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Inheritance of alleles at ten enzymatic loci of the freshwater snail *Stagnicola elodes* (Basommatophora: Lymnaeidae)

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Summary

The inheritance of alleles at ten enzyme loci of *Stagnicola elodes* (Lymnaeidae) was analysed by laboratory crosses. Alleles for alanine aminopeptidase (2 alleles), leucine aminopeptidase (2 alleles), aspartate aminotransferase (2 alleles), β-hydroxybutyrate dehydrogenase-1 (4 alleles), 6-phosphogluconate dehydrogenase (2 alleles), esterase-2 (2 alleles), esterase-3 (2 alleles), esterase-7 (2 alleles), esterase-13 (2 alleles) and esterase-18 (2 alleles) segregate in the F_2 generation according to Mendelian expectations. Contingency X^2 tests for linkage done on F_2 progeny from crosses between strains homozygous for alternate alleles at two loci showed that β-hydroxybutyrate dehydrogenase-1, esterase-13 and esterase-18 are presumably linked.

Introduction

The stagnicoline Lymnaeidae are a widespread and commonly encountered group of freshwater snails found primarily in North America. The biological relationships of the species of *Stagnicola* have not been extensively studied, due, in part, to difficulties in their systematics. An adequate taxonomy of the North American freshwater snail genus *Stagnicola* (Lymnaeidae) has, historically, been difficult to devise, because the most important characters used in the taxonomy of *Stagnicola* have been those of the shell. Shell morphology may be subject to ecophenotypic variation and, because genetic studies to determine the importance of shell characters are lacking, the schemes for defining species of the genus have been subjective. Many local races have been described based on minor shell variation. On the other hand, Walter (1968) has suggested that nearly all North American *Stagnicola* should be united under the species *Lymnaea* (= *Stagnicola*) *catascopium* Say, because the currently recognized species of *Stagnicola* are so conservative in their reproductive anatomies. Cytological investigations on *Stagnicola* have demonstrated that all members of the genus have a haploid chromosome number of $n = 18$ (Burch, 1965; Inaba, 1969), although certain differences in chromosome cytology have been demonstrated (Inaba, 1969). Except for Burch & Ayers (1973), who have demonstrated reproductive isolation between *Stagnicola elodes* (Say) and *S. emarginata* (Say), virtually nothing is known concerning breeding potential between races within a species or between nominal species.

Stagnicola elodes is the most common of the recognized species of *Stagnicola*, with its range being 'New England west to Oregon and California, south to New Mexico; widely distributed in the Canadian Basin' (Burch & Tottenham, 1980). During our investigations on the taxonomy of *Stagnicola*, we have investigated the allozymes of three stagnicoline species, using starch gel electrophoresis. We feel that breeding experiments will play an important role in the determination of the relationships between species of this genus and in the study of various aspects of their reproductive evolutionary ecology.

The snails of the genus *Stagnicola* are hermaphroditic, as are all freshwater pulmonates, and *S. elodes* is capable of self-fertilization (personal observation). Thus, after crosses performed to ascertain the extent of reproductive isolation, determining the paternity of any young would be complicated by the possibility that a parental snail may reproduce by self-fertilization rather than by cross-fertilization. Reproduction by self-fertilization rather than by cross-fertilization might give the false impression of interspecific fertility unless paternity is determined. In the absence of the phenotypic expression of a genetic marker which can be observed by inspection (such as albinism), it is possible to use electrophoretically detectable allozymes to determine the paternity of offspring produced from crosses.

Because *S. elodes* is so widespread and one of the more prominent members of the genus, we have

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determined the inheritance of 22 alleles at ten enzyme loci in order to make use of these enzymes as genetic markers in future studies concerned with the reproductive biology of the genus.

Materials and methods

The *S. elodes* used in the present study were collected at Dexter, Washtenaw County, Michigan, U.S.A. Laboratory strains homozygous for the following alleles were established: leucine aminopeptidase (LAP), 96, 100; alanine aminopeptidase (AAP), 100, 102; esterase-13 (EST-13), 100, 103; aspartate aminotransferase (AAT), 100, 106; β-hydroxybutyrate dehydrogenase-1 (HBDH-1), 100, 109, 116, 121. Crosses were made between homozygous lines for combinations of alleles listed above. Segregation of alleles was determined in the F_2 generation. In addition, inheritance of alleles at five other loci was determined from F_2 young during the same crosses. These loci were esterase-2 (EST-2), 100, 101; esterase-3 (EST-3), 97, 100; esterase-7 (EST-7), 95, 100; esterase-18 (EST-18), −98, −100, and 6-phosphogluconate dehydrogenase (PGDH), 96, 100.

F_1 siblings were crossed to provide F_2 young for analysis of segregation of alleles. The F_2 young were raised until the shell length was 8–10 mm or larger, and they were then frozen and stored at −70 °C prior to electrophoresis. Homozygous parental snails and the F_1 parental snails also were frozen and stored as above until analysis of their enzyme patterns was performed. The F_1 parental snails were analysed simultaneously with young from the F_2 generation to ensure that crosses between F_1 heterozygotes had been made. *Stagnicola elodes* is capable of self-fertilization and it is possible that self-fertilization and cross-fertilization might occur simultaneously, as has been reported for other freshwater pulmonates (Paraense, 1956; Rudolph & Bailey, 1985). For this reason, the analysis of F_2 progeny from F_1 heterozygotes will give a more reliable estimate of allelic segregation than will back-crosses (Mulvey & Vrijenhoek, 1984). The same ratios are theoretically expected in F_2 young from F_1 heterozygotes whether the F_2 were produced by self- or by cross-fertilization.

Homozygous parentals and F_1 parentals were homogenized by hand in 0·5 ml of distilled water. The F_2 young were homogenized by sonication in 100 μl of distilled water. All homogenates were centrifuged at 4 °C. Supernatent was absorbed on wicks cut from Whatmann 3 MM filter paper. Electrophoresis was performed at 4 °C on 12·5% starch gel slabs consisting of equal portions of Electrostarch (Lot no. 392) and Connaught starch. Following electrophoresis, the starch slabs were sliced and stained for the desired enzyme activity.

The electrophoresis buffer and staining solution used for each enzyme investigated are as follows.

Electrophoresis buffer: *N*-(3-aminopropyl) morpholine-citrate (pH 6·1) (Clayton & Tretiak, 1972). PGDH: 0·1 M Tris-HCl buffer (pH 8·0), 50 ml; phosphogluconic acid, barium salt, 20 mg; NADP, 10 mg; MTT, 10 mg; PMS, 2 mg.

Electrophoresis buffer: Tris-citrate/LiOH-borate (Ashton & Braden, 1961). Esterase: 0·1 M Tris-HCl buffer (pH 7·4), 50 ml; 1% α-naphthyl acetate in acetone, 1 ml; Fast Blue RR salt, 35 mg. AAT: 0·1 M Tris-HCl buffer (pH 8·0), 50 ml; L-aspartic acid, monopotassium salt, 150 mg; α-ketoglutaric acid, monosodium salt, 100 mg; pyridoxyl-5′-phosphate, 5 mg; Fast Blue BB salt, 100 mg. HBDH: 0·1 M Tris-HCl buffer (pH 8·0), 50 ml; DL-β-hydroxybutyric acid, sodium salt, 40 mg; NAD, 10 mg; MTT, 10 mg; PMS, 2 mg. LAP: 0·1 M phosphate buffer (pH 7·0), 50 ml; L-leucine-β-naphthylamide HCl, 20 mg; Fast Black K salt, 50 mg; 0·1 M $MgCl_2$, 1 ml.

Electrophoresis buffer: Tris-citrate (pH 6·8) (Selander *et al.* 1971). AAP: 0·1 M phosphate buffer (pH 7·0), 50 ml; DL-alanyl-β-naphthlyamide HCl, 20 mg; Fast Black K salt, 50 mg; 0·1 M $MgCl_2$, 1 ml.

Data from single loci in F_2 progeny were examined for their fit to the predictions of Mendelian inheritance using the X^2 statistic. To determine possible linkage, contingency X^2 statistics were calculated from genotypic data for pairs of loci in the F_2 progeny derived from double heterozygote F_1 crosses (Snedecor, 1966). When the null hypothesis of independence was rejected at the 0·01 level, the pair of loci were considered to be linked. A contingency X^2 test is a conservative test for association because it does not depend upon each locus being in perfect Mendelian ratio. Recombination fractions were determined by the maximum likelihood method for those pairs of loci which appeared to be linked (Allard, 1956).

Results

Inheritance of alleles at all ten loci occurs according to Mendelian expectations. X^2 tests showed that none of the observed ratios of F_2 progeny differed significantly from expected values (Table 1).

LAP, AAP, EST-2, EST-3, EST-7, EST-13 and EST-18 all behave as enzymes with monomeric structures. Heterozygotes at these loci have two bands, one corresponding to each electromorph found in the homozygous parental snails. AAT, HBDH-1 and PGDH behave as enzymes with dimeric structures, with heterozygotes possessing a heterodimer in addition to the homodimer found in each homozygous parental snail.

Tests for linkage revealed that HBDH-1, EST-13 and EST-18 do not segregate independently of each other and are presumably linked ($P < 0{\cdot}00001$ for each of the three combinations). X^2 values calculated for all other combinations of loci which could be tested showed that none differed from expected values, and thus those loci probably are not linked (Table 2). We detected no crossing-over between EST-13 and

Table 1. *Inheritance of alleles at ten enzyme loci of* Stagnicola elodes

Enzyme	Phenotype of F_1 siblings	Phenotype of F_2 offspring	Obs.	Exp.	Total	X^2	P† values
AAT	100/106 × 100/106	106/106	50	45	180	2·70	0·260
		100/106	79	90			
		100/100	51	45			
AAP	100/102 × 100/102	102/102	19	22·5	90	0·84	0·657
		100/102	46	45			
		100/100	25	22·5			
LAP	96/100 × 96/100	100/100	88	95	380	1·54	0·463
		96/100	202	190			
		96/96	90	95			
PGDH	96/100 × 96/100	100/100	21	19·25	77	0·22	0·896
		96/100	37	38·5			
		96/96	19	19·25			
	100/100 × 96/100	100/100	35	30	60	1·67	0·197
		96/100	25	30			
HBDH-1	116/121 × 116/121	121/121	24	30	120	2·05	0·359
		116/121	61	60			
		116/116	35	30			
	109/121 × 109/121	121/121	33	29·75	119	0·56	0·756
		109/121	56	59·5			
		109/109	30	29·75			
	100/121 × 100/121	121/121	39	42·5	170	1·15	0·562
		100/121	92	85			
		100/100	39	42·5			
	109/116 × 109/116	Not done					
	100/116 × 100/116	116/116	37	35	140	1·87	0·393
		100/116	75	70			
		100/100	28	35			
	100/109 × 100/109	109/109	44	45	180	0·04	0·978
		100/109	90	90			
		100/100	46	45			
EST-2	100/101 × 100/101	101/101	18	14·5	58	1·86	0·395
		100/101	24	29			
		100/100	16	14·5			
EST-3	97/100 × 97/100	100/100	50	57·75	231	2·22	0·330
		97/100	115	115·5			
		97/97	66	57·75			
	100/100 × 97/100	100/100	32	36	72	0·89	0·346
		97/100	40	36			
EST-7	95/100 × 95/100	100/100	9	7·5	30	0·60	0·741
		95/100	13	15			
		95/95	8	7·5			
EST-13	100/103 × 100/103	103/103	85	92·5	370	1·40	0·498
		100/103	196	185			
		100/100	89	92·5			
EST-18	−98/−100 × −98/−100	−100/−100	43	42·5	170	0·20	0·905
		− 98/−100	87	85			
		− 98/−98	40	42·5			
	−100/−100 × −98/−100	−100/−100	40	44·5	89	0·91	0·340
		− 98/−100	49	44·5			

EST-18. Recombination frequency between EST-13 and HBDH-1 was 3·5% (S.E. = ±0·8%) and recombination frequency between EST-18 and HBDH-1 was 2·7% (S.E. = ±0·9%). The EST-13, HBDH-1 recombination fraction and the EST-18, HBDH-1 fraction do not differ significantly.

Discussion

The inheritance of PGDH, esterases and HBDH-1 corresponds to that presented in reports on the genetic basis for inheritance of alleles for similar enzyme loci in *Biomphalaria glabrata* (Say) and *Helisoma duryi*

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Table 2. *Inheritance of alleles from crosses between* Stagnicola elodes *parents which were homozygous at two loci*

Phenotype of homozygous parentals	Phenotype of F_2 Progeny[a]										
	F/F[b]	F/H	F/S	H/F	H/H	H/S	S/F	S/H	S/S	Total	X^2
HBDH-1/EST-3											
116, 116/97, 97 × 100, 100/100, 100	3	12	9	14	34	13	5	19	11	120	3·5
116, 116/97, 97 × 121, 121/100, 100	6	11	6	12	27	17	3	6	6	94	0·96
HBDH-1/EST-13											
100, 100/103, 103 × 109, 109/100, 100 121, 121/100, 100	0	5	55	4	134	2	53	7	0	260	414·89*
HBDH-1/EST-18											
100, 100/−100, −100 × 109, 109/−98, −98	0	3	35	1	81	2	45	3	0	170	287·82*
HBDH-1/AAP											
109, 109/102, 102 × 121, 121/100, 100	6	11	9	9	22	9	4	12	8	90	1·83
HBDH-1/LAP											
100, 100/100, 100 × 109, 109/96, 96 121, 121/96, 96	21	47	15	41	91	50	19	53	13	350	6·64
HBDH-1/AAT											
121, 121/106, 106 × 116, 116/100, 100	7	12	3	17	27	19	11	13	11	120	3·07
EST-3/AAT											
97, 97/100, 100 × 100, 100/106, 106	6	8	8	19	28	18	10	15	8	120	1·02
EST-13/EST-18											
103, 103/−100, −100 × 100, 100/−98, −98	26	0	0	0	57	0	0	0	27	110	220·02*
EST-13/LAP											
103, 103/100, 100 × 100, 100/96, 96	17	37	9	38	76	44	21	39	9	290	8·84

* $P \leqslant 0{\cdot}01$; D.F = 4.

[a] F_2 progeny were produced from crosses between F, siblings which were heterozygous at both loci.

[b] For simplicity, the phenotype of each locus has been designated thus: F, homozygous for fast allele; H, heterozygous; S, homozygous for the slow allele.

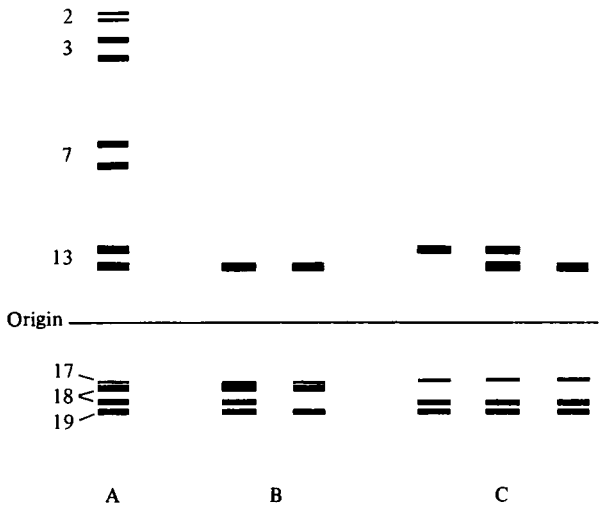

Fig. 1. Esterase alleles of *Stagnicola elodes*. (A) Alleles of EST-2, EST-3, EST-7, EST-13 and EST-18. EST-17 and EST-19 have been included to show their relationship to EST-18. (B) F_2 progeny homozygous for EST-13. The right lane is homozygous for EST-18 (−100, −100); the left lane is heterozygous for EST-18 (−98, −100). (C) F_2 progeny homozygous for EST-18. The left lane is homozygous for EST-13 (103, 103); the middle lane is heterozygous for EST-13 (103, 100); the right lane is homozygous for EST-13 (100, 100).

(Wetherby) (Mulvey & Vrijenhoek, 1981*a*; Jelnes, 1982). The subunit structures of the ten *S. elodes* enzymes correspond to those found in other freshwater pulmonate snails (Mulvey & Vrijenhoek, 1981*a*, *b*; Jelnes, 1982; Buth & Suloway, 1983). The Dexter population of *S. elodes* also contains a third allele at both the LAP and AAP loci. Heterozygotes containing these alleles and either of the other two alleles for each enzyme are double banded (MS in prep.), indicating that the inheritance of these alleles should correspond to that found for the alleles of LAP and AAP which were analysed in the present study.

The α-naphthyl acetate esterases of *S. elodes* are very complex, and we have been able to count 20 different bands of activity in an individual. The five esterase loci described in this report each show only two alleles. However, since natural populations of *S. elodes* have not been analysed at these loci, it is possible that other alleles may be present, since other workers have found more than two alleles for some esterase loci in other freshwater pulmonates (Mulvey & Vrijenhoek, 1981*a*; Jelnes, 1982; Buth & Suloway, 1983).

HBDH-1, EST-13 and EST-18 are presumably linked in *S. elodes*. Although the recombination frequency between EST-13 and EST-18 was 0%, phenotypes in two of the F_1 crosses indicate that the two isozymes are coded by distinct gene loci. In one cross, consisting of two F_1 young with EST-13/EST-18 alleles 103, 100/−100, −100, the alleles in the F_2 progeny segregated at the EST-13 locus and were homozygous at the EST-18 locus (Fig. 1C). The parents of the F_1 snails were 103, 103/−100, −100 and 100, 100/−100, −100. A second cross involved F_1 snails which were homozygous at the EST-13 locus (100, 100), but at the EST-18 locus, one snail was homozygous (−100, −100) and the other heterozygous (−98, −100). In Table 1, the last cross illustrated gives the results of the cross at the EST-18 locus. All F_2 young from that cross were homozygous at the EST-13 locus (Fig. 1B). These crosses would indicate that recombination may occur between these two loci, even though we did not detect any in the double heterozygote crosses reported here.

Two additional esterase loci (EST-12 and EST-16) also appear to be linked to HBDH-1, EST-13 and EST-18. However, the mobilities of the alleles at those esterase loci are such that they overlap the alleles of EST-13 in the case of one and are poorly resolved in the case of the second. Further work is necessary to determine the relationship of those loci to the HBDH-1, EST-13 and EST-18 loci.

Several linkage groups have previously been demonstrated in freshwater pulmonates. Mulvey & Vrijenhoek (1981*b*) suggested that two peptidase loci may be linked in *Biomphalaria obstructa* (Morelet). In *B. glabrata*, Mulvey & Vrijenhoek (1984) demonstrated linkage between esterase-1, esterase-2, esterase-4 and catalase (linkage group I) and between 6-phosphogluconate dehydrogenase and phosphoglucose isomerase (linkage group II).

For use as genetic markers, we anticipate that, of the ten loci analysed here, all except EST-2, EST-3 and EST-7 will be useful. The activities of these three esterases are not strong enough (with α-naphthyl acetate as substrate) for routine use as genetic markers. EST-2 is difficult to resolve in all preparations because it migrates very rapidly anodally and the alleles differ only slightly in their mobilities. EST-7 cannot be scored in all preparations due to its weak activity. Better resolution of these esterase loci might be possible with the use of other esterase substrates. EST-18 might also be less useful as a genetic marker because the slow allele (−98) is very similar in mobility to EST-17 (Fig. 1A) and often partially overlaps EST-17.

Since many freshwater pulmonate snails are capable of self-fertilization, as is *S. elodes*, the determination of paternity is especially important in controlled experimental crosses during breeding studies conducted to determine the extent of reproductive isolation between closely related groups. In addition to such studies, the determination of inheritance of enzyme alleles will be useful in elucidating the reproductive strategies of the snails under natural conditions in terms of the use of allosperm, consequences of multiple matings and other facets of mating strategies which require genetic markers in order to accurately determine paternity.

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