ALLOZYMIC VARIABILITY AND HETEROZYGOTE DEFICIENCY WITHIN AND AMONG MORPHOLOGICALLY POLYMORPHIC POPULATIONS OF *LIGUUS FASCIATUS* (MOLLUSCA: PULMONATA: BULIMULIDAE)

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ABSTRACT

Allozymic variability was examined within and among seven morphologically variable hammock populations of *Liguus fasciatus* (Müller). These populations contained representatives of 14 named varieties of this species; each hammock contained at least two phenotypic varieties. Among 24 gene loci examined, only one (glucose phosphate isomerase) was variable either within or among populations. These data substantiate the existence of a single polymorphic species within these populations.

Very narrow (25 m) separations of some hammocks by water represent significant barriers to gene flow between populations of *Liguus fasciatus*. However, recent woody growth between two adjacent hammocks has facilitated bidirectional immigration of snails. Reproduction between immigrant and resident snails appears to have been minimal, either because of the recent nature of the immigration or because of self-fertilization or assortative mating by the immigrants.

Most populations have significant heterozygote deficiencies at the glucose phosphate isomerase locus compared to the expectations of Hardy-Weinberg equilibrium, probably an indication of some degree of self-fertilization. The limited phenotypic combinations of shell patterns and colors present in the study populations are also not consistent with the proposed independence of a number of phenotypic characters if reproduction occurs by outcrossing. Interpretation of the inheritance of morphological characters is hampered by a lack of knowledge concerning the mode or modes of reproduction in *Liguus fasciatus*; further study of codominantly inherited allozymic alleles should facilitate such investigations.

Tree snails of the genus *Liguus* occur in southern Florida, Cuba, and Hispaniola. Currently, five species are recognized, although well over 150 trivial names are applied to various distinctive varieties (Clench, 1946, 1954, 1965; Jones, 1979). Most of the morphological and nomenclatural variability occurs in the species *Liguus fasciatus* (Müller), which occurs in Florida and Cuba (including the Isle of Pines). In Florida, approximately 58 named varieties of *L. fasciatus* occur (Roth and Bogan, 1984); these often have been divided into various numbers of subspecies (Clench and Fairchild, 1939; Pilsbry, 1899, 1912, 1946; Simpson, 1929).

Much of the morphological variation in *Liguus fasciatus* occurs among, rather than within, populations. In southern Florida, most populations are restricted to tropical hardwood hammocks isolated by water, sawgrass, buttonwood, cypress, or pine forest. In many of these populations, only one or a few phenotypes occur; furthermore, many phenotypes are restricted to single areas (Deisler, 1982).

Roth and Bogan (1984) devised a system for describing phenotypic variation in Floridian populations of *Liguus fasciatus*. They designated shells on the basis of twelve characters, each character with from two to four possible states. Roth and Bogan (1984) stated that they chose characters "...in which the alternate states can be seen to segregate in randomly selected material." Under their system, theoretically there are 33,280 possible phenotypic combinations (49,152 genotypic combinations, but 15,872 of these cannot occur as logical phenotypes, because they describe variation in bands that are not expressed). However, the vast majority of these combinations have never been reported. Roth and Bogan (1984) reported a total of 97 phenotypic combinations of *L. fasciatus* shells that have been grouped into the 58 nominal Floridian taxa. These represent 0.3% of the theoretically possible phenotypic combinations. The majority of these phenotypes are known from hundreds or thousands of museum specimens, so the absence of most phenotypic combinations is puzzling if the various characters are independent. Furthermore, in many populations, two

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phenotypes exist sympatrically that differ in numerous character states, and yet no other combinations of these states are known from the populations (Pilsbry, 1946).

One possible explanation for the above observations is that the characters described by Roth and Bogan (1984) are not independent and that entire phenotypes (or large portions of phenotypes) are under control of one or a few tightly linked gene loci. Another possiblity is that reproduction is not always accomplished through outcrossing in *Liguus fasciatus*, although mating does precede egg-laying (Brown, 1978; Jones, 1954; Pilsbry, 1946; Simpson, 1929; Solem, 1961). Even though mating occurs, reproduction could occur by gynogenesis, or mating could be required for ovulation before self-fertilization can occur. It is also possible that some phenotypes referred to the taxon *L. fasciatus* are reproductively isolated and specifically distinct. In order to discriminate among these possibilities, we examined the products of 24 enzyme loci in several morphologically variable populations of *L. fasciatus* by means of starch gel electrophoresis. Electrophoretic studies of codominantly inherited allozymes have proven to be a useful means of discriminating among reproductive modes in numerous organisms (Nevo, 1978). In addition, allozymic studies have been invaluable in determinig whether cases of morphological variation are the result of intraspecific polymorphism or reproductive isolation (see Hillis and Patton, 1982, for another example from molluscs).

MATERIALS AND METHODS

Seven populations of *Liguus fasciatus* were sampled from hammocks in the vicinity of Pinecrest, Big Cypress National Preserve, Florida (see Pilsbry, 1946, for hammock numbering system); 329 individuals were collected from these populations for allozymic analysis (Table 1). Samples were collected from throughout each hammock. All of the study populations contained at least two shell phenotypes, and one population (PC 88) contained nine named morphological varieties. Each of the varieties is described in Table 1 according to the system proposed by Roth and Bogan (1984). Some individuals classified under the *walkeri* phenotype could also be called *castaneozonatus,* depending on the degree of uniformity of the major bands. Because these two phenotypes seem to form a continuum in the study populations, the two categories were lumped under the *walkeri* class.

Initial screening of allozymic loci involved 20 to 40 individuals drawn from the various populations. Twenty-four presumptive gene loci were scored: creatine kinase (2.7.3.2), ten esterase loci (3.1.1.1), glucose phosphate isomerase (5.3.1.9), isocitrate dehydrogenase (1.1.1.42), two lactate dehydrogenase loci (1.1.1.27), two malate dehydrogenase loci (1.1.1.37), mannose phosphate isomerase (5.3.1.8), peptidase A, B, C, and S (3.4.11.13), peptidase D (3.4.13.9), and phosphoglucomutase (2.7.5.1) (Enzyme Commission numbers follow Bielka *et al.,* 1984). All individuals were then scored for variation at polymorphic loci.

Standard procedures of horizontal starch gel electrophoresis were employed (see Selander *et al.,* 1971). Snails were ground and diluted 1:1 in 0.01 M tris-0.001 M EDTA-0.001 M 2-mercaptoethanol, pH 7.5. Homogenates were centrifuged at 10,000 g for 5 min and then the supernatant was refrozen at −85°C for up to three months prior to use. Two buffer systems were used: TBE 9.1 (175.0 mM tris-17.5 mM boric acid-2.75 mM EDTA, pH 9.1) and Poulik (gel: 0.076 M tris-0.005 M citric acid, pH 8.7; electrode: 0.30 M boric acid, pH 8.2). Gels were prepared from 50% Sigma starch (lot 85F-0010) and 50% Otto Hiller electrostarch (lot 392). Gels were 12% starch for both systems. Two drops of

Table 1. Morphological characters of varieties of *Liguus fasciatus* examined and distribution of varieties within study populations. Shell phenotype characters follow Roth and Bogan (1984); C: ground color of shell (Y: yellow; W: white); B: *dryas* bands (B: brown; Y: yellow; BY: both brown and yellow; O: absent); S: spreading of *dryas* band pigment; E: vacant center of *dryas* bands (B: brown band; Y: yellow band); U: absence of one *dryas* band; M: marbling of *dryas* bands; L: sutural line (B: brown; Y: yellow; O: absent); P: peripheral line (B: brown; Y: yellow; O: absent); A: pink apex; O: pink columella; W: white suffusion; G: periostracal green lines.

	Pinecrest Hammock Co.							Shell Phenotype Characters											
Variety	1a	10	11	14	16	16a	88	C	B	S	E	U	M	L	P	A	O	W	G
aurantius	--	1	--	--	1	1	6	Y	Y	+	Y	—	—	O	O	—	—	—	+
barbouri	--	17	--	--	6	32	--	Y	BY	+	B	—	+	B	B	—	—	+,—	+
clenchi	--	--	--	--	--	--	3	Y	B	+	B	—	+	O	O	+	+	—	+
elegans	--	--	--	1	--	--	--	W	O	—	—	—	—	B	B	+	+	—	+
floridanus	--	--	--	--	--	--	5	Y	BY	+	B	—	+	B	B	—	—	—	+
livingstoni	--	5	--	--	--	--	22	W	Y	—	—	—	—	O	O	+	+	—	+
lossmanicus	--	--	12	--	--	--	47	Y	O	—	—	—	—	O	O	—	—	—	+
lucidovarius	--	--	1	--	--	--	--	W	BY	+	—	—	+	B	B	—	—	—	+
miamiensis	--	5	--	--	--	--	--	W	BY	—	—	—	+	O	O	+	+	—	+
mosieri	--	--	--	--	--	--	9	W	O	—	—	—	—	O	O	—	—	—	+
ornatus	--	--	--	--	--	--	8	Y	Y	—	—	—	—	Y	Y	+	+	—	+
roseatus	3	4	--	--	5	--	1	W	Y	—	—	—	—	Y	Y	+	+	—	+
testudineus	--	--	--	--	--	--	1	Y	B	+	B	—	+	B	B	+	+	—	+
walkeri	27	20	--	40	44	2	--	W	BY	—	B,—	—	+	B	B	+	+	+,—	+

2-mercaptoethanol were added to the gel mixture after boiling and degassing. Gels were electrophoresed for 10 to 14 hr at 12.5 V/cm. Histochemical staining procedures followed Harris and Hopkinson (1976), Siciliano and Shaw (1976), and Selander *et al.* (1971).

RESULTS

All of the loci examined were monomorphic for a single allele except for the glucose phosphate isomerase locus. Two alleles were present at this locus and were designated fast (F) and slow (S). Five of the populations (PC 10, 14, 16, 16a, and 88) were polymorphic for these two alleles, whereas the other two populations (PC 1a and 11) were fixed for the fast allele (Table 2).

Genetic distances (Hillis, 1984) between populations ranged from 0 to 0.03; genetic distances between varieties pooled across populations ranged from 0 to 0.04. Among populations polymorphic for glucose phosphate isomerase, observed frequencies of the heterozygous genotype were consistently lower than predicted for populations in Hardy-Weinberg equilibrium (Fig. 1). Deviations from Hardy-Weinberg equilibrium were significant in four of the five populations PC 10: $\chi^2 = 39.30$, df = 1, $p < 0.001$; PC 14: $\chi^2 = 20.22$, df = 1, $p < 0.001$; PC 16: $\chi^2 = 7.36$, df = 1, $p < 0.01$; PC 16a: $\chi^2 = 24.61$, df = 1, $p < 0.001$). Average individual heterozygosity ranged from 0 in PC 1a and 11 to 0.016 in PC 88.

Table 2. Genotypes for glucose phosphate isomerase of *Liguus fasciatus* by population and variety.

Population	Variety	Genotype		
		FF	FS	SS
PC 1a	*roseatus*	3	--	--
PC 1a	*walkeri*	27	--	--
PC 10	*aurantius*	1	--	--
PC 10	*barbouri*	1	1	15
PC 10	*livingstoni*	--	--	5
PC 10	*miamiensis*	--	1	4
PC 10	*roseatus*	--	--	4
PC 10	*walkeri*	5	--	15
PC 11	*lossmanicus*	12	--	--
PC 11	*lucidovarius*	1	--	--
PC 14	*elegans*	1	--	--
PC 14	*walkeri*	14	6	20
PC 16	*aurantius*	--	--	1
PC 16	*barbouri*	4	1	1
PC 16	*roseatus*	--	1	4
PC 16	*walkeri*	7	14	23
PC 16a	*aurantius*	1	--	--
PC 16a	*barbouri*	30	1	1
PC 16a	*walkeri*	--	--	2
PC 88	*aurantius*	1	2	3
PC 88	*clenchi*	1	1	1
PC 88	*floridanus*	--	5	--
PC 88	*livingstoni*	2	6	14
PC 88	*lossmanicus*	6	17	24
PC 88	*mosieri*	2	5	2
PC 88	*ornatus*	1	4	3
PC 88	*roseatus*	--	1	--
PC 88	*testudineus*	--	--	1

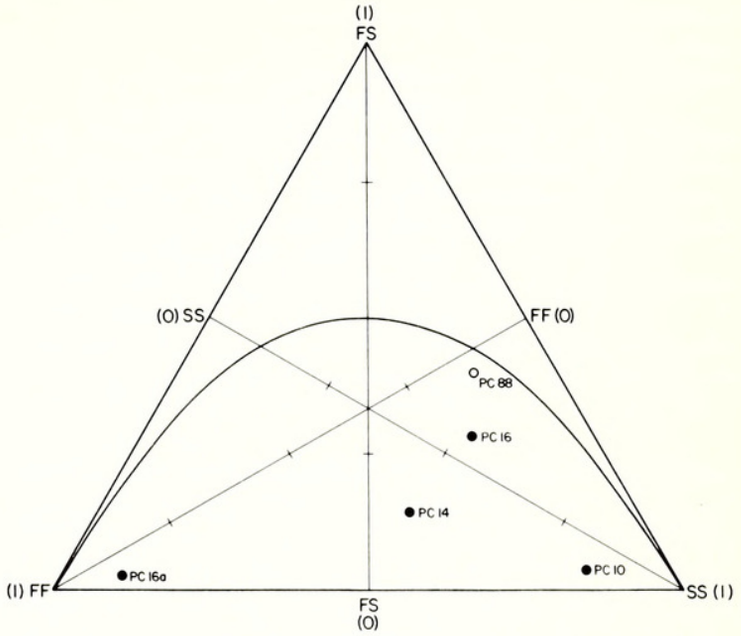

Fig. 1. Genotypic frequencies of populations of *Liguus fasciatus* variable at the glucose phosphate isomerase locus. Each of the three axes starts on one side of the triangle at a frequency of 0 and ends in a corner at a frequency of 1.0. The curve represents expected genotypic frequencies of populations in Hardy-Weinberg equilibrium. Populations represented by solid dots have a significant deficiency of heterozygous individuals ($p < 0.01$); the single population represented by an open dot does not differ significantly from Hardy-Weinberg expectations ($p > 0.05$).

DISCUSSION

Allozymic variation among morphotypes and populations of *Liguus fasciatus* is surprisingly low. The level of polymorphic loci per population in *L. fasciatus* (0 – 0.04) is lower than any other gastropod reported (Nevo, 1978), except for several self-fertilizing species (Selander and Kaufman, 1973a, b; McCracken and Selander, 1980). This is especially surprising because the normally highly polymorphic esterases and peptidases were included in this study. This low level of genetic differentiation clearly substantiates that the various phenotypes of *L. fasciatus* included in this study are conspecific.

Despite the low levels of genetic variability in *Liguus fasciatus* populations, variation at the glucose phosphate isomerase locus indicates that the water barriers between the hammock populations (Table 3) represent effective impediments to gene flow. With the exception of the two fixed populations (PC 1a and 11), all populations are significantly different in genotypic ratios at this locus (Fig. 1). Even very short water barriers appear to effectively isolate populations; for instance, PC 16 and 16a, separated by a narrow strip of water approximately 25 m wide (Table 3), support *L. fasciatus*

Table 3. Distances between hammocks in meters across water/sawgrass barriers.

	Pinecrest hammock number						
PC #	1a	10	11	14	16	16a	88
1a	----	950	1600	600	900	1050	4900
10		----	500	700	700	700	5900
11			----	1800	1900	2000	5800
14				----	45	250	5400
16					----	25	5600
16a						----	5750
88							----

populations that are significantly different in both phenotypic frequencies of the shells (Table 1) and genotypic frequencies at the glucose phosphate isomerase locus (Table 2). However, in this case there is some evidence of gene flow. In PC 16, shells are mostly of the *walkeri* phenotype (79%), with some *barbouri* (11%), *roseatus* (9%), and *aurantius* (1%) phenotypes. In contrast, PC 16a supports mostly *barbouri* (91%), with some *walkeri* (6%) and *aurantius* (3%). At the glucose phosphate isomerase locus, the S allele is dominant in PC 16, whereas the F allele is dominant in PC 16a (Table 2). For each phenotype except *barbouri* in PC 16, the dominant genotype is SS, whereas for *barbouri* it is FF (Fig. 2). Likewise, for each phenotype except *walkeri* in PC 16a, the dominant genotype is FF, whereas for *walkeri* it is SS (Fig. 2). In the 1930's and 1940's, the *barbouri* phenotype was not found in PC 16, and the *walkeri* phenotype was absent from PC 16a; dispersal apparently was occurring by the late 1970's, after some woody vegetation had grown up between the two hammocks (A. Jones, pers. comm.). This dispersal appears to have resulted in an influx of F alleles into PC 16 and S alleles into PC 16a (Fig. 2). Although dispersal by humans cannot be ruled out, it is likely that this represents natural dispersal, perhaps during periods of lowered water levels.

The genotypes of the suspected immigrant individuals in PC 16 and 16a are representative of their populations of origin (Fig. 2). Therefore, either these individuals represent first generation dispersals or the immigrants are mating preferentially among themselves (including the possibility of self-fertilization). The lack of observed genotypic differentiation among morphotypes in other hammocks reduces the likelihood of assortative mating.

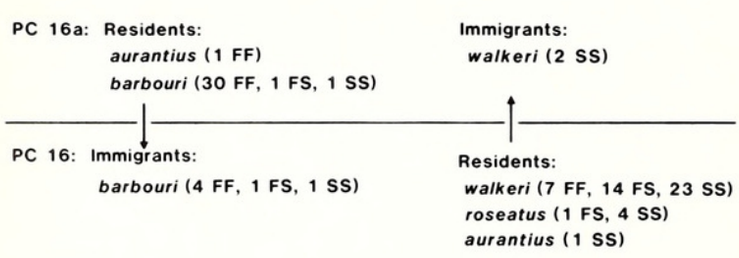

Fig. 2. Morphological phenotypes and glucose phosphate isomerase genotypes of resident and hypothesized immigrant *Liguus fasciatus* in hammocks PC 16 and 16a.

Although no studies have been conducted for confirmation, most investigators have assumed that individuals of *Liguus fasciatus* are obligate outcrossers. Clench (*In:* Young, 1960) and Brown (1978) considered parthenogeneis and self-fertilization to be unlikely in *Liguus*, for unspecifife reasons. The considerable deficiency of heterozygous individuals (Fig. 1), however, is indicative of some other mode other than outcrossing. Among other gastropods studied, degree of allozymic variation has been shown to be a strong indicator of the type of breeding system employed by the species. Among outcrossing gastropods, the percent of polymorphic loci and average individual heterozygosity are high, whereas in self-fertilizing species, average individual heterozygosity is very low and polymorphic loci are rare or absent (Selander and Kaufman, 1973a, b; McCracken and Selander, 1980). This pattern has also been observed in several other groups of hermaphroditic organisms (Brown, 1979; Harrington and Kallman, 1968; Nevo, 1978). The low levels of polymorphic loci in *L. fasciatus* (0 – 0.04) and the significant deficiencies of heterozygotes in four of five polymorphic populations are typical of self-fertilizing species. However, in one population (PC 88), there is no significant heterozygote deficiency ($\chi^2 = 1.11$, df = 1, $p > 0.05$). Several other pulmonates have been shown to consist of both self-fertilizing and outcrossing populations, or individuals may be facultatively self-fertilizing; furthermore, reproduction following copulation in *Philomycus* spp. can be either by self-fertilization or outcrossing (McCracken and Selander, 1980). The patterns of allozymic variability observed in this study indicate that multiple reproductive modes can be possible in populations of *L. fasciatus* as well.

A. Jones (pers. comm.) has made numerous introductions of *Liguus* into hammocks otherwise free of these snails. He has found that reproduction only occurs if two or more snails are introduced; single *Liguus* do not reproduce in isolation. These observations suggest that mating is essential for reproduction, but do not necessarily indicate outcrossing. Some reproduction could be by gynogenesis, in which spermatozoa from another individual are needed to stimulate embryonic development but make no genetic contribution. Alternatively, mating could stimulate ovulation, after which reproduction could be accomplished by self-fertilization. In either case, reproduction must include some outcrossing, because intermediate shells have been reported after a few generations of crosses of phenotypically distinct shells (Young, 1960).

Past attempts to study reproduction and inheritance in *Liguus fasciatus* have centered on morphological variation. However, until the potential reproductive modes of *L. fasciatus* are determined, analysis of inheritance of morphological variation will be hampered. The glucose phosphate isomerase locus, with two codominantly expressed alleles, provides a valuable tool for determining the mode or modes of reproduction in *L. fasciatus* populations. After this information is obtained, study of inheritance of morphological variation will be greatly facilitated.

In all of the study populations, it is clear that the morphological characters defined by Roth and Bogan (1984) are

not randomly segregating (Table 1). Instead, they exist as discrete combinations. Several of the characters always covary in these populations (e.g. characters L and P; also characters A and O; see Table 1). If the characters specified by Roth and Bogan (1984) are independent, then reproduction must be by self-fertilization or some form of parthenogenesis in these populations. Alternatively, the shell phenotypes of *Liguus fasciatus* could be specified by fewer loci than has been proposed.

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