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Note

Inheritance at Five Loci in the Freshwater Snail, *Physa heterostropha*

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INTRODUCTION

Interest in the genetics of pulmonate snails has arisen in at least three contexts. Population geneticists have often been attracted to snails as convenient organisms for basic research, from the classic studies of the banded land snail *Cepaea* (Jones *et al.,* 1977) to more recent isozyme studies of other land snails (Selander and Kaufman, 1975), slugs (McCracken and Selander, 1980), and a variety of freshwater species (Jarne and Delay, 1991). Interests of a more medical and parasitological nature have driven investigations into the genetics of *Biomphalaria* and other freshwater pulmonate populations serving as intermediate hosts of human schistosomes (Richards, 1970; Mulvey and Woodruff, 1985). Most recently, evolutionary biologists have been drawn to pulmonate snails as model organisms for the study of reproductive allocation (Rudolph and Bailey, 1985; Rollinson *et al.,* 1989; Schrag *et al.,* 1992; Jarne *et al.,* 1993).

Physa heterostropha pomilia (Conrad) is a freshwater pulmonate snail common in ponds and backwaters throughout North America. Genetic variability, reproductive plasticity, ease of culture, and rapid generation time combine to make it an ideal laboratory animal for general studies of sex allocation (Wethington and Dillon, 1993; in review). Our isofemale lines of *Physa* have their origins in 1989 collections from a pond at Charles Towne Landing State Park, within the city limits of Charleston, SC (site described by Dillon and Dutra-Clarke, 1992). We isolated 35 wild-caught snails and collected all offspring produced by each over a period of 60 days, in an effort to assess sperm storage capabilities (Wethington and Dillon, 1991). Small

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numbers of albino individuals were discovered among the progeny of four of these original 35 (numbers 7, 15, 27, and 29), perhaps the result of low, background levels of self-fertilization by heterozygotes. Albinism was subsequently shown to result from two separate recessive, nonallelic genes, $alb1^a$ in lines 15, 27, and 29, and $alb2^a$ in line 7 (Dillon and Wethington, 1992).

As markers in our 1989 sperm storage study we used isozymes of leucine aminopeptidase. Following convention, we designated the more common isozyme "100" and named the other isozyme according to its relative migration in millimeters, under standard gel conditions. Natural *Lap* polymorphism at the Charles Towne Landing population is interpretable as resulting from two codominant alleles in Hardy–Weinberg equilibrium, with the frequency of putative alleles $lap^{100} = 0.58$ and $lap^{103} = 0.42$. We also discovered that individuals from the Charles Towne population displayed a wide variety of esterase isozyme phenotypes. We hypothesized that variation at three esterase loci was involved, with putative alleles at a faster cathodal locus, $est2^{100} = 0.92$, $est2^{103} = 0.07$, and $est2^{106} = 0.01$, a slower cathodal locus, $est3^{96} = 0.15$ and $est3^{100} = 0.85$, and an anodally migrating locus, $est6^{100} = 0.65$, $est6^{102} = 0.04$, and $est6^{104} = 0.31$. (A rationale for our locus nomenclature is described below.) In this paper we establish the Mendelian nature of inheritance at *Lap* and three *Est* loci in *P. heterostropha* and examine evidence for their linkage to *Alb2,* as a preliminary step toward a fuller understanding of the complex reproductive biology of pulmonate snails.

MATERIALS AND METHODS

Through several generations of directed breeding we were able to isolate two lines of *Physa* fixed for alternative alleles at *Alb2,* breeding true for different *lap* and *est* isozyme phenotypes. Albino line 7-15-13 was $alb2^a alb2^a$ and fixed for isozyme bands $est2^{100}$, $est3^{96}$, $est6^{104}$, and lap^{103}. Pigmented line 35-8-2 was $alb2^+ alb2^+$ and fixed for bands $est2^{103}$, $est3^{100}$, $est6^{100}$, and lap^{100}. We paired 10 individual snails from each of these two cultures at age 2 weeks, well in advance of their sexual maturity, and reared them together until several pairs were producing viable eggs. We then isolated the members of each pair and (to ensure an outcross) collected 20 pigmented F_1 individuals from three albino parents. When the F_1 reached 2 weeks of age, we initiated test crosses by placing them with 2-week-old individuals from the 7-15-13 pure line.

The pairs of F_1 individuals were separated when egg masses first appeared in their culture vessels and isolated for a period of 1 week. Thereafter a schedule was maintained alternating weeks of pairing and weeks of isolation. We saw no evidence of self-fertilization. All pairs of F_1 snails reproduced at about 8 weeks of age, as would be expected for

outcrossing, and all F_2 litters examined contained both pigmented and albino snails. We separately analyzed the first F_2 offspring of seven 7-15-13 albino individuals, comprising 2–4 egg-laying weeks each. In only one of the seven sets of F_2 sibships (23 cultures, 428 F_2 animals combined) was the ratio of albino-to-pigmented individuals different from 1:1 at the (nominal) 0.05 level. This is clearly well within expectation for Type I statistical error and, hence, constitutes no evidence of selfing.

We randomly subsampled from among the 428 F_2 offspring 138 pigmented individuals and 138 albino offspring. Whole-animal homogenate was subjected to horizontal starch gel electrophoresis using techniques detailed by Dillon (1985, 1992). Gels were 14%, a mixture of 3 parts starch for electrophoresis (S-4501; Sigma Chemical, St. Louis, Missouri) and 1 part electrostarch (Otto Hiller, Madison, Wisconsin). We used a TEB8 buffer (III of Shaw and Prasad, 1970) to resolve esterases (est) and stained gels with fast blue BB salt, using α-naphthyl acetate as substrate. Leucine aminopeptidase (lap) isozymes were resolved with an AP6 buffer (Clayton and Tretiak, 1972), using L-leucine β-naphthylamide as substrate and black K salt as stain. Results were analyzed using GMENDEL 1.0, a Fortran-77 program using log-likelihood ratios (G statistics) to examine Mendelian segregation and linkage data (Liu and Knapp, 1990).

RESULTS

Figure 1 shows example electrophoretic results from the two pure lines and their F_1 hybrid. All expected combinations of isozyme phenotype could be found among the F_2 testcross offspring. Although not apparent in the figure, isozyme bands ascribed to the *est2* locus stained redder than all others—a characteristic useful in helping distinguish bands encoded by this locus from those surrounding.

Summing over the first 2 to 4 egg-laying weeks of the first seven F_2 sibships, we counted 231 pigmented and 197 albino individuals. This is not significantly different from the expected test-cross ratio (Table I). In the 276-individual subsample for electrophoretic analysis, the results at all four of the putative enzyme loci were also indistinguishable from the 1 homozygote:1 heterozygote ratio expected. Thus we conclude that inheritance is Mendelian at all five of the loci under study here.

Table I shows two-locus G statistics testing 1:1:1:1 expectation for all pairs of loci. With $N = 276$, such tests would be expected to detect recombination frequencies of 38% or less. None of the 10 two-locus tests resulted in a significant value of G, however. We therefore conclude that these five loci assort independently.

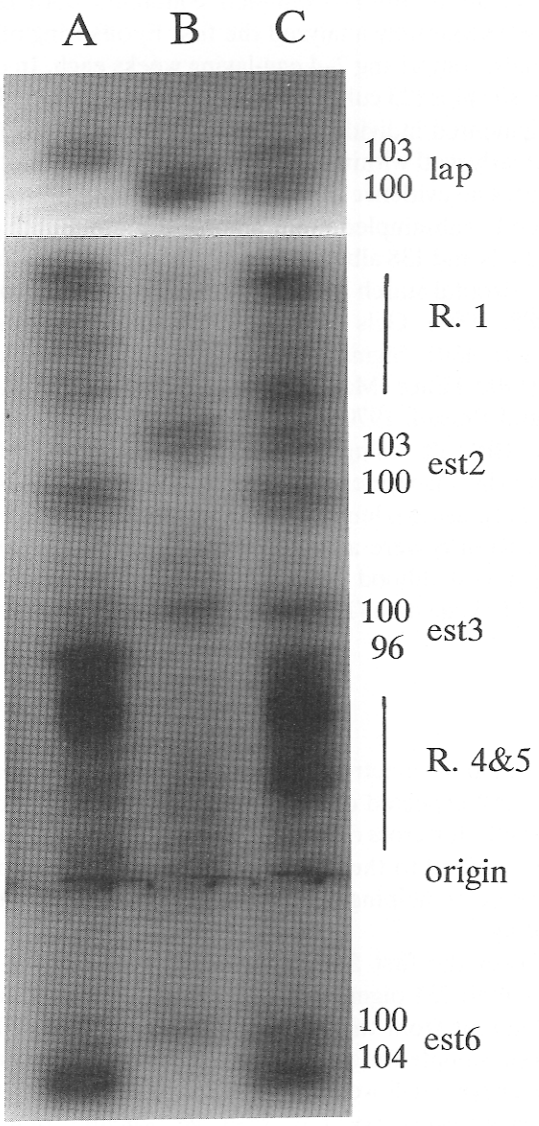

Fig. 1. Gels showing whole-animal homogenate of *Physa* stained for leucine aminopeptidase (top) and esterase (bottom). Line 7-15-13 is shown in lane A, line 35-8-2 is in lane B, and their F_1 hybrid is in lane C. R, uncharacterized "regions" of the gel.

Table I. Log-Likelihood *G* Statistics[a]

Locus	One-locus statistic	Two-Locus			
		Alb2	*Lap*	*Est2*	*Est3*
Alb2	1.35 (0.24)				
Lap	0.00 (1.0)	0.93 (0.33)			
Est2	2.87 (0.09)	0.00 (0.96)	1.20 (0.27)		
Est3	2.66 (0.10)	0.04 (0.85)	0.37 (0.54)	0.57 (0.45)	
Est6	1.61 (0.21)	2.30 (0.13)	1.78 (0.18)	0.30 (0.58)	2.68 (0.10)

[a]The test is of goodness of fit to 1:1 expectation in one-locus cases or 1:1:1:1 in two-locus cases. $N = 428$ for albinism alone; $N = 276$ for all other entries. *P* values in parentheses.

DISCUSSION

Most terrestrial snails and slugs belong to the pulmonate order Stylommatophora, rather distantly related to *Physa* and the other basommatophoran pulmonates of fresh waters. A considerable body of genetic information is available for the stylommatophoran *Cepaea nemoralis,* the banded European land snail upon which much evolutionary work has focused (Jones *et al.,* 1977). Using polyacrylamide "disc" electrophoresis and two substrates, Oxford (1973) distinguished 23 zones of esterase activity in *Cepaea.* He demonstrated that alleles segregating in Mendelian fashion at three (apparently unlinked) loci accounted for some of these zones but that other zones were food-induced phenocopies (Oxford, 1975). Brussard and McCracken (1974) reported two putative loci encoding *Lap* in *Cepaea* and verified Mendelian inheritance at *Lap2.* Johnson (1979) could detect no linkage between *Lap2* and an esterase locus, "*Est-F.*" While the control of shell coloration is complex and well characterized in *Cepaea,* no body color locus or albinism locus is known. The body color of stylommatophoran pulmonates is often much lighter than that characteristic of basommatophorans.

Some data comparable to ours are available for the basommatophoran family Lymnaeidae. Rudolph and Burch (1987) described the esterase zymograms of the *Lymnaea (Stagnicola) elodes* as "very complex," with up to 20 bands resolvable in some individuals. They were able to attribute some isozyme variation in *Lymnaea* to Mendelian inheritance at five loci, *Est2, Est3, Est7, Est13,* and *Est18,* and detected close linkage between *Est13* and *Est18.* Rudolph and Burch also confirmed the Mendelian nature of inheri-

tance at a single, independently assorting, *Lap* locus. A single albinism locus has been described in *Lymnaea peregra* (Boycott and Diver, 1927).

The basommatophoran family Planorbidae is the best-characterized of the pulmonate taxa. Mulvey and Vrijenhoek (1981a) interpreted esterase zymograms as the product of seven gene loci in the North American planorbid *Biomphalaria obstructa,* and demonstrated Mendelian inheritance at a rapidly migrating locus *Est1.* These same authors recognized only four zones of esterase activity in *B. glabrata,* the most prominent intermediate host of *Schistosoma* in the new world (Mulvey and Vrijenhoek, 1981b). By intercrossing laboratory strains, Mulvey and Vrijenhoek were able to confirm Mendelian inheritance at loci encoding three of those zones, *Est1, Est2,* and *Est4.* Mulvey and Vrijenhoek (1984) showed tight linkage between *Est2* and *Est4* and found some evidence of *Est1–Est4* linkage.

Body pigmentation is influenced by two loci in *B. glabrata,* a "basic" or "*C*" locus (three alleles known, including albinism) and an unlinked "*S*" locus controlling the distribution of pigment in the mantle (Richards, 1985). The existence of an *S* locus has not been specifically verified in the lesser-known *B. obstructa.* Locus *C* (also called "*pig*") assorts independently of the esterase loci in *B. glabrata* (Mulvey and Vrijenhoek 1984). Mulvey *et al.* (1988) confirmed Mendelian inheritance at two *Lap* loci and detected no linkage among them, *Est2,* or the pigmentation locus *S.*

The situation in the planorbid *Helisoma duryi* seems similar to that in *B. glabrata.* Jelnes (1982) was able to verify Mendelian inheritance at three esterase loci, *Est-A, Est-B,* and *Est-d,* which, to judge from his figures, seem roughly comparable to the *Est1, Est2,* and *Est4* of Mulvey. Jelnes could find no evidence of linkage among any of these loci, however, nor did the esterase loci appear linked to the *alb* locus in *Helisoma.*

In sum, the genetic diversity displayed by pulmonates is such that generalizations regarding the inheritance of esterase, leucine aminopeptidase, and body pigmentation are difficult. As was the case in *Biomphalaria glabrata* and *Helisoma duryi,* we have described in *Physa* two polymorphic esterase loci migrating cathodally and one locus migrating anodally. We have directly compared *Physa* to *Biomphalaria obstructa* under our gel conditions and, in consultation with M. Mulvey, propose the system of nomenclature shown in Figure 1. It is based loosely upon the *B. obstructa* system (Mulvey and Vrijenhoek, 1981a), without a second anodal locus, "*Est-7.*" We set aside "regions" 1, 4, and 5 against the possibility that Mendelian variation will be recognized there in *Physa.*

It should be cautioned, however, that there seems to be little homology among the esterase loci of *Biomphalaria obstructa* and *B. glabrata,* let alone *Physa.* Putative *obstructa* loci *Est-2, Est-3,* and *Est-4* are smeared and diffuse in *glabrata,* so that *glabrata Est-2, Est-3,* and *Est-4* would be roughly equiva-

lent to *obstructa* (and *Physa*) *Est-5, Est-6,* and *Est-7,* respectively. None of the three esterase loci described here in *Physa* occupies a position comparable to the loci apparently linked in *B. glabrata.* When added to our observation that *Biomphalaria* esterase isozymes rather uniformly seem to migrate more rapidly under our gel conditions than *Physa* isozymes, the evidence for homology between the *Physa* esterases and any *Biomphalaria* esterase loci is tenuous at best.

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