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Title: Genetic variation in Macrobrachium lar on Guam.

Approved: Lynn Raulerson
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Vertical polyacrylamide gel electrophoresis was used to assess protein variation in three stream subpopulations of the freshwater prawn Macrobrachium lar. Homogenates of tail muscle tissue were examined.

Twelve enzymes and 19 loci were determined to be under independent genetic control. Four loci exhibited genetic variation. Each stream subpopulation was polymorphic at 21.1% of the loci examined. Mean heterozygosity per locus ranged from 6.6% to 11.9%; mean heterozygosity per individual ranged from 10.1% to 13.3%. Genetic heterozygosity is evenly distributed among the subpopulations studied. Slight differences in frequency of loci could have been attributed to genetic drift. Estimates of genetic similarity and genetic distance also indicated that the subpopulations were part of a panmictic population.

TO THE GRADUATE SCHOOL AND RESEARCH

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GENETIC VARIATION IN *MACROBRACHIUM LAR* ON GUAM

By

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INTRODUCTION

The mechanisms of selection, mutation, and genetic drift are instrumental in the perpetuation of genetic variability. However, different theories place varying degrees of emphasis on these elements of change. The modern synthetic theory advocates natural selection and emphasizes long term interruption of gene flow which creates a transition across species as a result of accumulated allelic substitutions. The significance of molecular variations is a point of continued controversy. Selectionists believe evolution is controlled by directional selection of advantageous alleles which result in superior genotype (Ayala, 1979; Gould, 1980). Neutralists believe molecular mutations may be adaptively neutral evolutionary noise not subject to selection; evolution is a result of "random walk" or genetic drift (Dobzhansky, 1970; Kimura, 1968; King and Jukes, 1969). Another theory, the punctuational model, first proposed by Ernst Mayer (1954), suggests species and higher order taxa arise only through sudden allele changes without interruption of gene flow and natural selection acts to produce only lesser modifications within species. Therefore, strong selection and rapid fixation of chromosomal variants produce sizable evolutionary steps from a small amount of large scale change (Eldredge and Gould, 1972). Particularly in small breeding populations, major chromosomal changes can give rise to fixation in a few generations. Stanley (1979) states that a group of 10 breeding individuals can have fixed variations in two or three generations.

In the absence of disturbing factors like mutation, migration and selection, gene frequencies will remain constant (Wright, 1931). However, genetic drift or random chance would cause the abundance of different alleles to fluctuate until an allele is fixed and genetic variation at that locus is lost. Genetic drift would cause high levels of variation between populations but low levels of variation within populations. For random genetic drift to be important, populations must be isolated sufficiently to restrict migration (Crow and Kimura, 1970). Migration increases genetic variability and counteracts genetic drift. Under certain circumstances, a single migration can prevent populations from differentiating into different species. Migration will increase levels of variation within populations but reduce variation between populations.

The same principle holds if a species is distributed not in discrete populations but uniformly over a landscape. In this isolation-by-distance model, the variation in genetic frequency from place to place is small; but as the average distance of migration increases, variation in genetic composition becomes less pronounced (Futuyma, 1979). Isolated subpopulations with high incidences of inbreeding have great genetic variation when compared to one another, but low within-population variation. Genetic studies by Ayala (1979), Fuller (1977), Hedgecock (1978), Hedgecock et al. (1979), Trudeau (1977), and others show wide ranges of genetic variation between isolated populations.

The ultimate fate of genes in a population depends on which has the greater effect; the systematic forces (selection, mutation, migration) or the action of genetic drift (Futuyma, 1979). Because mutation rates are low, their effect on the genetic variability of a population

is likely to be overwhelmed by stronger factors (Futuyma, 1979). Selection in different environments will increase adaptation of populations in their own environment and promote genetic divergence. If the environments are similar, selection will increase adaptation and reduce genetic divergence (Hartl, 1980).

Macrobrachium lar is distributed throughout the tropical Indo-Pacific from Africa eastward to the islands of the Tuamotus and Marquesas; the Ryukyu Islands are the northern limit of its natural range. In the Pacific basin, it is indigenous to all archipelagos with inhabitable streams, except Hawaii, where it was introduced from Guam in 1956 (Maciolek, 1972). Macrobrachium lar inhabits most free flowing streams in southern Guam. It is highly mobile, a strong swimmer and crawler, and nocturnally active. Extended intrastream movement is not greatly hampered by obstacles such as waterfalls and debris dams; individuals have been observed crawling on stream banks. It is tolerant of wide ranges in environmental conditions such as temperature, salinity and oxygen. M. lar is a randomly mating species, reported to reproduce throughout the year. The larvae are flushed downstream where they remain planktonic for up to 3 months. After 12 molts, postlarvae presumably settle in the stream discharge area (Atkinson, 1973).

The genus of freshwater shrimp, Macrobrachium, has been suggested as a potential resource for aquaculture (Atkinson, 1973; Ling, 1969; Maciolek, 1972; Trudeau, 1977, 1978). To date there is no information on the genetic profile of this prawn. Knowledge of the extent of genetic variation and inheritance of certain genetic markers is a useful tool for assessing aquaculture potential and establishing selective breeding programs. Species are frequently geographically subdivided

into local populations or demes that have limited gene exchange and potentially high levels of genetic variance. It is essential to know whether broodstocks of Macrobrachium obtained from natural populations are part of one large panmictic population or many variable demes.

The objective of this study was to investigate the effects of migration and genetic drift on genetic variation in insular populations of M. lar found in essentially identical environments.

At the molecular level, the best means for directly determining genetic variability is gene mapping of DNA sequences. Since this approach is both time-consuming and expensive, estimates of genetic variability are usually determined biochemically, based on assessment of heterosis in structural gene products, e.g., enzymes. Structural genes determine the amino acid sequences of enzymes.

The molecular technique of electrophoresis and histochemical staining was introduced as a tool in population biology by Lewontin and Hubby (1966). Electrophoresis provided both a quantitative and qualitative technique for the analysis of isozymes, which were multiple molecular forms of enzymes that catalyze the same reactions. Electrophoresis could theoretically detect approximately 1/4-1/3 of amino acid substitutions in proteins, e.g., those substitutions which alter the protein electrostatic charge or conformation (Lewontin, 1974; Selander, 1976). Polyacrylamide gel electrophoresis was used to separate protein products of structural genes to determine the genetic variation in populations of M. lar. Population geneticists and evolutionists have applied a similar methodology toward a better understanding of the biological and evolutionary significance of protein polymorphisms in

natural populations (Ayala et al., 1974; Gillespie and Kojima, 1968; Lester, 1979; and Skibinski et al., 1980).

MATERIALS AND METHODS

Three populations of the freshwater prawn Macrobrachium lar were examined for genetic variability with electrophoretic protein separation. Specimens were collected from 3 southern Guam streams: Asmafines and Sella streams, which feed into Sella Bay, and the Asalonso stream, which feeds into Paicpouc Cove. All streams originated from separate headwaters. An electroshocker and baited minnow traps were used to collect prawns. A total of 167 individuals were collected. Sample sizes examined were as follows:

1) Asmafines 55 specimens; 26 males, 29 females
2) Sella 52 specimens; 30 males, 32 females
3) Asalonso 60 specimens; 28 males, 32 females.

Specimens were transported to the lab packed in grass cuttings and water. The water was changed every 30 minutes. Specimens were immediately processed upon return to the laboratory. The weight and sex were recorded for each individual. Sex was determined by the ratio of carapace length (eyesocket to end of cephalothorax segment) to 2nd abdominal segment width. Individuals with a ratio of 2.2 or less were considered female, 2.3 or greater were considered male. Males were also identified by the presence of a "reproductive flap" adjacent to the 3rd walking appendage.

After removal of the exoskeleton, tail muscle was flash frozen in liquid nitrogen. Frozen tissue was either stored at -70±C or immediately homogenized. The tissue was ground with a mortar and pestle

and homogenized with 2-3 volumes of grinding solution (0.01 M Tris, 0.79 EDTA with 40 mg NADP, Lester, J., pers. comm.). Cell structure was further disrupted with a tissue sonicator to facilitate release of intracellular enzymes. Throughout processing, samples were chilled in an icebath to reduce frictional heating and retard the action of lytic enzymes. Samples were centrifuged at 15,000 rpm at 10°C for 20 minutes. The supernatants were stored at -70°C.

Although electrophoretic technique is commonly used (Avise, 1975; Brewer, 1970; Lewontin, 1974; Selander, 1976) the polyacrylamide gel electrophoresis (PAGE) technique for separation of Macrobrachium sp. proteins has not been reported in the literature. In previous studies of other invertebrates a starch gel support medium was used. PAGE has several advantages over starch gel. The acrylamide gel forms a tougher, more flexible medium which is transparent and makes isozyme bands easier to quantify. Gels can be made with acrylamide concentrations as low as 3% and as high as 50% (the lower the concentration the larger the pore size). Acrylamide solutions of several pore sizes can be included in the same gel slab thereby establishing a gradient with varying sieving effects. Acrylamide gels do not have to be sliced and keep better than starch gels. Appendix A presents a description of the electrophoresis technique.

Vertical PAGE was used to assess genetic variability in M. lar populations. Staining techniques were adapted from Tracey et al. (1975). Gels with a 5-20% gradient were cast daily, or 1-3 days before use and refrigerated. Prior to an electrophoretic run, samples were prepared with a 50% sucrose solution to increase density and an indicator dye, bromophenol blue. A variable volume of sample (20-30 ml) was

applied to each gel sample well, depending on the enzyme assayed. Power was supplied by a Buchler 3-1500 Constant Power Supply.

Gels were treated with a histochemical staining solution containing a specific substrate for each enzyme system and a colored salt which reacted with the reaction products catalyzed by the enzyme (Table 1). The resultant zymogram banding pattern produced by enzymatic activity provided a visual record of isozymes. The term allozyme was used to describe different forms of an enzyme coded by the same locus.

Gels were fixed with either 50% ethanol or 10% acetic acid. Zymograms were either scored immediately and dried or wrapped in plastic and stored at room temperature.

Isozyme bands were labeled by a system adapted from Brewer (1970). Zymogram patterns were divided into regions with number designations and letters designating bands within a region. The region closest to the anodal end was labeled I, the farthest migrating band within a region was designated as A. Numbering and lettering continued sequentially toward the cathodal end of the gel. Interpretations of these zymograms were made according to Tracey et al. (1975).

Estimates of genetic variability can be made for both populations and individuals. The incidence of heterozygotes is the most informative measure of genetic variation (Ayala and Valentine, 1978). The overall amount of variation in a population is established by the average frequency (or proportion) of heterozygotes per locus. This is the average observed heterozygosity of each locus (number of heterozygous individuals divided by all loci sampled). Average heterozygosity is the best estimator of genetic variability in outbreeding populations (Nei and Roychoudbury, 1974). It is dependent on the

number of loci assayed per individual rather than the number of individuals sampled. The sampling variance can be divided into the variation among individuals (inter-locus variance) and the variation among loci (intra-locus variance). The inter-locus variance depends on the genetic structure of populations, which is determined by evolutionary forces, mutation, selection, random drift and other factors. These forces are difficult to quantify. The intra-locus variance depends on the sample size and gene frequencies of studied loci.

Indices of heterozygosity in the average individual are based on the frequency of heterozygous loci per individual and the frequency of individuals heterozygous per locus. The sampling error is dependent on the number of loci examined, not the number of individuals sampled. The arithmetic means are identical for the same number of loci sampled. In randomly mating populations the proportion of heterozygous individuals per locus is normally distributed, while the proportion of heterozygous locus per individual does not approach a normal curve. Loci are considered polymorphic when the common allele frequency is no greater than 0.950 (Ayala and Valentine, 1978). It would be expected that a population in Hardy-Weinberg equilibrium would have low intra-locus heterozygosities as allele frequencies became fixed (approached frequencies of either 1.0 or 0). Significant differences in frequencies for specific loci could indicate little or no migration between subpopulations. Intra-locus frequency values were tested with a one way anova and a chi-square test for goodness of fit (Sokal and Rohlf, 1969).

In a randomly mating population the expected and observed frequencies of heterozygotes are usually very similar. Expected

heterozygosity is calculated directly from the allele frequencies. For the 4 polymorphic loci exhibiting a total of 8 alleles (2 per locus; A and A') with frequencies f_1, f_2, f_3 ---- f_8, the expected frequency of heterozygotes is:

$$H = 1 - (f_1^2 + f_2^2 + f_3^2 + \text{----} f_8^2).$$

The observed heterozygosity is calculated by dividing the number of heterozygous individuals by the total number of individuals sampled. Differences between these frequencies can result from natural selection, mutation, drift and other factors.

In estimating average heterozygosity and genetic identity it was more critical to sample a large number of loci (>30) than a large number of individuals per locus. However, a large number of individuals per locus (20-30) were required to determine a Hardy-Weinberg equilibrium. A total of 167 individuals were assayed for each locus. A large number of enzymes were screened to determine which systems showed the best resolution with the electrophoresis set up (PAGE) and experimental animal (M. lar). The 19 selected enzymes provided the most usable information from the resources available. Sampling error producing differences between populations might have been reduced if a greater number of loci had been assayed.

The amount of differentiation among populations can be determined by Nei's (1972) measures of genetic similarity (I) and genetic distance (D). Genetic similarity is the average probability that two alleles, randomly chosen, will be from different populations. The equation is:

$$I = \Sigma x_i y_i / (\Sigma x_i^2 \, \Sigma y_i^2)^{1/2}$$

where x, y are populations and X_i, Y_i are frequencies of the ith allele in populations x and y. Genetic distance (D) is a measure of

subdivision between populations: $D = -\log_c I$. It is the mean number of electrophoretically detectable amino acid substitutions per locus for the time period the two populations being compared have diverged from a common ancestor.

RESULTS

Three subpopulations of Macrobrachium lar from southern Guam were compared to determine genetic variation within and between populations in similar environments. Twelve enzymes and proteins from tail muscle of M. lar were assayed (Table 1). Nineteen zones of activity were under independent genetic control (Table 2). At 15 of these zones all individuals exhibited bands of nonvariable mobility. They were assumed to be encoded by a single monomorphic locus at which every individual was homozygous for the same allele. Four zones exhibited multiple allozymes of single and double band phenotypes: Est I, Got I, Ndh and Suc. The protein designated as Ndh was described by Tracey et al. (1975) as "tetrazolium reductase". This multiple allozyme banding pattern was expected if the protein or enzyme was a monomer and the double band phenotypes were heterozygotes.

Each stream subpopulation was polymorphic at 21.1% of the zones of activity or loci. The average value of polymorphic loci falls within ranges found for other invertebrate species (Table 3). The number of genomes assayed per locus is twice the number of individuals sampled in each population. All genetic variability was found at Est I, Got I, Ndh, and Suc loci.

Genetic variation was calculated for three subpopulations of Macrobrachium lar, which were sampled from the Asalonso, Asmafines and Sella streams (Table 4). The total heterozygosity found in M. lar subpopulations was 9.4% ± 0.196 observed frequency and 9.5% ± 0.187

Table 1. List of enzyme systems and buffers; symbols and references.

	ENZYME SYSTEMS	SYMBOL	REFERENCE
1.	Acid phosphatase	Acph	Brewer 1970
2.	Alcohol dehydrogenase	Adh	Selander et al. 1971
3.	Aldehyde dehydrogenase	Ao	Selander et al. 1971
4.	Esterase	Est	Selander et al. 1971
5.	Glutamate dehydrogenase	Gdh	Selander et al. 1971
6.	Glutamic oxaloacetic transaminase	Got	Selander et al. 1971
7.	Indophenol oxidase	Ipo	Selander et al. 1971
8.	Leucine amino peptidase	Lap	Selander et al. 1971
9.	Malate dehydrogenase	Mdh	Selander et al. 1971
10.	Mannose phosphate isomerase	Mpi	Selander et al. 1971
11.	Nothing dehydrogenase	Ndh	Selander et al. 1971
12.	Phosphoglucomatase	Pgm	Selander et al. 1971
13.	Succinate dehydrogenase	Suc	Selander et al. 1971

BUFFER	ENZYME SYSTEM	REFERENCE
System A	Ao	Poulik 1957
System B	Got, Ipo, Mpi	Tracey et al. 1975
System D	Pgm	Tracey et al. 1975
Lioh	Lap, Acph	Selander et al. 1971
Tris-Hcl/Borate	Adh, Idh, Mdh, Ndh	Selander et al. 1971
Tris-Hcl/Glycine	Est, Gdh, Ipo, Suc	Cheney and Babbel 1978

Table 2. Proportion of loci found to be heterozygous in each subpopulation of Macrobrachium lar.

LOCUS	STREAM SUBPOPULATION		
	ASALONSO	ASAMAFINES	SELLA
Acph	N	N	N
Adh	N	N	N
Ao	N	N	N
Est I	0.467	0.273	0.326
Est II	N	N	N
Est III	N	N	N
Est IV	N	N	N
Gdh	N	N	N
Got I	0.700	0.491	0.558
Got II	N	N	N
Ipo I	N	N	N
Ipo II	N	N	N
Lap I	N	N	N
Lap II	N	N	N
Mdh	N	N	N
Mpi	N	N	N
Ndh	0.467	0.273	0.519
Pgm	N	N	N
Suc	0.617	0.218	0.558

N = no heterozygotes observed

Table 3. Estimates of genetic variation for selected invertebrate species.

SPECIES	HETEROZYGOSITY TOTAL	INDIVIDUAL	LOCUS	REFERENCE
Drosophila affinis		0.253		Ayala, 1979
Drosophila simulans		0.800		Ayala, 1979
Drosophila tropicalis			0.152	Ayala, 1979
Drosophila willistoni		0.177	0.170	Ayala, 1979
Euphausia superba	0.057	0.058		Ayala and Valentine, 1978
Homarus americanus		0.220	0.039	Tracey et al., 1975
Homarus gammarus			0.033	Hedgecock et al., 1977
Limulus polyphemus	0.067			Ayala and Valentine, 1978
Macrobrachium ohione	0.098	0.170	0.171	Trudeau, 1977
Macrobrachium rosenbergii	0.028	0.028		Hedgecock et al., 1979
Macrobrachium sp. (4 sp.)		0.161	0.076-0.195	Trudeau, 1977
Mytilus edulis		0.130		Skibinski et al., 1980
Palaemonetes pugio		0.235	0.068	Fuller, 1977
Tridacna maxima	0.216	0.202		Ayala and Valentine, 1978
Decapod crustaceans (8 sp.)		0.014-0.082		Hedgecock et al., 1976
Decapod crustaceans (50 sp.)	0.055			Nelson and Hedgecock, 1980
Invertebrates (24)	0.150	0.250-0.300		Selander and Kaufman, 1973

Table 4. Genetic variation in 3 subpopulations of Macrobrachium lar.

	STREAM SUBPOPULATION			
	Asalonsa	Asamafines	Sella	Total
Number of individuals	60	55	52	167
Number of loci	19	19	19	19
Mean number of heterozygotes per individual	0.101 ± 0.053	0.133 ± 0.056	0.116 ± 0.061	0.116 ± 0.053
Mean number of heterozygotes per locus	0.119	0.066	0.103	
Total number of heterozygotes observed	129	69	102	
Total number of heterozygotes expected	111	91	101	
Genetic similarity	0.970	0.950	0.966	0.962
Genetic distance	0.030	0.051	0.035	0.039
Total heterozygosity observed				0.0941 ± 0.196
expected				0.0951 ± 0.187

expected frequency. Total heterozygosity is calculated from the combined subpopulation frequencies for each locus. A significant statistical difference ($P<0.05$) occurred between these expected and observed frequencies (Sokal and Rohlf, 1969; chi-square test for goodness of fit). The chi-square test was run on the heterozygosity values for each locus and subpopulation. Significant differences ($P<0.05$) occur between observed and expected heterozygote frequencies at the Asalonsa and Sella streams for the Got I and Suc loci (Table 5). Both locations have greater numbers of heterozygotes than would be expected based on the Hardy-Weinberg equilibrium equation. The observed value for the entire population was typical of heterozygosity levels recorded for other species of Macrobrachium and decapod crustaceans (Table 3).

Another measure of genetic variability is the average heterozygosity per locus. Since only 4 of the 19 loci sampled were polymorphic, the average heterozygosity was low (9.6%). Average subpopulation heterozygosities ranged from 6.6% in the Asmafines stream to 11.9% in the Asalonso stream. These values fall within the range of mean heterozygosities per locus recorded for other crustaceans (1.4%-19.5%, Table 3).

Heterozygosity values can be quite variable within invertebrate species (Table 3). The mean heterozygosity per individual for M. lar has a range from 10.1% in the Asalonso stream to 13.3% in the Asmafines stream, with a mean value of 11.6%. This average value is slightly lower than the 16.6% found by Trudeau (1977) for 4 Macrobrachium species but is within the 7.6-20.8% range.

Table 5. Gene frequencies of heterozygous loci for stream subpopulations.

	STREAM SUBPOPULATION											
	Asalonsa		Asamafines		Sella		Total		Mean		Standard Error	
Locus	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Est I	0.467	(0.498)	0.273	(0.397)	0.326	(0.440)	0.359	(0.449)	0.100	(0.051)	0.355	(0.445)
Got I	0.700	(0.420)	0.491	(0.500)	0.558	(0.493)	0.589	(0.473)	0.107	(0.044)	0.583	(0.471)
Suc	0.617	(0.470)	0.281	(0.341)	0.558	(0.493)	0.467	(0.437)	0.215	(0.082)	0.464	(0.435)
Ndh	0.376	(0.465)	0.273	(0.397)	0.519	(0.499)	0.383	(0.455)	0.124	(0.052)	0.389	(0.454)
Total	0.538	(0.425)	0.314	(0.409)	0.490	(0.611)						
Mean	0.540	(0.463)	0.314	(0.414)	0.490	(0.481)						
Standard Error	0.146	(0.032)	0.121	(0.066)	0.110	(0.028)						

The intra-locus variance was calculated to make comparisons between subpopulations and loci possible (Table 5). Although there was a wide range of variation between loci, no significant differences ($P<0.05$) were observed. Heterozygosity for Est I, Got I and Suc was highest in the Asalonso stream. This stream had a greater than expected number of observed heterozygous M. lar. The Sella stream exhibited the highest Ndh heterozygosity value. All heterozygosity values for the Asmafine stream were intermediate between the Asalonsa and Sella stream values. A significant difference between locations ($P<0.05$) occurs for 2 of the 4 variable loci, Got I and Suc. However, the total intra-locus heterozygosity values did not differ significantly between streams. The Asalonso stream exhibited the highest mean intra-locus value (54.8% ± 0.146) and the Asmafines stream had the lowest values (31.4% ± 0.121). The highest heterozygosity occurred within the Asalonso stream in the Got I locus (58.3% ± 0.107).

Fifteen loci were nonvariable. The Acph, Adh, Ao, Gdh, Mpi and Pgm loci each appeared to be composed of a band of very high molecular weight which migrated very slowly and aggregated near the site of application at the cathodal end of the gels. The Est III and Est IV and Got II were single fast moving bands. The Lap and Ipo enzymes appeared to have 2 independent zones of activities, one fast and one slow; these were assumed to be 2 loci. The Est II and Mdh loci appeared to be fast moving double bands of activity which were probably dimeric molecules.

Each polymorphic locus exhibited a two-allele system symbolized as A and A'. Phenotypes homozygous for the A' allele were not observed. Table 6 presents for each subpopulation the sample size, allele

Table 6. Genotype proportions and allele frequencies for heterozygous loci.

ENZYME	SUB-POPULATION	SAMPLE SIZE	GENOTYPES Observed (Expected) A/A	A/A'	A'/A'	ALLELE FREQUENCY A	A'	CHI SQUARE
EST	Asa	60	32(35.2)	28(21.5)	0(3.3)	0.767	0.233	5.5602
	Asm	55	40(41.0)	15(13.0)	0(1.0)	0.864	0.135	1.3321
	S	52	35(36.4)	17(14.2)	0(1.4)	0.837	0.164	2.0056
	Total	167	0.641	0.359	0			2.8249
GOT	Asa	60	18(25.3)	42(27.3)	0(7.4)	0.650	0.350	17.4279*
	Asm	55	28(31.3)	27(20.4)	0(3.3)	0.755	0.245	5.7832
	S	52	23(27.0)	29(20.9)	0(4.0)	0.721	0.279	7.7318*
	Total	167	0.423	0.587	0			13.1899*
NDH	Asa	60	38(40.0)	22(18.0)	0(2.0)	0.817	0.183	2.9889
	Asm	55	40(41.0)	15(13.0)	0(1.0)	0.864	0.136	1.3321
	S	52	25(19.0)	27(30.0)	0(3.0)	0.740	0.260	5.1947
	Total	167	0.677	0.383	0			1.4966
SUC	Asa	60	23(28.7)	37(25.6)	0(5.7)	0.692	0.308	11.9123*
	Asm	55	43(43.6)	12(10.7)	0(0.7)	0.891	0.110	0.8662
	S	52	23(27.0)	29(20.9)	0(4.0)	0.721	0.280	7.7318*
	Total	167	0.473	0.527	0			

* Significant difference ($P<0.05$).

frequencies, observed and expected number of phenotype and genotype frequencies. Genotype frequencies between subpopulations were tested with a test of equality for two percentages (Sokal and Rohlf, 1969). All loci exhibited a double banded phenotype for heterozygotes and only "A" allele homozygous individuals. In the Ndh locus the frequency of heterozygotes and the allele ratios between streams did not differ from the Hardy-Weinberg expectation. The frequency of heterozygotes for the Suc locus was significantly different between the Asmafines and Sella streams and the Asmafines and Asalonso streams. The number of heterozygotes exceeded the expected value which indicated a lack of interbreeding between subpopulations. In all 3 subpopulations the Est I locus did not differ from the expected Hardy-Weinberg value of heterozygous individuals. However, the Asalonso and Asmafines streams were significantly different with respect to the frequency of heterozygotes within each subpopulation. The Got I locus differed from the Hardy-Weinberg expected number of heterozygous individuals. An excess of heterozygotes occurred for both the Sella and Asalonso streams. The frequency of heterozygotes differed significantly between the Asmafines and Asalonso subpopulations which indicated a lack of interbreeding between subpopulations (Table 7).

Heterozygosity values for the 19 loci assayed reveal that the 3 subpopulations have very similar genotypic frequencies (Tables 2 and 5). Variance is evenly distributed within and between subpopulations. To evaluate the effect of genetic drift the amount of reproductive isolation (subdivision) between subpopulations must be determined. The amount of subdivision and differentiation among subpopulations is quantified by Nei's (1972) measures of genetic identity or similarity

Table 7. Paired comparisons of subpopulation heterozygosity frequencies, test of equality of two percentages.

	Asa/Asm	Asa/S	Asm/S
Est I	2.24*	1.61	0.58
Got I	2.81*	1.94	0.80
Ndh	1.07	1.80	2.79*
Suc	4.76*	0.78	3.84*

* Significant difference ($P<0.05$).

(I) and genetic distance (D). Genetic identity is the average probability per locus of selecting two electrophoretically identical alleles from two different populations. Genetic identity is calculated directly from gene frequency data (Table 5). Genetic distance, based on the identities within and between populations, is a negative logarithmic transformation of I (genetic identity). It is theoretically the mean number of electrophoretically detectable amino acid substitutions per protein that have occurred since the 2 populations diverged from a common ancestor.

Macrobrachium lar subpopulations are genetically similar (Table 4). Genetic identity and genetic distance average 0.962 and 0.039, respectively. The genetic identity values fall within the 0.95-1.0 range which Ayala et al. (1974) considered to be identical. Each subpopulation exhibited approximately the same degree of amino acid substitutions.

To determine whether sex or size influenced genetic variation, frequency data of the four variable loci for all three subpopulations were pooled and each factor tested with a one way anova (Sokal and Rohlf, 1969). No significant statistical difference ($P<0.05$) was found for size or sex classes.

The amount of genetic divergence among subpopulations was no greater than that expected between independent random samples from a single large population. Macrobrachium lar appears to be genetically homozygous over the range of loci sampled, based on the lack of significant differences between allele frequencies for specific loci. However, this did not conclusively indicate gene flow between subpopulations. Since only a small number of loci were sampled, it was

possible that further investigation could have revealed gene frequencies significantly different between subpopulations.

DISCUSSION

In the modern synthesists' view the evolution of populations occurs by changes in frequency of genes and genotypes. The process of genetic variation in a population is a complex interaction of forces which include mutation, migration, random drift, and natural selection. These forces act on the gene pool to increase, decrease, or stabilize the amount of variation. Mutation, migration (gene flow between populations), and genetic drift are random or stochastic processes which bring about changes independently of the effects these changes have on populations. Natural selection, a deterministic process which increases the frequency of useful variants, is the only adaptive force of evolutionary change.

The process of evolution has two dimensions: anagenesis or phyletic evolution and cladogenesis or splitting (Ayala, 1975). Anagenic or phyletic evolution is changes with time occurring in a population or group of populations. These changes result in increased adaptation to the environment and often reflect changes in the physical or biotic conditions of the environment. Cladogenesis occurs when a phyletic line splits into two or more independently evolving lineages. Most evolution has been associated with splitting of lineages or the process of speciation. Species are discrete and independent evolutionary units. The speed of divergence is variable relative to morphological change. Evolution makes use of already available variants and is not limited by the appearance rate of novelties which are caused by new

mutations (sibling species may differ by as a little as 10% of their genome). Considerable evolutionary change can occur without the appearance of novel genes. The chief contributor to the process of speciation is the immense array of genetic variation that exists in populations of sexually reproducing organisms.

The question of how much differentiation occurs during the speciation process is the cardinal problem of evolutionary genetics. Estimates of genetic differentiation measure the amount of change in cladogenesis and anagenic evolution.

Variance of gene frequencies within and between populations depends on the magnitude of differences between evolutionary forces. If the environments inhabited by populations are very similar, it can be assumed that genetic variance does not result mainly from adaptations to that habitat. Similarly, gene frequencies would not vary if mutation rates are equal or negligible and population sizes are similar. Variations in gene frequencies would result from random chance or the introduction of new alleles by migration.

The selection of sampling streams in southern Guam was designed to minimize the effects of selection, mutation and population size. Since each stream was approximately the same length, population size was assumed to be equal for all locations. The environments were assumed to be similar since all streams originate at similar altitudes, receive comparable amounts of annual rainfall and open to the ocean. The mutation rate was assumed to be minimal, so low that the average heterozygosity of the population would not be changed. Two of these streams were close to allow for possible short distance migration and the third stream was geographically isolated to allow for possible long

range migration. If there were migration between streams, genetic variation within populations would be high with low variation between populations. If no migration occurred between streams, genetic drift would cause low variation within stream populations, but high variation between them.

Populations of oceanic islands were often founded by a small number of individuals. Guam, located in the Kuroshio Current, could have originally been populated by few Macrobrachium lar swept north and west from the Eastern Caroline Islands. The number of alleles in the newly founded colony on Guam would have been reduced compared to the source population. This was because rare alleles in the source population were likely to be missing from the new colony, which contributed to the level of heterozygosity. If a colony remained small, heterozygosity declined rapidly because of inbreeding. The genotype frequency changed but not the allele frequency. Therefore heterozygosity could build slowly by mutation and drift unless selection increased the frequency of rare alleles (Futuyma, 1979). However, if a population expands rapidly after colonization, inbreeding will be low and heterozygosity levels will be maintained.

Genetic variance is controlled by random drift in small isolated subpopulations with high incidences of inbreeding. Heterozygosity is lost through inbreeding because of the genetic similarity between mating individuals. In infinitely large, randomly breeding populations, mating usually occurs between close neighbors, which causes the formation of subpopulations. Local differentiation would continue until one allele or another becomes fixed and variation at a locus is lost. The homogeneity within subpopulations is balanced by the

variation between populations. Therefore, the variation of the population as a whole remains the same.

For random drift to be effective, isolation between subpopulations must be sufficient to effectively restrict migration (Crow and Kimura, 1970). Conversely, when sufficient migration occurs (as infrequently as one migrant in alternate generations) the fixation of alleles is slow and the distribution of allele frequencies becomes less variable between subpopulations. Rates of migration are not only a function of geographic proximity but also of the vagility (mobility or migratory power) of the species and rates of dispersal. The values for M. lar reflect a trend noted by Selander and Kaufman (1973), Tracey et al. (1975) and Trudeau (1977). Large, more mobile animals tend to be less heterozygous than smaller, more stationary species. Selander and Kaufman (1973) reported that highly mobile marine species with pelagic larvae are less heterozygous than more sessile species with nonpelagic larvae. This correlation is based on a theoretical model presented by Levins (1968), which suggests the heterozygosity of a species varies as it perceives its environmental conditions. A species which perceives its environment as highly variable (coarse-grained) will exhibit a high degree of heterozygosity to cope with a wide range of conditions. A more fine-grained environment would be perceived as less variable and the adaptive strategy would be to maintain fewer alleles and exhibit lower levels of heterozygosity. Fuller (1977) found lower heterozygosity values in small isolated populations of the grass shrimp Palaemonetes pugio than in the large, bay populations open to migration.

In the absence of migration data the best way to determine whether natural populations were subdivided was to look for indications of genetic differentiation. Genetic differences between subpopulations are measured with the chi-square test of observed frequencies, test of equality of two percentages, one-way anova and Nei's measures of genetic similarity and genetic distance. The M. lar population on Guam is not subdivided if the three subpopulations studied for this report are typical of all Guam subpopulations. The chi-square values for the 4 polymorphic loci are significant for Got I and Suc ($P<0.05$). Both loci have an excess of heterozygotes at the Asalonso and Sella stream subpopulations. However, the majority of variation is homogeneously distributed in these 3 locations. Paired comparisons of heterozygosity values between streams reveal significant differences ($P<0.05$) between the Asmafines and Asalonso streams at the Est I and Suc loci. The Asmafines and Sella streams differ at the Ndh and Suc loci. The occurrence of greater than expected numbers of heterozygotes may result from heterosis or the heterozygote superiority over the homozygous geneotype. The frequencies of heterozygotes between the Asalonso and Sella streams do not differ at any of the polymorphic loci. These differences between the subpopulations would indicate that some local differentiation is occurring within streams. However, mean heterozygosity values within and between streams do not significantly differ. This lack of variance could result from migration between and within subpopulations which is sufficient to maintain similar levels of heterozygosity, thereby effecting an overall homozygous population. Any differences in frequency could be attributed to random chance, i.e., genetic drift (Lewontin, 1974). Polymorphisms in human blood groups

(Lewontin, 1974), shell characteristics in the snail Cepea nemoralis (Lewontin, 1974) and patchy frequency of genes in the house mouse Mus musculus (Selander et al., 1969) have been attributed to genetic drift.

Although only homozygous "A" individuals were found, it was not possible to ascertain if alleles for variable loci were fixed in the population. A larger sample size would be required to make this distinction. It was not known whether the departures from Hardy-Weinberg equilibrium for a randomly mating population were a result of chance alone, sampling error or scoring error.

Evidence of a panmictic population can be further substantiated by using Nei's statistics. Genetic similarity or identity values for paired sets of heterozygosity data between streams show no location differentiation. These values fall within the 0.95-1.00 range of population identity (Ayala et al., 1974). The amounts of amino acid substitutions (genetic distance) between each pair of streams are also approximately equal which reveals little or no differentiation between populations.

Based on the genetic identity values it would appear that the geographic range of M. lar in southern Guam is not greater than its migratory ability (vagility and dispersal). Statistical theories such as the island (Wright, 1943), stepping stone (Dobzhansky, 1970) and isolation-by-distance models, predict that migration rates should be correlated with the distance between each subpopulation and the degree of differentiation. This does not apply to Guam since the population is genetically not subdivided. Distance does not appear to inhibit migration.

Genetic similarity of Macrobrachium lar subpopulations is probably maintained by migration through vagility and dispersal. The Macrobrachium lar population in southern Guam is essentially a panmictic association of randomly breeding individuals. However, differences in heterozygosity levels between loci reveal local differentiation without the interruption of gene flow.

CONCLUSIONS

Three subpopulations of *Macrobrachium lar* from southern Guam were assayed for electrophoretically detectable variations in enzyme/protein composition. Within and between subpopulations comparisons were made. The following conclusions were made from this study:

1. The three subpopulations of *M. lar* appear to be genetically similar.

2. Although a small amount of local differentiation was found, genetic variation is evenly distributed over the range of loci sampled.

3. Lack of significant differences between subpopulations may result from migration and larval dispersal. However, the findings of this limited study can not be presented as conclusive evidence of gene flow between subpopulations.

4. Slight differences in frequency of loci can be attributed to genetic drift.

5. Heterozygosity values for *M. lar* fall within ranges found for other invertebrate species.

6. The sex and size of individuals does not influence genetic variability.

7. The amount of genetic divergence among subpopulations is no greater than that expected between independent random samples from a single large panmictic population.

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APPENDIX A

ELECTROPHORESIS TECHNIQUE

Electrophoretic techniques have been widely used by population geneticists to measure the levels of heterozygosity found in natural populations (Avise, 1975; Ayala and Valentine, 1978; Brewer, 1970; Lewontin and Hubby, 1966; Lester, 1979; Lewontin, 1974; Selander, 1976; and Trudeau, 1977). The technique applies the physiochemical properties of proteins to the characterization of gene frequencies. The observation of alternate alleles of various locus can be used to determine differences between individuals, populations, species and to a limited extent higher order taxa.

Electrophoresis detects variation in the primary configuration of proteins and enzymes, which are products of structural genes. Structural genes code for the amino acid sequence of proteins.

Alternate forms of a gene are alleles. A locus is a position on a chromosome where there is a gene for a particular trait. A locus can have any allele which codes for a protein or enzyme. Loci identification are made by their coded products, e.g., the esterase locus code for the enzyme esterase. Isozymes are different molecular forms of enzymes which share a common substrate or catalyze the same reaction. Allozymes are multiple protein products of a single gene locus (Avise, 1975). A different amino acid sequence in one or more of the polypeptide chains that make up proteins and enzymes is an allele difference or allozyme.

Electrophoresis detects allozyme differences by measuring mobility of an enzyme or protein in an electric field. Migration rates vary depending on the net electrical charge, size and shape of the protein or enzyme molecule. Proteins which migrate different distances differ by at least one amino acid.

Separation of proteins by electrophoresis is done by the establishment of an electric current through an electrophoretic media. Proteins migrate with this current from the anode to cathode poles. In order to transmit current, the electrophoretic media must contain an ionized solution or buffer.

Electrophoresis can only detect amino acid substitutions which cause changes in the net electrostatic charge of a polypeptide. However, proteins and enzymes with identical mobility do not necessarily have identical amino acid sequences. This is because 16 of the 20 common amino acids have nonionizable side chains and are electrophoretically neutral in the pH range of buffers. Only four amino acids have charges; argine and lysine are basic (positive) and glutamine acid and aspartic acid are acidic (negative).

Positive amino acids, arginine and lysine, have an ammonia group which is in dynamic equilibrium between a neutral and a positively charged form:

$$H^+ + -C-NH_2 \rightleftharpoons -C-NH_3^+$$

The negative amino acids, glutamic acid and aspartic acid have a carboxylic acid R group which is in dynamic equilibrium between neutral and negatively charged forms:

$$H^+ + -C-\overset{\overset{O}{\|}}{C}-O^- \rightleftharpoons -C-\overset{\overset{O}{\|}}{C}-OH$$

A polypeptide made up of positive, negative and neutral amino acids will have a net electrostatic charge, (+ or -), which is dependent on the balances of charges and the folding conformation of the molecule. In globular proteins (enzymes), amino acid charges tend to be on the surface, while hydrophobic neutral amino acids have charges in the interior (Brewer, 1970; Lewontin, 1974; and Selander, 1976).

The charge on the polypeptide as a whole depends on the pH or hydrogen ion concentration of the buffer in the support media. The protein is neutral as its isoelective point. It has the same number of charged carboxyl and ammonia groups. As the pH is lowered (increasing the hydrogen ion concentration), the ammonia groups (NH_2) are progressively ionized and become positively charged ions (NH_3^+), while the acidic carboxyl ions (COO^-) are at saturation and netural. The polypeptide as a result assumes a positive charge. The reverse will happen with an increase in pH. The isoelectric point of most proteins is around pH8. The net electrical charge on a protein at a specific pH will depend on the number of ion groups on the surface of the amino acids. The greater the charge on a protein the faster it will move in an electrical field toward the electrode with opposite charge.

If an allele change at a locus results in the replacement of an amino acid in one group with one of an opposite charge, there is an alternation of the protein isoelectric point. These changes in net charge separate proteins and thus identify allozymes. Separation of proteins can also be affected by molecular sieving in the electrophoretic medium. Acrylamide electrophoretic media has pore sizes

similar to protein molecules. Therefore, the size and shape of the protein molecule will affect its rate of migration.

Variation in the concentration of acrylamide, between 5-30% can change the gel pore size. A decrease in acrylamide concentration causes an increase in pore size, while an increase in concentration produces smaller pore sizes. This property allows for the construction of gel sieves with variable pore sizes.

I used a vertical slab gel electrophoresis apparatus for my experiments. The support media was a polyacrylamide gel of graduated concentration from 5-20%. The gradient was established by controlling the mixture of a separate acrylamide solutions (5% and 20%). The dense 20% solution settled toward the slab bottom, while the lighter less concentrated mixtures the top. A pore size gradient was established, in this manner.

Opposite poles of a power source were connected to the ends of the slab. The anode at the top and the cathode at the bottom samples were applied to wells in the top of the gel. The gel buffer pH was adjusted to the alkaline side of the isoelectric point of the protein studied. Therefore, the proteins were negatively charged and migrated toward the positive cathode pole. Protein or enzyme migration rates were dependent on the net electrical charge, molecular size and shape, and magnitude of the the electrical current.