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Inheritance of Isozyme Phenotype at Three Loci in the Freshwater Snail, *Goniobasis proxima:* Mother–Offspring Analysis and an Artificial Introduction

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A good deal of interest has recently focused on the genetics of Goniobasis *populations, with isozyme frequencies often a primary tool. I sampled 72* Goniobasis proxima *from a population naturally polymorphic at three enzyme loci,* Gpi, Opdh, *and* Est, *reared each in isolation, and electrophoretically analyzed 14 sibships produced. Since females apparently mate but once in their lifetimes, it was possible to infer paternal genotype and demonstrate that inheritance is Mendelian at these three loci. The three loci appear to assort independently. A pair of* G. proxima *populations fixed for alternative alleles at the three loci has been introduced into a small spring. Triply heterozygous F_1 hybrids have been recovered, although at a low frequency.*

KEY WORDS: isozymes; inheritance; introductions; *Goniobasis proxima;* snails.

INTRODUCTION

Snails of the genus *Goniobasis* (also often referred to as *Elimia*) have recently become the object of a growing number of investigations in evolution and population genetics (Chambers, 1978, 1980, 1982; Dillon, 1982, 1984a, b, 1985a; Dillon and Davis, 1980). Most of the several hundred species described from North America are restricted primarily to small streams, with population densities falling off markedly as the stream size increases. The isolation resulting from this unusual population structure can be so profound that

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hundreds of distinct populations may be available for study in a small geographic area.

Researchers have typically found striking isozyme differences between even neighboring conspecific populations. For example, in 25 populations of the southern Appalachian species, *Goniobasis proxima* (Say), the average pair of populations showed entirely different isozymes at between two and three of seven putative loci examined (Dillon, 1984a). Divergence of this magnitude raises a question about species concepts in *Goniobasis*. So several years ago I initiated a number of artificial introduction experiments to establish whether any reproductive isolation exists between populations currently considered to be *Goniobasis proxima*, using unique isozymes at several putative loci as genetic markers (Dillon, 1985b).

Thus it became important to establish not only the mechanism of inheritance of particular isozyme phenotypes, but also whether linkage relationships exist. Only Chambers (1980) has successfully bred and reared *Goniobasis* to verify the Mendelian interpretations given isozyme frequencies. Unfortunately, the results of only three of Chambers' crosses were available, and the low numbers of offspring (12, 8, and 7) rendered any conclusions tentative.

A primary obstacle to studies of this type has been the relatively long generation time seen in most species. For example, individual *G. proxima* hatching from eggs laid in the early spring of one year reach sexual maturity the spring or summer of the following year. Apparently mating takes place shortly after maturation and seems to occur but a single time in the life of each female (Dillon, 1985b). Females do not lay their first eggs until the spring 2 years after their own birth. Thus well over a year is required to carry a cross through one generation, and should a researcher wish to examine assortment between loci using classical techniques, over 3 years would be necessary.

The purpose of this paper is twofold. First, I examine inheritance at three enzyme loci in *G. proxima:* octopine dehydrogenase (*Opdh;* EC 1.5.1.11), glucose phosphate isomerase (*Gpi;* EC 5.3.1.9), and an arylesterase locus (*Est;* EC 3.1.1.2). I employ a mother-offspring method to circumvent the 3-year waiting period, taking advantage of the fact that females seem to mate only once. These three loci are among the six serving as genetic markers in an artificial introduction experiment initiated in 1982. Thus I also present *Opdh, Gpi,* and *Est* allele frequencies after one generation of hybridization between two diverse populations of *G. proxima* in the wild.

METHODS

Mother–Offspring Analysis

Heterozygosity is generally quite low in natural populations of *Goniobasis* (Dillon and Davis, 1980). However Dillon (1984a) discovered one population

Table I. Gene Frequencies at Three Polymorphic Enzyme Loci in *G. proxima* Population YAD2, Revised from Dillon (1984a)

1984 name	New name	Frequency
Gpi 98	G^S	0.066
Gpi 102	G^M	0.915
Gpi 105	G^F	0.014
Odh 106	O^S	0.297
Odh 110	O^M	0.308
Odh 111	O^F	0.390
Est 100	E^S	0.205
Est 103	E^M	0.510
Est 106	E^F	0.286

in northwestern North Carolina ("YAD2") substantially polymorphic at the *Gpi*, *Opdh*, and *Est* loci. Revised frequencies for the three most common alleles at each of these loci, based now on a sample of approximately 100 individuals, are given in Table I. Each allele has also been assigned a new symbol, simplifying the nomenclature used by Dillon (1984a).

On February 23, 1985, 72 adult *G. proxima* estimated to be approximately 2 years old were collected from population YAD2. These were placed individually in 72 one-quart (0.95-liter) jars, with aeration, and held in an environmental chamber at 15°C with 16 hr light and 8 hr dark. The diet was naturally occurring periphyton augmented by commercial fish food. Although temperature and photoperiod were manipulated to induce egg laying, the first juveniles did not hatch until April 15. Hatching was completed by June, and juveniles reached sufficient size for electrophoretic analysis in September.

Artificial Introductions

Considering all possible comparisons of the 25 *G. proxima* populations examined by Dillon (1984a), the most divergent pair was PHLP and LAUR. Population PHLP inhabits a tributary of the Dan River, an Atlantic drainage, in Franklin County, Virginia. Population LAUR inhabits a tributary of the Holston River, a Mississippi drainage, in Washington County, Virginia, about 150 km distant. As detailed by Dillon (1984a), the two populations do not have any isozyme bands in common at the *Opdh*, *Gpi*, or *Est* loci. In late August 1982, I introduced 500 snails from each of these two populations into Coyner Springs, near Waynesboro, Virginia, approximately 200 km to the northeast. There are no native *Goniobasis* in Coyner Springs.

An effort was made to transplant only 1-year-old snails, estimating from the small size. Any error would be to the inclusion of older snails, with no young-of-the-year snails transplanted. Thus all females transplanted can be

assumed to have been previously inseminated, and the snails born in the spring of 1983 should be pure PHLP and pure LAUR. In 1984 this generation was 1 year old and mated, presumably with some chance of outcrossing, and thus the young-of-the-year snails in 1985 could be considered the first filial generation. So in late June 1985, I collected 57 obviously newborn *G. proxima* from Coyner Springs and a like number of adult, roughly 2-year-old parental snails for analysis.

Analysis

Whole snails were ground in a 7% sucrose solution buffered at *p*H 7.4 with 0.05 M tris(hydroxymethyl)aminomethane and H_3PO_4. Samples were centrifuged and the supernatant was separated electrophoretically in gels of 14% hydrolyzed starch, a mixture of 3 parts Sigma starch to 1 part Electrostarch. A TEB 8.0 buffer system was used to resolve the esterase loci, while TC 6.0 was found most suitable for *Gpi* and *Opdh* (Dillon, 1985). Gels were sliced and stained using the recipes given by Dillon and Davis (1980) and Dillon (1982).

Goniobasis proxima esterase zymograms are qualitatively identical when α-naphthyl acetate, β-naphthyl acetate, or α-naphthyl propionate is supplied as substrate. Tissue preparations typically show approximately 6 to 10 isozyme bands. Although the origin of most of these bands is unknown, the products of several esterase loci are doubtless represented. Isozymes produced by the single esterase locus examined in this study are the slowest of the anodally migrating zones on TEB 8 gels. They are also by far the most darkly staining bands, often overdeveloping before most of the outer zones of activity have even appeared. Activity is not noticeably inhibited by 10^{-4} Hg^{2+} or 10^{-3} Diamox. However, the products of this locus show very little, if any, activity when α-naphthyl butyrate is provided as substrate.

RESULTS

Mother–Offspring Analysis

A total of 29 parental snails laid eggs over the course of the study, although in some cases only two or three offspring survived to a size suitable for electrophoretic analysis. I eventually examined only the 14 sibships of 20 or more individuals (Table II). The 43 snails that did not lay eggs included 20 obviously parasitized individuals, 13 apparently healthy females that laid no eggs, and only 7 males. Three snails died during the experiment.

Table II shows the maternal genotype, the genotypes of the offspring for each locus, and the inferred paternal genotype. All sibships could be

Table II. Individual Offspring Categorized by Genotype, Each Locus Examined Separately

Cross no.	Maternal genotype			Number of offspring	*Gpi*				*Opdh*						
	Gpi	*Opdh*	*Est*		FM	MM	MS	χ^{2a}	SS	MS	FS	MM	FM	FF	χ^{2a}
1	MS	SS	MM	37		17	20	0.24		37					
2	MM	FF	FM	22		9	13	0.73			11			11	0.0
3	FM	FS	FM	30	13	17		0.53		9	5		8	8	1.17
4	MS	FS	MS	40		23	17	0.90	19		21				0.10
5	MM	SS	MM	20		20				8	12				0.80
6	MM	FF	FS	30		30							15	15	0.0
7	MM	FS	FS	26		26			13		13				0.0
8	MM	FM	FM	41		41				18	23				0.61
9	MM	FS	MS	36		36			10	6	12		8		2.22
10	MM	FS	FM	21		21			4		9			8	1.95
11	MM	SS	MS	26		26				8	18				3.85*
12	MM	SS	MS	35		35				21	14				1.40
13	MM	SS	MM	20		20				20					
14	MM	FS	MM	25		25					14			7	1.08

Table II. (Continued)

Cross no.	*Est*							Paternal genotype		
	FF	FM	FS	MM	MS	SS	χ^{2a}	*Gpi*	*Opdh*	*Est*
1		16		21			0.68	MM	MM	FM
2		5		17			6.55*	MS	FS	MM
3	9	18		3			3.60	MM	FM	FM
4				15	25		2.50	MM	SS	MM
5				7	13		1.80	MM	FM	MS
6	8	15	2		5		12.40*	MM	FM	FM
7		6	4		7	9	2.00	MM	SS	MS
8		10	12	14	5		4.37	MM	SS	MS
9		14	9	5	8		4.67	MM	MS	FM
10			10		11		0.05	MM	FS	SS
11				5	13	8	0.69	MM	FM	MS
12				10	15	10	0.71	MM	FM	MS
13		8		12			0.80	MM	MM	FM
14		25						MM	FS	FF

[a]Testing goodness of fit to a 1:1, 1:2:1, or 1:1:1:1 ratio, as appropriate.
*Significant at the 95% confidence level or greater.

interpreted as the results of a single mating. Of the 29 values of chi-square given in Table II, only 3 were significant at the 95% confidence level or above. It seems clear that inheritance is Mendelian at these loci. The three unusually high values of chi-square are probably best ascribed to type I statistical error.

The frequencies of the various genotypes considering pairs of loci together are given in Table III. Examining first the question of linkage between *Opdh* and *Est*, we note that four crosses (numbers 1, 2, 13, and 14) do not involve parents heterozygous at the appropriate loci. However, two crosses (numbers 4 and 5) could be considered ideal for testing independent assortment between *Opdh* and *Est*. One parent is homozygous at both loci, while the other parent is doubly heterozygous. Independent assortment should result in four genotypic classes represented in equal proportions. In contrast, two other crosses (numbers 5 and 9) involve a pair of doubly heterozygous parents. The 12 or 16 distinct genotypic classes resulting from these crosses are considerably more than can be analyzed given the small number of offspring available.

Between these two extremes, six crosses involve one parent heterozygous at both *Opdh* and *Est* and one singly heterozygous parent. This may result in either eight distinct genotypes or six distinct genotypes, in the case where both parents are heterozygous for the same two alleles. The latter situation is seen at the *Opdh* and *Est* loci in crosses 10, 11, and 12. Analysis of these three

Table III. Individual Offspring Categorized by Genotype, Pairs of Loci Considered Together

Cross no.	Parental genotypes	Numbers of offspring in each genotypic class								X^{2a}
2	$G^MG^MO^FO^F \times G^MG^SO^FO^S$	$G^MG^MO^FO^S$	3	$G^MG^SO^FO^S$	8	$G^MG^MO^FO^F$	6	$G^MG^SO^FO^F$	5	2.36
3	$G^FG^MO^FO^S \times G^MG^MO^FO^M$	$G^FG^MO^MO^S$ $G^FG^MO^FO^S$	4	$G^MG^MO^MO^S$ $G^MG^MO^FO^S$	10	$G^FG^MO^FO^M$ $G^FG^MO^FO^F$	9	$G^MG^MO^FO^M$ $G^MG^MO^FO^F$	7	2.80
4	$G^MG^SO^FO^S \times G^MG^MO^SO^S$	$G^MG^MO^SO^S$	12	$G^MG^SO^SO^S$	7	$G^MG^MO^FO^S$	11	$G^MG^SO^FO^S$	10	1.40
4	$G^MG^SE^ME^S \times G^MG^ME^ME^M$	$G^MG^ME^ME^M$	8	$G^MG^SE^ME^M$	7	$G^MG^ME^ME^S$	15	$G^MG^SE^ME^S$	10	3.80
4	$O^FO^SE^ME^S \times O^SO^SE^ME^M$	$O^SO^SE^ME^M$	8	$O^SO^SE^ME^S$	11	$O^FO^SE^ME^M$	7	$O^FO^SE^ME^S$	14	3.00
5	$O^SO^SE^ME^M \times O^FO^ME^ME^S$	$O^MO^SE^ME^M$	4	$O^MO^SE^ME^S$	4	$O^FO^SE^ME^M$	3	$O^FO^SE^ME^S$	9	4.40
6	$O^FO^FE^FE^S \times O^FO^ME^FE^M$	$O^FO^ME^FE^F$ $O^FO^ME^FE^S$	6	$O^FO^ME^FE^M$ $O^FO^ME^ME^S$	9	$O^FO^FE^FE^F$ $O^FO^FE^FE^S$	4	$O^FO^FE^FE^M$ $O^FO^FE^ME^S$	11	3.87
7	$O^FO^SE^FE^S \times O^SO^SE^ME^S$	$O^SO^SE^FE^M$ $O^SO^SE^FE^S$	4	$O^SO^SE^ME^S$ $O^SO^SE^SE^S$	9	$O^FO^SE^FE^M$ $O^FO^SE^FE^S$	6	$O^FO^SE^ME^S$ $O^FO^SE^SE^S$	7	2.00
8	$O^FO^ME^FE^M \times O^SO^SE^ME^S$	$O^MO^SE^FE^M$ $O^MO^SE^FE^S$	9	$O^MO^SE^ME^M$ $O^MO^SE^ME^S$	9	$O^FO^SE^FE^M$ $O^FO^SE^FE^S$	13	$O^FO^SE^ME^M$ $O^FO^SE^ME^S$	10	1.05

[a]Goodness of fit to a 1:1:1:1 ratio.

sibships is complicated by the fact that there are two ways to arrive at two of the genotypic outcomes. On the other hand, the three crosses involving one double heterozygote and a unique single heterozygote (numbers 6, 7, and 8) result in eight genotypic classes that can be unambiguously combined into four classes and tested against a 1:1:1:1 ratio. So in summary, Table III shows the results of five tests for independent assortment between *Opdh* and *Est*, two that naturally result in four genotypic classes and three others than can be combined to do so.

Only the first four crosses listed in Table III involved polymorphism at the *Gpi* locus. However, cross 1 did not involve polymorphism at the other two loci appropriate to test linkage between *Gpi* and either *Opdh* or *Est*. The remaining three crosses were all suitable to test linkage between *Gpi* and *Opdh*, resulting either in four genotypic classes or in eight classes that could be combined unambiguously into four. Cross 4, resulting in 40 offspring, was also suitable to test linkage between *Gpi* and *Est*.

It should be cautioned that sample sizes of 30 to 40 are adequate to detect linkage only between loci 23 to 28 map units apart or closer. But none of the values of chi-square in Table III is significant at the 95% confidence level. All three of the loci under study here seem to assort independently.

Artificial Introduction

Results were consistent with expectation based on the life history of *G. proxima* and simple Mendelian inheritance at the three enzyme loci. The 57 young-of-the-year snails collected at Coyner Springs included 53 individuals completely homozygous for the LAUR alleles and 4 individuals who were triple heterozygotes, apparently PHLP/LAUR hybrids. Parental snails collected included 54 pure-LAUR 2-year-olds, 1 very old (aged 4 years or older) LAUR snail, and 1 very old pure-PHLP individual.

DISCUSSION

Although a considerable body of literature exists regarding isozyme frequencies in populations of snails, demonstrations of Mendelian inheritance of isozyme phenotype are rather rare. Chambers (1980) found no variation either at the *Gpi* locus or at any of the three esterase loci in his successful crosses of *Goniobasis* and, thus, could reach no conclusion regarding their inheritance. However, *Gpi* and 3 esterase loci were among 11 enzyme loci with variants segregating in a Mendelian fashion in *Biomphalaria*, a freshwater pulmonate snail only distantly related to *Goniobasis* (Mulvey and Vrijenhoek, 1981, 1984). (*Goniobasis* belongs to another subclass, the Prosobranchia.) Mulvey and Vrijenhoek found that their three esterase loci seemed to be

linked but that these loci assorted independently of *Gpi*. Jelnes (1982) also found no evidence of linkage between *Gpi* and two esterase loci in another freshwater pulmonate, *Helisoma duryi*.

A good deal of research has focused on the inheritance of esterases in the terrestrial pulmonate *Cepaea nemoralis*. Linkage between several esterase loci has been reported by Selander and Foltz (1981). Oxford (1973) and Johnson (1979) have also demonstrated Mendelian inheritance at individual esterase loci in *C. nemoralis*. It is interesting to note, however, that one of the rare cases of non-Mendelian determination of isozyme phenotype was also discovered in the esterases of *Cepaea* (Oxford, 1975, 1978).

To my knowledge, no direct evidence of Mendelian inheritance at the *Opdh* locus in gastropods has been reported prior to this work. Baldwin and England (1982) inferred that a single locus with several codominant alleles was responsible for isozyme phenotype in a wild population of the marine prosobranch *Strombus luhuanus* by comparing observed genotype frequencies to Hardy–Weinberg expectation.

It is not surprising that *Gpi*, *Est*, and *Opdh* seem to assort independently in *G. proxima*, given the diploid number of 34 reported for this species by Dillon (1982). The significance of these findings lies more in their application to ongoing transplant studies. For example, thus far there is no evidence of reproductive isolation between the PHLP and the LAUR populations, in spite of their genetic differences. The LAUR population seems to be much better adapted to Coyner Springs, however. Although PHLP individuals can obviously survive in this environment, no pure-PHLP individuals were among the 2-year-olds sampled. This suggests that the introduced PHLP females did not lay any eggs? The low frequency of hybrid individuals among the F_1 may be due to introduced PHLP males surviving to mate with locally born LAUR females.

However, the fecundity of the hybrid snails produced has yet to be tested. It may develop that PHLP/LAUR hybrids are subvital or sterile. This question can be resolved in 1987, with the passage of another generation, for since it has been established that the three marker loci assort independently, F_2 hybrids should be identifiable. Results will be forthcoming.

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