	0	0	0	2	5	0	0
Route 219 Spring	12	0	1	11	0	0	0	0
Buckeye Creek Cave	7	0	0	7	0	0	0	0
Spring Creek Spring	5	0	0	2	2	1	0	0
The Hole	6	0	0	6	0	0	0	0
Higginbotham's Cave	10	0	0	10	0	0	0	0
Coffman Cave	10	0	0	10	0	0	0	0
Ludington Cave Spring	7	0	0	7	0	0	0	0
McClung's Cave	14	0	0	13	1	0	0	0
Benedict's Cave	12	0	0	12	0	0	0	0
Fort Spring	3	0	1	2	0	0	0	0
General Davis Cave	8	2	4	2	0	0	0	0
South, Greenbrier River								
Organ Cave	28	0	0	0	5	23	0	0
Dixon's Spring	7	0	0	7	0	0	0	0
Spring South of Second Creek	7	0	0	7	0	0	0	0
Burnside Branch Cave	10	10	0	0	0	0	0	0
Ordovician Limestone Belt								
Zenith Spring	5	0	0	5	0	0	0	0
Patton's Cave Spring	7	0	0	0	0	2	4	1
Kitchen Creek Spring	7	0	0	5	2	0	0	0
Ward's Cove Karst								
Fallen Rock Cave	34	32	2	0	0	0	0	0
Hugh Young Cave	11	8	3	0	0	0	0	0
Maiden Spring	39	36	1	2	0	0	0	0

Table 5 Indices of probability of genotypic identity for MDH-1 locus of 27 populations of Gammarus minus

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
2			1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
3				1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
4					1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
5						1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
6							1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
7								1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
8									1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
9										1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
10											1.00	1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
11												1.00	1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
12													1.00	1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
13														1.00	1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
14															1.00	1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
15																1.00	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
16																	0.94	1.00	0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
17																		0.94	0.95	0.94	0.20	0.00	0.00	0.78	0.16	0.16	0.16
18																			0.98	1.00	0.00	0.00	0.00	0.76	0.00	0.00	0.00
19																				0.98	0.00	0.09	0.00	0.87	0.00	0.00	0.00
20																					0.00	0.00	0.00	0.76	0.00	0.00	0.00
21																						0.00	0.00	0.00	0.94	0.93	0.96
22																							0.76	0.33	0.00	0.00	0.00
23																								0.00	0.00	0.00	0.00
24																									0.00	0.00	0.00
25																										0.97	0.96
26																											0.96
27																											

Table 6 Indices of probability of genotypic identity for PEP-1 locus of 27 populations of Gammarus minus

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1		1.00	0.00	0.00	0.00	0.00	0.99	1.00	0.59	1.00	1.00	1.00	1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
2			0.00	0.00	0.00	0.00	0.99	1.00	0.59	1.00	1.00	1.00	1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
3				0.98	0.98	0.96	0.00	0.00	0.36	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.99	0.00	0.00	0.00	0.00	0.41	0.05	0.00	0.00	0.00
4					1.00	0.90	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.96	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00
5						0.90	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.96	0.00	0.00	0.00	0.00	0.40	0.00	0.00	0.00	0.00
6							0.00	0.00	0.54	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.98	0.00	0.00	0.00	0.00	0.40	0.14	0.00	0.00	0.00
7								0.99	0.61	0.99	0.99	0.99	0.99	0.99	0.99	0.91	0.44	0.00	0.99	0.99	0.00	0.00	0.40	0.91	0.01	0.03	0.06
8									0.59	1.00	1.00	1.00	1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	0.00	1.00	0.90	0.00	0.00	0.05
9										0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.27	0.44	0.59	0.59	0.00	0.59	0.14	0.84	0.00	0.00	0.03
10											1.00	1.00	1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
11												1.00	1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
12													1.00	1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
13														1.00	0.99	0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
14															0.99	0.87	0.37	0.02	0.99	0.99	0.00	0.99	0.00	0.94	0.00	0.00	0.05
15																0.86	0.36	0.00	1.00	1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
16																	0.72	0.00	0.86	0.86	0.00	0.86	0.00	0.83	0.03	0.16	0.06
17																		0.00	0.36	0.36	0.36	0.36	0.00	0.37	0.42	0.65	0.42
18																			0.00	0.00	0.00	0.00	0.41	0.08	0.00	0.00	0.00
19																				1.00	0.00	1.00	0.00	0.90	0.00	0.00	0.05
20																					0.00	1.00	0.00	0.90	0.00	0.00	0.05
21																						0.00	0.00	0.00	0.99	0.90	0.99
22																							0.00	0.90	0.00	0.00	0.05
23																								0.00	0.00	0.00	0.05
24																									0.00	0.00	0.05
25																										0.94	0.99
26																											0.93
27																											

Pj . x and Pj . y represent the recorded frequencies of the j^{th} genotype in populations x and y, and n is the number of genotypes at the locus. The probability of drawing identical genotypes from two populations is represented by the numerator, while the denominator is the average probability of drawing identical genotypes from the same population on two successive independent draws. The indices range in value from 0.00 to 1.00 in which 1.00 indicates the populations compared are genetically identical, while 0.00 indicates they are completely dissimilar. Thus a gradation of genetic similarity is presented. The numbers from 1 to 27 in Tables 5 and 6 represent the 27 population in which MDH-1 and PEP-1 were analyzed. These numbers correspond to the populations in Table 2. Thus, 1 represents Linwood Spring, 2 represents Tub Spring, 3 represents Martha's Cave, etc. These numbers are also grouped by geographic area for easy inter- and intra-area comparisons.

An examination of all data elicits a number of trends. First, in populations surveyed at all six protein systems, most show low levels of genetic variability. None of the 12 loci are polymorphic in six populations consisting of three Form I, two Form II, and one Form III. In six other populations, one of either MDH-1 or PEP-1 is polymorphic (eight percent polymorphism). MDH-1 is polymorphic in one Form III population, while PEP-1 is polymorphic in two Form I, one Form II, and two Form III populations. Fallen Rock Cave (Form I) in WCK is the only population to have two of the 12 loci polymorphic - both MDH-1 and PEP-1 (18 percent polymorphism). See Table 1.

Second, the amount of genetic variability is similar in all morphotypes. Spring populations exhibit no more genetic variability than Form I cave populations. Although the Form I population of Fallen Rock Cave (WCK) has the highest level of polymorphism of the 13 populations analyzed for all six proteins, a comparison of allelic frequencies for both MDH-1 and PEP in WCK populations shows similar levels of polymorphism in both Form I and Form III.

Third, examination of polymorphic loci MDH-1 and PEP-1 reveals geographic variation in allelic frequencies as well as indices of genotypic identity. The sampled populations in each of the six geographic areas have a distinguishable set of genetic characteristics. NPC populations are fixed for

MDH-I$^{0.87}$ and PEP-I$^{0.78}$. PEP-I$^{1.00}$ frequency in SPC populations north of the shale and sandstone barrier created by Droop Mountain is high. MDH-I$^{0.78}$ is fixed in SPC populations. All GSK populations except General Davis Cave are fixed for MDH-I$^{0.87}$ and most are fixed, or nearly so, for PEP-I$^{0.78}$. SGR populations are separated from GSK area by the Greenbrier River, a habitat alien to G. minus. Populations of G. minus in the SGR show a great degree of variability both in alleles present and their frequencies. Here three alleles for PEP-I are in high frequency: PEP-I$^{0.74}$ (Burnside Branch Cave); PEP-I$^{0.78}$ (Dixon's Spring and Spring South of Second Creek) and PEP-I$^{1.00}$ (Organ Cave). Variability occurs at MDH-I also. Burnside Branch Cave has MDH-I$^{1.00}$ in high frequency and MDH-I$^{0.75}$ is found, although rare, in Dixon's Spring. In OLB localities genetic variability again is present to a high degree. These populations are unique in being characterized by a high frequency of MDH-I$^{0.75}$ allele. PEP-I$^{1.09}$ is unique in one population (Patton's Cave Spring) with PEP-I$^{0.78}$ and PEP-I$^{1.00}$ also present. WCK populations are geographically isolated from all other areas. These are characterized by high frequencies of the previously rare alleles MDH-I$^{0.94}$ and PEP-I$^{0.74}$. MDH-I$^{1.09}$ is unique to these populations.

Fourth, there are no consistent allelic differences that can be used to indicate genetic differences among morphotypes. Allelic frequencies for both MDH-I and PEP-I loci in Form I and Form III populations within Ward's Cove karst area are practically identical as are Martha's Cave (Form II) and Lobelia Spring (Form III) in SPC. In GSK five Form I, four Form II and three Form III populations are, with one exception, monomorphic for MDH-I$^{0.87}$ and most are monomorphic or nearly so for PEP-I$^{0.78}$.

Cytogenetic Results

Figure 4 shows metaphase figures examined from Form I Gammarus minus. The apparent 2N number in G. minus varied between 51-54 with no particular morphotype having a consistent count. 2N numbers of 53-54 were most frequently encountered. There appears to be 20 metacentric, 29 sub-metacentric, two acrocentric, two sub-acrocentric, and one very small metacentric chromosome in 2N=54 individuals.

FIGURE 4 Metaphase Figure of Form I Gammarus minus

DISCUSSION

Taxonomic Implications

Holsinger and Culver (1970) in their study of morphological variation in G. minus found no diagnostic characters that warranted division of the species into separate taxa. However, there may be undetected differences between these forms of G. minus that would warrant the division of the species into separate taxa. Others have reported electrophoretic techniques helpful in resolving systematic problems of many animal groups (Prakash et al., 1969; Johnson and Selander, 1972; Hubby and Throckmorton, 1968; Avise, 1974). Within many animal genera, almost all populations of different species showed genetic differences at 20-50% of their loci. Twelve loci were analyzed in the present study. These electrophoretic data may help resolve the question whether or not G. minus should be divided into separate taxa, especially if the number of loci at which genetic differences are identifiable falls within the above mentioned range. The genetic data thus far collected provide no support for the forms of G. minus representing separate species.

Electrophoresis is less concise below the species level. Generally most conspecific populations show a high degree of similarity which makes subspecific identification by biochemical analysis difficult (Avise, 1974). It is possible to distinguish subspecies only where they have undergone an exceptional amount of differentiation (e.g., Hunt, 1970; Avise and Smith, 1974a). More evidence of genetic differentiation in G. minus populations must be collected before subspecific analysis can be attempted.

Genetic Variability in G. minus populations

Using electrophoresis, estimates of polymorphism in outbreeding terrestrial and aquatic organisms range between 20 to 60 per cent polymorphic loci (See tables in O'Brien and MacIntyre, 1969; Kojima, et al., 1970; Selander, et al., 1970). Compared to these high levels of polymorphism, the levels in most G. minus populations is low, zero to 17 per cent. There should, however, be some variation among species in the degree of genetic variability. Obviously, some species will have less variability than others. A major fraction

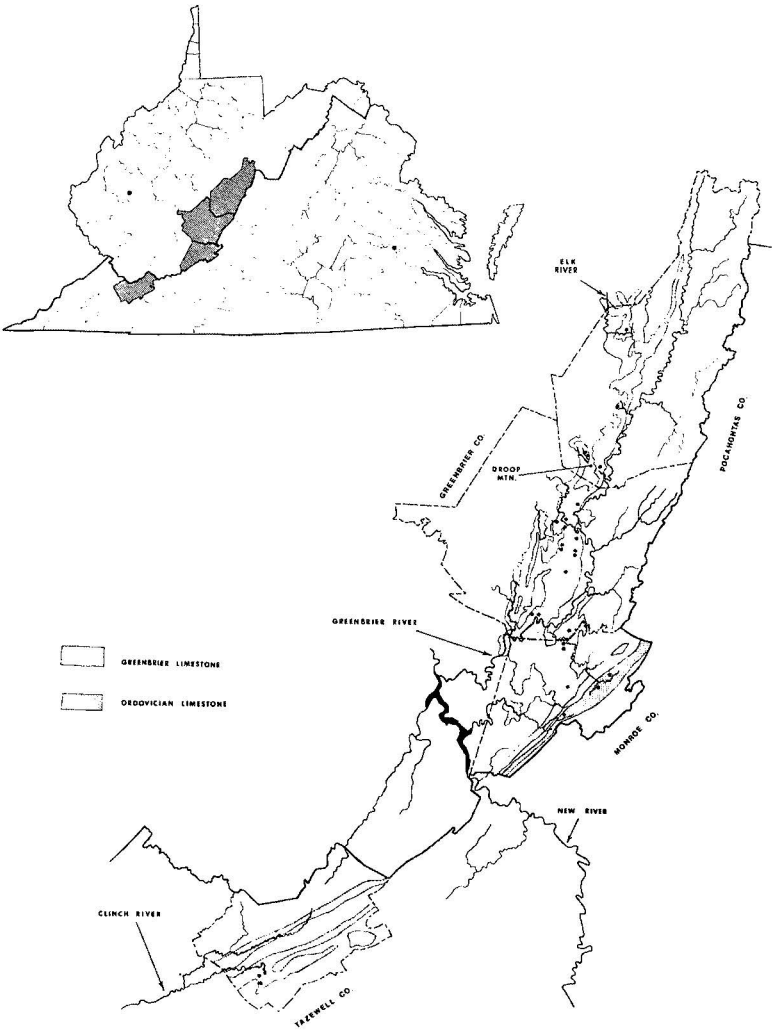
of the genome should be monomorphic in species that have recently expanded from a small genetic base, or have undergone severe selection for certain specialized genotypes, or whose breeding pattern gives rise to high local inbreeding (Lewontin, 1974). Genetic drift also is frequently cited for its role in reducing genetic variability. Random drift should cause different alleles to be fixed or eliminated in the various populations studied. On the other hand a surprisingly small amount of migration is sufficient to swamp out differentiation brought on by random drift (Lewontin, 1974). Thus, considering drainage patterns and potential for dispersal, it is strongly probable that the homogeneity of allele frequencies over broad areas is due to the process of migration. Initially, however, drift in ancestral populations may be responsible for some of the basic allele differences associated with the various geographic areas and also generally low genetic variability.

Hydrology and Gene Flow

Species may maintain gene flow throughout a karst area by dispersal through interconnected caves and water filled solution channels (Culver 1970b; Culver, 1971b; Holsinger, 1967; Holsinger, 1969; Holsinger, 1972; Holsinger and Culver, 1970; Poulson and White, 1969; Avise and Selander, 1972). Considering the extent of cave development (over 900 caves are reported for the area studied) and subterranean drainage patterns in the Greenbrier Valley, the potential for this occurring is tremendous. Distributional patterns of allele frequencies and indices of genetic similarity are recognizable for different geographic areas and are primarily associated with the hydrology (i.e., drainage patterns) and geology of the area. To illustrate this, the physical features and implications for dispersal and gene flow are discussed.

Northern Pocahontas County area (NPC) has two widely separated spring populations. Both springs are located in Greenbrier limestone. Linwood Spring lies in a disjunct section and is within the Elk River drainage, whereas Tub Spring is in the Greenbrier River basin (see figure 5). Allelic types are identical and allele frequencies are fixed for both sites, yet dispersal between these two sites is improbable based on the marked differences in surface drainage

FIGURE 5 Hydrology and Geology of the Study Area


ELK RIVER
GREENBRIER CO.
DROOP MTN.
POCAHONTAS CO.
GREENBRIER RIVER
GREENBRIER LIMESTONE
ORDOVICIAN LIMESTONE
MONROE CO.
NEW RIVER
CLINCH RIVER
TAZEWELL CO.

between the two populations. Since these alleles are common throughout Greenbrier Valley populations, it may be a chance occurrence that the allele frequencies are identical at these two geographically different sites. No concrete statement on dispersal patterns can be made because of the small number of NPC populations sampled. Some speculations, however, can be made based on knowledge of the geology and hydrology of the Swago Creek area of which Tub Spring is a part. There is extensive surface and subterranean drainage with subterranean drainage routes following closely the surface routes. (White and Schmidt, 1966). The potential for both surface and subterranean dispersal is great. Therefore, populations of this area should be genetically homogeneous. Some evidence for surface dispersal has been noted in the Swago Creek area (Holsinger and Culver, 1970).

Three Southern Pocahontas County (SPC) sites are also developed in Greenbrier limestone. Marten's Cave and Lobelia Spring are in the Lobelia section (Alderson limestone of the Greenbrier Series) that crops out on the west side of Droop Mountain, while Martha's Cave is in the main NE-SW section cropping out to the west (see figure 5). Droop Mountain would appear to act as a major dispersal barrier for SPC populations on either side. The drainage of the Lobelia side consists of a number of small tributaries that unite to form Bruffey's Creek and Hills Creek. The streams form as run-off along the Alderson limestone and Greenville shale and flow a short distance along the Union limestone before sinking (Wolfe, 1964). These streams both flow under a spur of Droop Mountain, the major surface barrier of the area, and ultimately emerge at Locust Creek Spring (Wolfe, 1964; White and Schmidt, 1966). The waters from Marten's Cave and Lobelia Spring enter Bruffey's Creek which has been shown to flow through Martha's Cave (White and Schmidt, 1966). Therefore, it is easy to envision amphipod dispersal under the spur of Droop Mountain, and this is supported by homogeneity of allele frequencies at both PEP-I and MDH-I loci (see figures 6 and 7). The hydrology, as well as PEP-I alleles that characterize this area, indicate that these SPC populations have been relatively isolated from populations of surrounding geographic areas. This isolation has allowed the SPC populations to become genetically divergent from

FIGURE 6 Allele Frequency Distribution for Malate Dehydrogenase-1 Locus in the Study Area

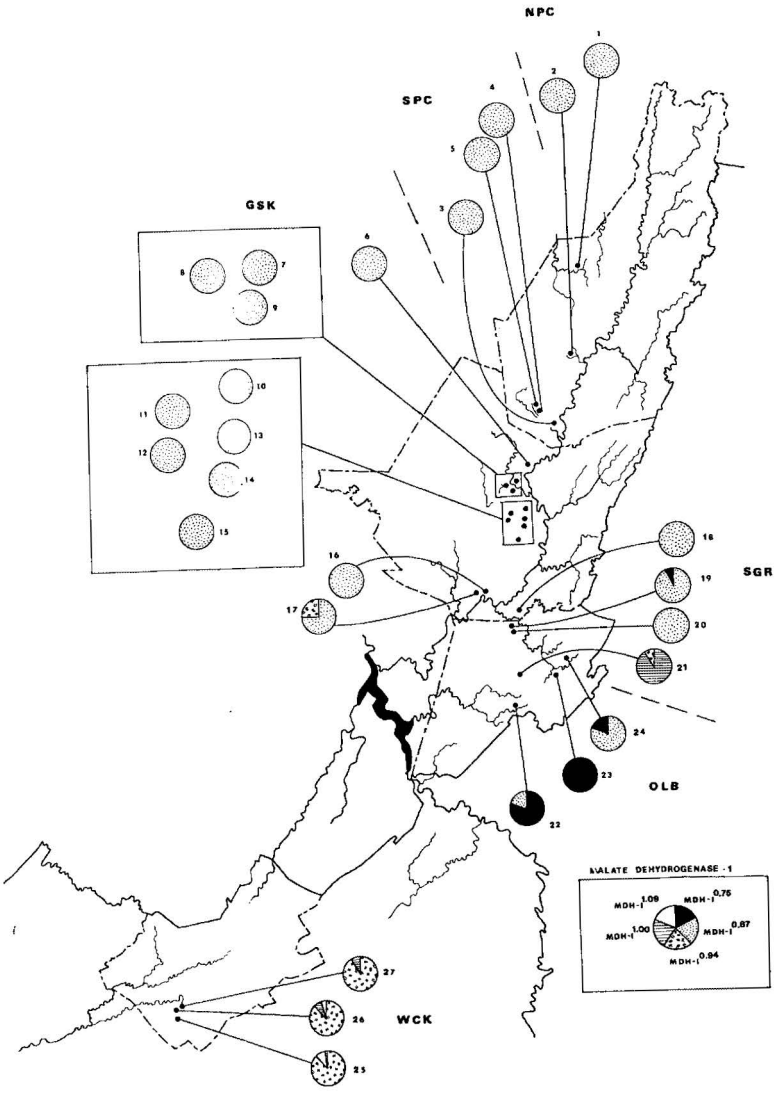
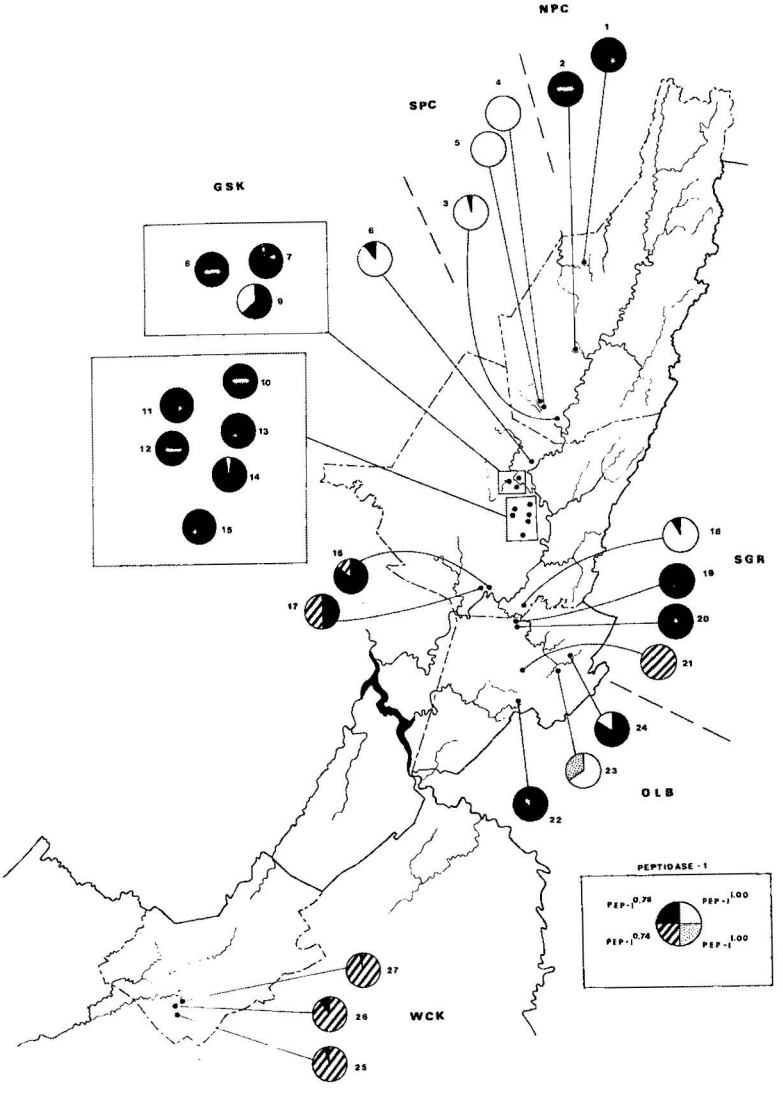

NPC
SPC
GSK
SGR
OLB
WCK
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
MALATE DEHYDROGENASE -1
MDH-1 1.09
MDH-1 0.75
MDH-1 1.00
MDH-1 0.87
MDH-1 0.94

FIGURE 7 Allele Frequency Distribution for Peptidase-1 Locus in the Study Area


NPC
SPC
GSK
SGR
OLB
WCK
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
PEPTIDASE - 1
PEP-1 0.78
PEP-1 1.00
PEP-1 0.74
PEP-1 1.00

populations of surrounding geographic areas, but dispersal within the SPC has maintained homogeneity among the populations.

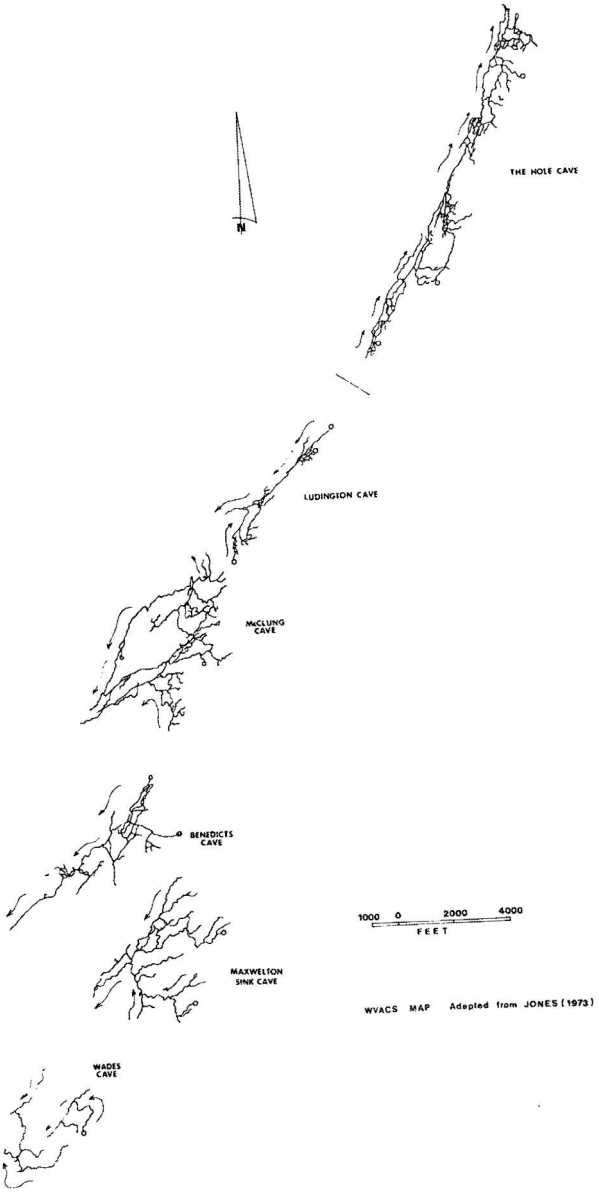
The Great Savannah karst (GSK) was the most extensively studied of the geographic areas. A number of hydrological sub basins are encountered in GSK (Jones, 1973). The northern-most GSK sample is the Bone-Norman Cave population. Although the hydrology of the Bone-Norman Cave system is not completely understood, the high frequency of PEP-$I^{1.00}$ allele seems to indicate close relationship with SPC populations. One might speculate, then, that the water entering the Bone-Norman system may originate on the west side of the Droop Mountain near the SPC populations.

The unique LAP-$I^{0.98}$ allele in Route 219 Spring population indicates some local differentiation, although MDH-1 and PEP-1 alleles are similar to those of the majority of GSK populations. Route 219 Spring waters discharge into Spring Creek, but its subterranean origins are ill defined.

The portion of GSK from Spring Creek south to the Greenbrier River has the highest degree of cave interconnectivity (see figure 8). A number of caves have several miles or more of passage. As described by Jones (1973), subsurface drainage of this area is broken up into several sub-basins. Buckeye Creek sub-basin consists of surface and subterranean components, of which Buckeye Creek Cave is a part. Water from this basin resurges at a point close to where Route 219 Spring flows into Spring Creek. Dispersal between the Buckeye Creek Cave population and Route 219 Spring is possible and would account for similarities at MDH-1 and PEP-1 loci. Not all 13 loci were analyzed in the Buckeye Creek Cave population. Perhaps if they had been analyzed, sub-basin differences in genetic structure may have been identified.

"The Hole" Cave Basin is purely subterranean and consists of passages and stream channels with flow in a NNE direction. Dye tracing shows that its water resurges in two springs along Spring Creek (Jones, 1973). This cave is one of five so called "contact caves" developed at or near the contact of the Hillsdale limestone and Maccrady Formation. "The Hole" has three known entrances and over 15 miles of mapped passages. The population in this cave is marked by the unique GP-$3^{0.94}$ allele which indicates that some degree of differentiation

FIGURE 8 The " Contact Caves " of the Great Savannah Karst in West Virginia



WVACS MAP Adapted from JONES (1973)

from other GSK populations has occurred. Hydrologically, this population is disjunct from the other "contact caves".

The largest GSK sub-basin is Davis Spring Basin (Jones, 1973). The majority of sampled GSK populations are within this basin. The northern segment of the basin drains through Higginbotham's Cave and then through Coffman Cave. Coffman Cave has been dye traced to Fort Spring (Jones, 1973). Drainage in the eastern part of Davis Spring Basin is primarily subsurface through extensive, well integrated cave systems. The West Virginia Association for Cave Studies (WVACS) has studied caves within this part of the basin extensively; Ludington, McClung, Maxwelton Sink, Benedicts, and Wades (figure 8) are the principal cave systems. Populations associated with each of these sites except Maxwelton Sink and Wades were sampled in the present study. The water in Ludington Cave (thus also Ludington Cave Spring) has been traced through McClung's Cave and then to Fort Spring (Jones, 1973). Evidence also strongly indicates that water within the other caves flows to Fort Spring. Although these caves have a dendritic pattern, their general orientation and water flow through them are southwest. Fort Spring appears to be the point of resurgence for this entire drainage basin (Jones, 1973). One important gap in our knowledge of this basin is the nature of the flow systems between the "contact caves" and Coffman Cave and the resurgence point at Fort Spring. Also, where above Fort Spring do these systems merge? This knowledge would go far in answering questions on dispersal of amphipods. Obviously this area has a well integrated subterranean drainage system and the potential for dispersal and consequent gene flow is tremendous. All populations of this area have practically identical allele frequencies (see figures 6 and 7). Both electrophoretic and hydrological data lends considerable weight to the idea that at least intermittent gene flow is occurring or has occurred between populations in this area. Within the "contact" cave areas of the basin especially, it is easy to envision the presence of one large Form I amphipod population.

Indices of genetic similiarity for PEP-I indicate that General Davis Cave is distinct from all other GSK populations except perhaps Fort Spring. This is

probably a reflection of General Davis Cave being in a separate drainage sub-basin--Davis Hollow Basin (Jones, 1973). Two possible explanations might account for genetic similarity between the populations in Fort Spring and General Davis Cave. Fort Spring is only about one-half mile from the spring where subterranean water in Davis Hollow Basin resurges and flows into the Greenbrier River. Thus rafting of individuals from Fort Spring to this point might allow for gene flow into General Davis Cave aquifer. This situation would be tenuous at best considering the foreign nature of the Greenbrier River habitat in reference to G. minus. More likely, however, this similarity may be due to sampling error of the PEP-1 locus in Fort Spring population.

The area south of the Greenbrier River (SGR) is, like GSK, a mature karst area with numerous sinkhole plains and coalesced sinkhole valleys (uvalas). Although there are numerous caves in the area, extensive cave interconnecting is not present. Cave systems are more truncated and the area appears to lack the well integrated subterranean drainage system present in GSK. Surface drainage also is proportionately greater. These features seem to be reflected in a heterogeneous distribution of allele frequencies, and wide ranging gene flow is not a predominant feature throughout the area.

Organ Cave lies in a major finger of Hillsdale limestone (Greenbrier Series) which projects north and lies east of the Greenbrier River (see figure 5). It is the most extensive cavern system in the Virginias with over 45 miles of explored passage. PEP-$1^{1.00}$ is the dominant peptidase allele and within the SGR populations this is unique. Dixon Spring and spring south of Second Creek have nearly identical frequencies for alleles MDH-$1^{0.87}$ and PEP-$1^{0.74}$, although Dixon Spring has MDH-$1^{0.75}$ in low frequency. These two sites are close to one another, and they may be hydrologically related, although no dye tracings have been performed. Burnside Branch Cave is strikingly different at both loci from other populations in SGR. The water from Burnside Branch Cave, however, emerges at Dixon Spring (W. K. Jones - pers. comm.). The differences in allele frequencies seem to preclude the possibility of dispersal between Burnside Branch Cave and Dixon Spring. Features of this particular

aquifer that would prevent amphipod dispersal are unknown but considerable intra-area variation in allele frequencies is evident in SGR (see figures 6 and 7).

Genetic differences in populations north and south of the Greenbrier River indicate that the river may act as a major dispersal barrier. As one can see by driving between Lewisburg, West Virginia to Organ Cave on Route 219, the Greenbrier River is deeply entrenched. Although G. minus is encountered in springs along the Greenbrier River, the river itself is a foreign habitat. Holsinger, et al. (in press) have found that the Greenbrier River has acted as a dispersal barrier for a number of cave related species. Considering these factors, gene flow between GSK and SRG populations could not be easily accomplished. In fact, considering the geology and the degree of entrenchment of the Greenbrier River, these two areas have probably been isolated for some time.

The Ordovician Limestone Belt (OLD) consists of a narrow band of Ordovician limestones cropping out in the eastern part of Monroe County, W. Va. (see figure 5) occupying the valley floor on the west side of Peters Mountain and Back Valley (Davies, 1965). Two major surface drainage systems are found in OLB: Greenbrier River and New River. Both Kitchen Creek Spring and Patton's Cave Spring are within the Greenbrier drainage while Zenith Spring is in the New River drainage. Although Kitchen Creek Spring and Patton's Cave Spring are only about three miles apart, they are on two distinct tributaries of Second Creek. Both alleles and frequencies vary considerably between these sites, indicating the populations are most probably isolated from each other. Consequently, it is probable that no gene flow occurs between these two populations. The presence of Zenith Spring population within the New River drainage is believed to be responsible for allelic differences that distinguish it from other OLB populations. OLB populations demonstrate a pattern of heterogeneity which corresponds with surface drainage patterns (see figures 6 and 7). Considering the geology and hydrology of this area, further sampling of cave and spring populations would probably reveal further heterogeneity in genetic structure.

If one expects a high potential for gene flow within the Greenbrier Valley, there should be some similarities overall. On the other hand, some major differences should occur in areas geographically and hydrologically distinct from this area. The Ward Cove karst (WCK) is one such area (see figure 5). It is some 75 miles southwest of Monroe County, W. Va.; geologically and hydrologically it is well isolated from Greenbrier Valley populations by several high ridges of clastic rocks. These ridges should be effective dispersal barriers and so genetic differences should be present when Greenbrier Valley and WCK populations are compared (Holsinger, 1969). However, WCK is a mature karst area with the potential for subterranean dispersal and, therefore, within this area there should be genetic homogeneity.

In WCK populations, the degree of polymorphism at both MDH-1 and PEP-1 is higher than in Greenbrier Valley populations. Dominant alleles are quite different and a unique allele ($MDH\text{-}1^{1.09}$) is present. Nearly identical allele frequencies throughout the area point to a well integrated drainage allowing dispersal and gene flow (See figure 6 and 7). Fallen Rock and Hugh Young caves lie near a synclinal axis with water flowing through them and resurging at Maiden Spring. These caves are components of an extensive subterranean drainage system. Considering this and the fact that Form I G. minus has been found in Maiden Spring during high water, the potential for dispersal within this aquifer is good.

Genetic similarity of Greenbrier Valley populations suggests either common descent from an ancestral population with the currently identified alleles fixed or in high frequency, or the existence of intermittent gene flow among populations, or both. That gene flow may occur has been proven. Based on electrophoretic differences in Form I populations of the Great Savannah and Ward Cove karst areas, and also on subtle morphological differences that have been observed by Holsinger (pers. comm.), it is probable that this form has evolved twice. It also appears likely that Form II populations have evolved separately at several different times. Why Form I amphipods are restricted to certain caves in these areas is still unknown.

Another question is why have Form I G. minus not evolved in other karst areas of North America where surface forms are present? G. minus is scarce in cave streams in karst areas of southwestern Virginia. Tennessee, Kentucky, Indiana, Georgia and Alabama, although it is often encountered in springs within these areas (Holsinger and Culver, 1970). Troglobitic species of the amphipod Crangonyx are common to caves of these same areas, however, and Holsinger and Culver (1970) suggest that the scarcity of G. minus in these cave streams is probably due to its inability to successfully compete with Crangonyx. This may also have prevented the evolution of Form I amphipods in these areas.

Avise and Selander (1972) and Culver (1970) argue that stochastic processes, including genetic drift play an important part in the evolution of cave organisms. There are some allele fixation differences in cave versus spring populations (involving the unique alleles in Route 219 Spring and The Hole populations - See Table 1) but, on the whole, other factors argue against drift as a mechanism for producing regressive cave features. For example, Form II morphotypes usually inhabit small caves, and populations are generally much smaller than Form I populations, averaging per cave about 10^3 individuals with bottlenecking to 10^2 during spring floods (D. C. Culver, pers. comm.). Form I populations might average about 10^5 individuals per cave with bottlenecking questionable. In GSK and WCK areas, eye regression has been greatest in larger population, i.e., Form I G. minus populations. Random drift is more effective in small populations, whereas natural selection should be more effective in larger populations where there is a larger pool of selectable alleles and the severity of selection is greater because of increased competition. Alternatively, eye regression may be less pronounced in Form II populations because they represent more recent colonization of caves. Although drift may help explain generally low levels of genetic variability in G. minus, by itself, it does not adequately explain regressive evolution of G. minus cave forms.

The pattern of regressive evolution of eyes appears to be similar in most amphipod cave populations studied. First the facets around the periphery

become irregular and less numerous, and this then continues centripetally. Individual facets ultimately disappear, although pigmentation remains. This parallelism in regression suggests a common selective mechanism, not the action of mutation and drift which would probably produce unique schemes of regression for each isolated cave population.

Can the observed morphological differences of the three types of G. minus be attributed to environmental factors in the different habitats? This aspect has been noted by Minckley and Cole (1963) and Holsinger and Culver (1970). Holsinger and Culver (1970) suggest that the variation in G. minus may be genetic or ecophenotypic or a combination of both. Ecophenotypic variation is the response to an environmental condition resulting in a nongenetic modification of the phenotype (Mayr, 1969). Ecophenotypy poses some problems, however. If ecophenotypic differences are not genetic, as Mayr (1969) claims, then morphological changes should occur in a relatively short period of time if the population was removed from the particular habitat influencing the modification.

Two populations studied seem to indicate that this morphological variation is not purely ecophenotypic. The surface stream entering Ludington Cave (Ludington Cave Spring) has Form I amphipods. Assuming this population represents immigrants from a subterranean system in the area, the morphology should change through time if it is ecophenotypically controlled. No morphological differentiation has appeared in this seemingly healthy, breeding population from the time of Holsinger's original observations in 1966 to the present. A similar situation occurs at Hugh Young Cave, where the cave stream flows out of the entrance. Form I amphipods are found in both the cave and surface portions of the stream. There are obviously a number of unknowns, but the implication is that some genetic basis exists for these different morphotypes. Although the morphological variation in G. minus does not appear to be due purely to ecophenotypic responses, they may represent genetic assimilation of environmentally induced change similar to what Waddington (1956) found in Drosophila melanogaster.

The genetic basis for eye degeneration probably involves only a small portion of the genome. Thus these differences may not be picked up by the electrophoretic technique. The surveyed portion of the G. minus genome may represent a non-divergent segment, similar to that encountered by Turner (1974) in the Death Valley pupfish. A large electrophoretic survey involving a greater number of polymorphic loci might improve our understanding of this problem.

Morphological variation has also posed problems of interpretation within the Gammarus pulex group (Goedmaker, 1972; Pinkster, 1972) in Europe. Certain populations of G. pulex in France are found in low pH waters, and these differ from the normal pulex in one character. Both variant and normal populations inhabit the same stream systems, and the variant can live in normal pH waters. Following Pinkster's (1972) treatment of the pulex group problem, G. minus populations might maintain their morphological character, even though gene flow between the three forms is possible. Selection pressures corresponding to environmental factors may interfere with regular gene exchange between caves and springs.

A possible example of this occurs at Bone-Norman Cave. The water that enters the cave comes from a surface spring (Taylor's Spring) which has a large population of G. minus Form III with an average density of about $2000/.09m^2$. This water sinks into the ground and appears inside Bone-Norman Cave. Here G. minus Form II is present with an average density of about $7/.09m^2$. Obviously some features of the cave habitat are preventing the maintenance of a large population, and a combination of drift and selection may account for the alteration in morphology.

Changes in stream gradients and diversion of surface waters into subterranean channels through underground stream capture also may act in isolating populations in caves (Holsinger, et al., in press). Form I populations may represent populations of G. minus that have been effectively isolated from surface forms by hydrological and/or habitat barriers. This is possible considering that they are restricted to caves which are parts of extensive subterranean drainage systems (e.g., the "contact" caves of GSK, Organ Cave, and certain WCK area

caves). Within these areas there is practically no surface drainage. Practically all Form II and Form III populations occur along the periphery of these areas. Also, although Form III populations are encountered in resurgences for aquifers having Form I populations, they are not present at insurgent points. Thus, Form I populations may have undergone a period of isolation, divergence, and adaptation to the cave environment. Form II morphotypes may represent borderline populations of more recent cave colonization.

One plausible conclusion that can be drawn from these data is that adaptation to cave existence and regression of eye structure have left no mark on the surveyed portion of the genome. These data corroborate the findings of Avise and Selander (1972) who found no major genetic differentiation between the cave and stream populations of Astyanax. According to Pinkster (1972), morphological variation can be considered the first step towards speciation. The morphological change demonstrated in Form I populations in the Great Savannah and Ward Cove karst areas may be an example of a preliminary stage in the evolution of an aquatic troglobite. If so, my genetic data do not support the hypothesis of Barr (1967, 1968), which states that a major "genetic revolution" accompanies incipient speciation of troglobitic populations. Preliminary chromosomal analyses did not yield consistent chromosomal differences between morphotypes. If these results persist, they would corroborate the electrophoretic data.

Environmental heterogeneity versus genetic variability

An offshoot of this study is the question of the effect of environmental heterogeneity on genetic variability. Environmental heterogeneity is thought to promote genetic variability by mechanisms of diversifying selection (Dobyhansky, 1970). Thus genetic variability is maintained in populations as an adaptation to environmental heterogeneity in time and space (Gooch and Schopf, 1973). A number of attempts have been made to relate genetic variability with environmental heterogeneity (Agnew, 1968; Levins, 1971;

Avise and Selander, 1972; Johnson and Selander, 1971; Selander et al. 1970; Schopf and Gooch, 1971; Gooch and Schopf, 1973). Results of these studies are generally inconclusive and even contradictory.

Two features attributed to environmental heterogeneity may affect genetic variability (Soule, 1971). (1) The niche width-variation hypothesis states that populations having more niches or broader niches of central areas of the species range should be relatively more polymorphic, because different complexes of environmental factors will probably select for different phenotypes. (2) The gene flow-variation hypothesis emphasizes the role of migration among populations adapted to varied habitats in maintaining polymorphism in populations. Compared to surface environment, the cave environment is generally considered relatively stable and uniform in time and space. The spring environment might also be considered rather stable and uniform since there are a number of similarities between cave streams and springs - i.e., water temperature, oxygen concentration, pH, water flow, and cover. If we accept the tenant that these two environments are relatively stable and uniform in time and space, both hypotheses lead to predictions of low levels of genetic variability. Lacking further support for these hypotheses, however, drift and migration seem to be the more tenable causes for the low levels of genetic variability throughout G. minus.

SUMMARY

This study deals with the investigation of the degree of genetic differentiation between three morphological forms of the freshwater amphipod Gammarus minus. Geographic distribution of allele frequencies of polymorphic loci are also examined and the results correlated with the geology and hydrology of the specific geographic area. Two disjunct limestone areas were examined.

Vertical polyacrylamide and horizontal starch gel electrophoresis and histochemical staining were utilized in characterizing gene loci from six protein systems in individuals of Gammarus minus representing nine cave populations (Six Form I and three Form II) and four surface populations (Form III). A total of 27 populations were examined for the two polymorphic loci. Cytogenetic examinations were also made.

A number of trends were elicited. First, in populations surveyed at all six protein systems, most show low levels of genetic variability. Second, the amount of genetic variability is similar in all three morphotypes. Third, there are no consistent allele differences that can be used to indicate genetic differences among the morphotypes. Also, no consistent chromosomal differences were detected, with 2N= 53-54 most commonly encountered in all three morphotypes. Random drift and migration are probably responsible for the generally low levels of genetic variability. This genetic study corroborates the taxonomic study by Holsinger and Culver (1970) which found no characters that warranted the division of the species into separate taxa. Fourth, examination of the polymorphic loci MDH-1 and PEP-1 reveals geographic variation in allele frequencies as well as indices of genotypic identity. The sampled populations in each of six geographic areas have a distinguishable set of genetic characteristics. The distributional patterns are associated with the surface and subterranean drainage patterns. Areas having well integrated subterranean drainage systems are genetically homogeneous, while those with poorly integrated systems are genetically heterogeneous.

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