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Congruence between starch gel and polyacrylamide gel electrophoresis in detecting allozyme variation in pulmonate land slugs

The predominantly selfing slug species *Arion (Carinarion) fasciatus*, *A. (C.) silvaticus* and *A. (C.) circumscriptus* are native in Europe and have been introduced into North America, where each species consists of a single, homozygous multilocus genotype (strain), as defined by starch gel electrophoresis (SGE) of allozymes. In Europe, the "one strain per species" hypothesis does not hold since polyacrylamide gel electrophoresis (PAGE) of allozymes uncovered 46 strains divided over the three species. However, electrophoretic techniques may differ in their ability to detect allozyme variation. Therefore, several *Carinarion* populations from both continents were screened by applying the two techniques simultaneously on the same individual slugs and enzyme loci. SGE and PAGE yielded exactly the same results, so that the different degree of variation in North American and European populations cannot be attributed to differences in resolving power between SGE and PAGE. We found four *A. (C.) silvaticus* strains in North America indicating that in this region the "one strain per species" hypothesis also cannot be maintained. Hence, the discrepancies between previous electrophoretic studies on *Carinarion* are most likely due to sampling artefacts and possible founder effects.

Keywords: Arionidae / Land slugs / Polyacrylamide gel electrophoresis / Population genetics / Starch gel electrophoresis EL 5250

1 Introduction

Allozyme electrophoresis refers to the separation of allelic variants of enzymes in an electric field. Several support media are used for this purpose, such as cellulose acetate, agarose, polyacrylamide and starch, the latter of which being the most popular one [1]. However, the amount of allozyme variation that can be detected may differ between support media. Hence, variation revealed by one method may remain undetected by another. The occurrence of such "cryptic" or "hidden" variation is quite pervasive [2–5] and should be evaluated carefully when population genetic studies involving the same organisms but different electrophoretic techniques yield inconsistent results. Against this background, the present study reports on a methodological evaluation of discrepancies between previous allozyme analyses on the terrestrial slugs of the arionid subgenus *Carinarion* Hesse, 1926 in North America and Europe.

In 1980, McCracken and Selander [6] used starch gel electrophoresis (SGE) to assess genetic variation and breeding systems in North American populations of the three *Carinarion* taxa, *viz. Arion (Carinarion) fasciatus* (Nilsson, 1823), *A. (C.) circumscriptus* Johnston, 1828 and *A. (C.) silvaticus* Lohmander, 1937. They confirmed that the three taxa are "good" species, since each taxon consisted of a single homozygous multilocus genotype (*i.e.*, "strain") (Table 1). The lack of allozyme variation within each species and the complete absence of heterozygotes was interpreted to be the result of a uniparental mode of reproduction, most probably self-fertilization [6].

Subsequently, Foltz *et al.* [7] also used SGE to examine several British *Carinarion* populations. The "one strain per species" idea of McCracken and Selander [6] was supported for *A. circumscriptus.* Yet, *A. silvaticus* revealed two phosphoglucomutase (Pgm) alleles, the most common being electrophoretically identical to the North American Pgm allele [7] (Table 1). Again, the absence of heterozygotes was interpreted as the consequence of a uniparental breeding system.

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Abbreviations: Alat, alanine transaminase; **EstQ**, carboxylesterase; **Fumh**, fumarate hydratase; **Lap,** leucyl aminopeptidase; **MDS**, nonmetric multidimensional scaling; **MST**, minimum spanning tree; **Pep**, aminopeptidase (leucyl-alanine); **Pgm**, phosphoglucomutase; **SGE**, starch gel electrophoresis

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Table 1. Numbers of animals, populations, loci and strains in the studies of McCracken and Selander [6], Foltz *et al.* [7] and Jordaens *et al.* [10]

Species	Study	Animals (*n*)	Populations (*n*)	Loci (*n*)	Strains (*n*)
A. fasciatus	McCracken and Selander (1980)	814	10	18	1
	Foltz *et al.* (1982)	/	/	/	/
	Jordaens *et al.* (2000)[a)]	676	31	16	13
	Present study (North America)	172	6	14	1
A. silvaticus	McCracken and Selander (1980)	29	3	17	1
	Foltz *et al.* (1982)	48	4	15	2
	Jordaens *et al.* (2000)[a)]	582	90	16	24
	Present study (North America)	70	3	14	4
A. circumscriptus	McCracken and Selander (1980)	312	4	18	1
	Foltz *et al.* (1982)	46	5	15	1
	Jordaens *et al.* (2000)[a)]	285	46	16	9
	Present study (North America)	169	4	14	1

a) Number of animals, loci and strains combined with Backeljau *et al.* [8] and Jordaens *et al.* [9]

In contrast, the "one strain per species" hypothesis was later rejected in electrophoretic studies which surveyed Central and Northwest European *Carinarion* populations and which detected at least 46, often co-occurring strains, *i.e.*, 13 in *A. fasciatus*, nine in *A. circumscriptus* and 24 in *A. silvaticus* [8–10] (Table 1). Moreover, in several Central European populations genotype frequencies at several loci did not deviate significantly from Hardy-Weinberg equilibrium expectations, suggesting that outcrossing may not be infrequent in this region. However, because Backeljau *et al.* [8] and Jordaens *et al.* [9, 10] used polyacrylamide gel electrophoresis (PAGE) instead of SGE, it remained unclear to what extent these contrasting results represented meaningful biological patterns or merely reflected differences in the resolving power of SGE and PAGE. Therefore, the present study assesses this possible methodological bias by applying SGE and PAGE simultaneously on the same individual slugs and enzyme loci to screen allozyme variation in several North American and European *Carinarion* populations.

2 Materials and methods

We collected 411 specimens from nine North American *Carinarion* populations in Connecticut and Massachusetts, USA (Table 2). Species identifications followed Lohmander [11] and Waldén [12]. Individuals were transported alive to the laboratory where they were frozen at −80°C upon arrival. Animals were dissected on ice and the hepatopancreas were homogenized in a 20% w/v sucrose solution (1 g tissue/5 mL solution) to which protease inhibitors 7 mg/10 mL DTT and 8 mg/10 mL amino caproic acid and an antioxidant 19 mg/10 mL EDTA were added. Crude homogenates were centrifuged at 15 000 rpm and 4°C for 40 min. Clear individual supernates were divided in two fractions, one for PAGE and one for SGE, and stored at −80°C until electrophoresis. Fourteen enzymes, including those studied by McCracken and Selander [6] (personal communication) and Foltz *et al.* [7], were screened with vertical PAGE according to the procedures outlined by Backeljau [13] (Table 3). Staining recipes were adapted from Harris and Hopkinson [14]. The quaternary structure of an enzyme was determined if there was at least one heterozygote individual. Afterwards, the same 14 enzymes were screened by applying SGE on the second fraction of the supernates of 166

Table 2. Numbers of individuals of *A circumscriptus* (1), *A. fasciatus* (2) and the four *A. silvaticus* strains (3–6) collected in North America

Population	Strain					
	1	2	3	4	5	6
Pomfret (CT)	53	–	–	–	–	–
Mansfield (CT)	–	40	–	–	–	–
Woods Hole (MA)	14	–	–	–	–	–
North Carvon (MA)	92	3	–	–	–	–
Bellingham (MA)	–	65	–	–	–	–
West Barnstable 1 (MA)	–	49	4	1	–	–
West Barnstable 2 (MA)	–	7	2	–	–	–
West Barnstable 3 (MA)	10	8	–	–	–	–
Sandwich (MA)	–	–	–	–	2	61

CT = Connecticut, MA = Massachusetts

Table 3. Enzymes assayed, enzyme abbreviations, E.C. numbers, quaternary structure of the enzymes and buffer systems used for PAGE and SGE procedures in *Carinarion*

Enzyme	Abbreviation	E.C. No.	Buffer system PAGE	SGE	Ref.	Quaternary structure
Alanine transaminase	Alat	2.6.1.2	a	d	[13, 16]	Dimeric
Aminopeptidase (leucyl-alanine)	Pep	3.4.11	b	e	[13, 17]	Monomeric
Aspartate transaminase	Aat	2.6.1.1	a	d	[13, 16]	?
Carboxylesterase	EstQ	3.1.1.1	a	d	[13, 16]	?
Fumarate hydratase	Fumh	4.2.1.2	a	d	[13, 16]	?
Glucose-6-phosphate isomerase	Gpi	5.3.1.9	a	d	[13, 16]	?
Glycerol-3-phosphate dehydrogenase	α-Gpd	1.1.1.8	a	d	[13, 16]	?
L-Lactate-dehydrogenase	Ldh	1.1.1.27	c	e	[13, 17]	Monomeric
Leucyl aminopeptidase	Lap	3.4.11.1	a	d	[13, 16]	?
Malate dehydrogenase	Mdh	1.1.1.37	a	d	[13, 16]	?
Mannose-6-phosphate isomerase	Mpi	5.3.1.8	a	e	[13, 17]	?
Phosphoglucomutase	Pgm	5.4.2.2	c	f	[13, 15]	Monomeric
Phosphogluconate dehydrogenase	Pgd	1.1.1.44	a	d	[13, 16]	?
Superoxide dismutase	Sod	1.15.1.1	a	e	[13, 17]	?

a, Tris/citric acid pH 8.0 (6% gels); b, gel buffer: Tris/HCl pH 9.0, tray buffer: Tris/glycine pH 9.0 (7% gels); c, gel buffer: Tris/HCl pH 9.0, tray buffer: Tris/glycine pH 9.0 (6% gels); d, Tris/citric acid pH 8.0 (12% gels); e, phosphate/citric acid pH 7.0 (12% gels); f, citric acid/*n*-3-aminopropyl morpholine (12% gels)

individuals, such that all strains and heterozygous types detected with PAGE (Jordaens *et al.* [10] and this study) were represented. Horizontal SGE in 12% starch (Sigma S-5651) was performed using three different buffer systems [15–17] according to Murphy *et al.* [17] (Table 3). Enzyme systems were resolved in different buffer systems and the buffer system which yielded the best resolution was used (Table 3). All gels were photographed. Alleles were designated alphabetically according to increasing electrophoretic mobilities (A = fastest allele). Previously typed specimens were included in each run. Differences between electromorphs were confirmed by running them simultaneously on single gels. Nei's (1978) genetic distance [18] was calculated between all pairs of North American and European strains [8–10] and ordinated with nonmetric multidimensional scaling (MDS) and minimum spanning trees (MST) using NTSYS version 1.80 [19].

3 Results

PAGE and SGE yielded exactly the same relative band positions in the gels and allelic combinations. Yet, banding patterns of PAGE were sharper and therefore easier to interpret (Figs. 1A–H). Five enzymes were polymorphic in North American populations, *i.e.*, alanine transaminase (Alat), carboxylesterase (EstQ), fumarate hydratase (Fumh), leucyl aminopeptidase (Lap) and (Pgm). With PAGE, we observed three zones of activity for Pgm. Pgm often yields several zones of activity, each the product of a separate locus [20]. Similarly, we interpreted our Pgm profiles as three separate loci. The most anodal locus, Pgm-1, showed the highest activity and was most easy to interpret. Activity at the two other loci was sometimes low and therefore, those loci were excluded from the results. SGE resolved only one locus for Pgm that correspond with Pgm-1 of PAGE. As for Pgm, PAGE of aminopeptidase (Pep) resolved three loci whereas only one locus was resolved with SGE. Both loci seemed homologous, yet, with SGE some individuals showed very low reactivity for Pep such that not all individuals could be typed. Nonspecific esterases are a complex of enzymes, with a large and unknown number of loci [20]. Furthermore, some esterase variants may be food-induced [21–26]. However, due to its highly characteristic position (*i.e.*, most cathodal) and high intensity, the Q band seems suitable for a locus-by-locus analysis [21]. For all other enzymes, no problems were observed. Heterozygotes for Pep, Ldh and Pgm showed two bands and were therefore interpreted as monomers [20]. Heterozygotes for Alat showed the typical three bands of a dimer [20]. The quaternary structure of EstQ could not be determined since for this locus no heterozygotes were observed (Table 3).

A total of six strains were found (Tables 2, 4). *A. circumscriptus* and *A. fasciatus* were represented by single strains (*i.e.*, 1 and 2) corresponding with the strains Q and B of Backeljau *et al.* [8]. *A. silvaticus* comprised four strains (*i.e.*, 3, 4, 5 and 6) (Table 1). Strains 5 and 6

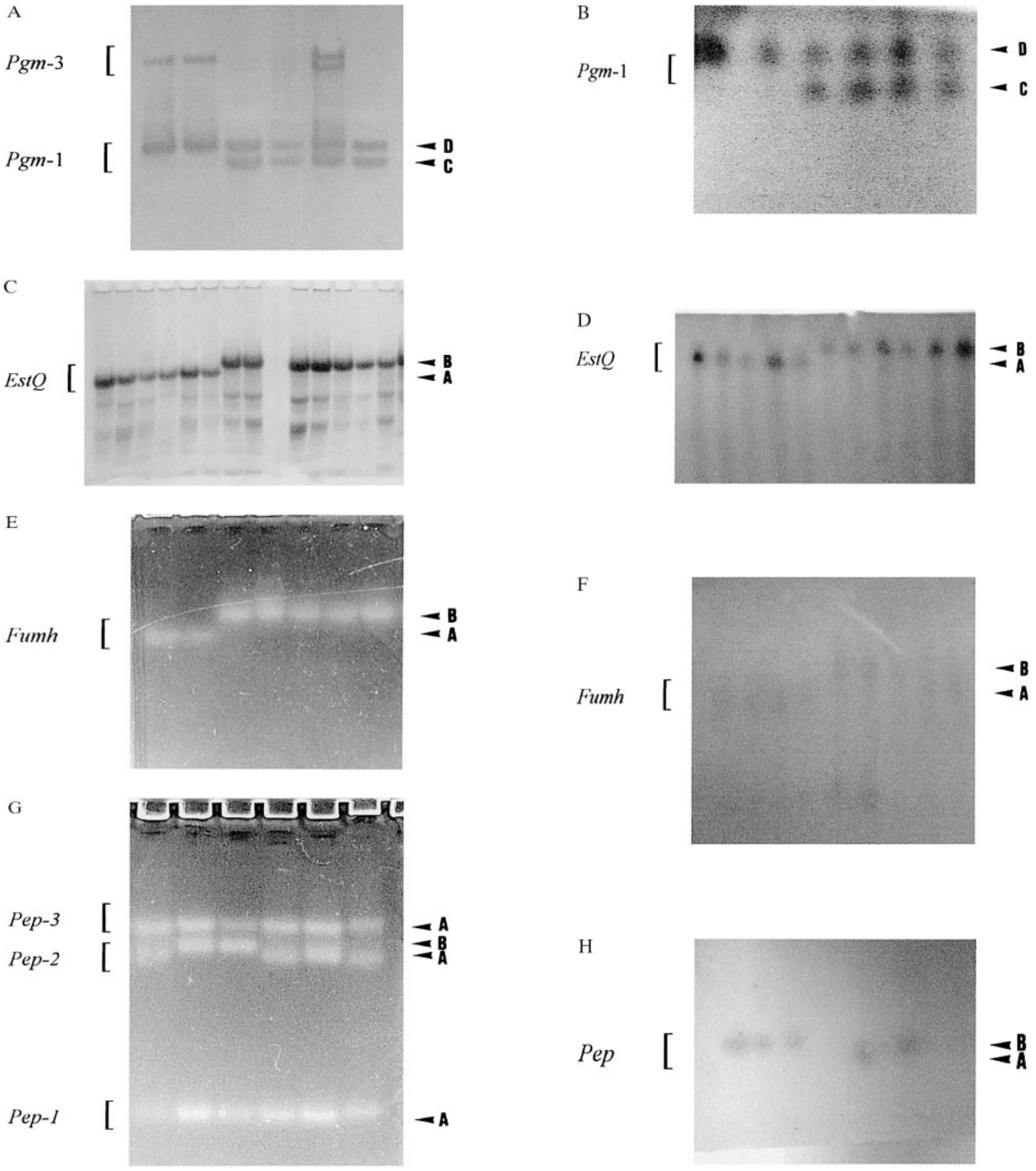

Figure 1. (A) PAGE banding pattern of Pgm showing two zones of activity that correspond with loci Pgm-1 and Pgm-3. Pgm-1 shows two alleles, *i.e.*, C and D. (B) SGE banding pattern of Pgm showing one zone of activity that corresponds with locus Pgm-1. Pgm-1 shows two alleles, *i.e.*, C and D. (C) PAGE banding pattern of EstQ showing several zones of activity that correspond with loci EstQ and several unspecified loci. EstQ has two alleles, *i.e.*, A and B. (D) SGE banding pattern of EstQ showing one zone of activity that corresponds with locus EstQ. EstQ has two alleles, *i.e.*, A and B. (E) PAGE banding pattern of Fumh PAGE showing one zone of activity that corresponds with locus Fumh. Fumh shows two alleles, *i.e.*, A and B. (F) SGE banding pattern of Fumh showing one zone of activity that corresponds with locus Fumh. Fumh shows two alleles, *i.e.*, A and B. (G) PAGE banding pattern of Pep showing three zones of activity that correspond with loci Pep-1, 2 and 3. Pep-1 shows one allele, *i.e.*, A, Pep-2 shows two alleles, *i.e.*, A and B and Pep-3 shows one allele, *i.e.*, A. (H) SGE banding pattern of Pep showing one zone of activity that corresponds with one Pep locus. Pep has two alleles, *i.e.*, A and B.

Table 4. Allelic composition of the six North American strains with 0 = allele absent and 1 = allele present. For the full names of the enzymes we refer to Table 3

Locus/allele		Strain					
		1	2	3	4	5	6
Alat	A	0	0	1	0	1	1
	B	1	1	0	1	0	0
EstQ	A	1	0	0	0	0	0
	B	0	1	1	1	1	1
Fumh	A	1	0	0	0	0	0
	B	0	1	1	1	0	0
	C	0	0	0	0	1	1
Lap	A	0	1	1	1	0	0
	B	1	0	0	0	1	1
Pgm	A	0	0	0	0	0	0
	B	0	0	1	1	0	1
	C	1	0	0	0	1	0
	D	0	1	0	0	0	0

corresponded with strains R and N of Backeljau *et al.* [8] and Jordaens *et al.* [9], while strains 3 and 4 were new. In four populations species co-occurred and in two populations two strains of *A. silvaticus* were found together (Table 2). Yet, no heterozygotes were observed. The MDS/MST analysis clustered the strains of *A. fasciatus* and *A. silvaticus* together (Fig. 2), irrespective of their origin (see also Jordaens *et al.* [10]).

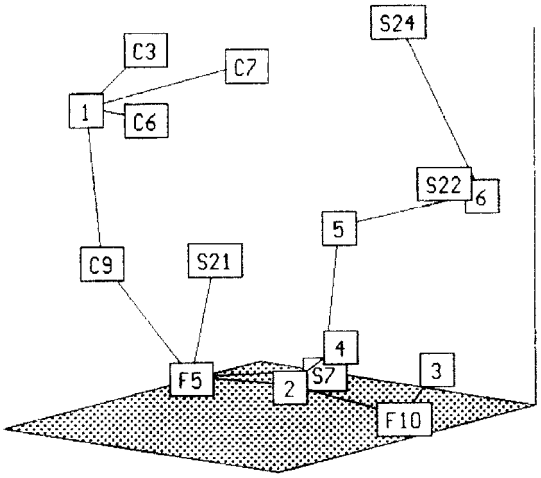

Figure 2. Ordination of Nei's (1978) genetic distances by means of three-dimensional plots of nonmetric multidimensional scalings with superimposed minimum spanning trees. Numbers refer to the six North American strains. C3, C6, C7, C9, S7, S21, S22, S24, F5 and F10 refer to European strains of *A. circumscriptus*, *A. silvaticus* and *A. fasciatus*, respectively.

4 Discussion

Although the presence of hidden electrophoretic allozyme variation is a common phenomenon (*e.g.*, [20, 27]) that may depend on the supporting medium [2–5], the present data show that at least in our study PAGE and SGE yielded identical results for *Carinarion* spp. Therefore, SGE [6, 7] and PAGE ([8–10]; this work) studies on these animals are fully comparable. Yet, despite this congruence between SGE and PAGE, we are still unable to homologize the Pgm^b allele of Foltz *et al.* [7] with the Pgm alleles reported for PAGE [8]. Possibly, the British Pgm^b allele is a rare variant that is absent from the European mainland and North America. Other electrophoretic techniques, such as isoelectric focusing (IEF), may be helpful to find additional variation. For instance, Jordaens *et al.* [26] examined environmental effects on Est variation in 47 *Carinarion* families. In most families, PAGE and IEF resolved an equal amount of variation. Yet, in eight families Est variation was found with IEF but not with PAGE. Whether this also holds for other, less complex enzyme systems, remains to be investigated.

In this study, we showed that PAGE and SGE give similar results in *Carinarion* slugs. Yet, with PAGE, three different loci were resolved for Pgm and Pep whereas a single locus was found with SGE. Moreover, SGE of Pep sometimes yielded weak reaction, so that not all individuals could be typed. Therefore, it is recommended to evaluate different electrophoretic techniques before extensive electrophoretic studies are carried out in order to apply the electrophoretic technique and buffer systems that will yield the best results.

The three *Carinarion* species have been introduced into North America around 1870 [28] and were reported to consist of single strains [6]. Because British *A. silvaticus* and *A. circumscriptus* showed little or no genetic variation, the lack of polymorphism in North American *Carinarion* was not attributed to colonization events (*i.e.*, founder effects), but rather to a uniparental breeding system [29]. We provisionally confirm the lack of allozyme variation in North American *A. fasciatus* and *A. circumscriptus*. However, the "one strain per species" hypothesis [6] was not supported for *A. silvaticus*, since we detected four different strains among 70 individuals from three populations. Hence, the (near) absence of allozyme variation in *A. silvaticus* in previous studies [6, 7] probably reflects nothing more than sampling bias, since these analyses involved only 29 and 48 individuals from three and four populations, respectively.

The absence of allozyme variation in North American *A. fasciatus* and *A. circumscriptus* is less likely the result of sampling bias, since considerable numbers of speci-

mens of both species have been screened (Table 1). Nevertheless, the numbers of North American populations surveyed for both species remain limited (Table 1). Hence sampling bias can still not be ruled out. Alternatively, founder effects could also have depleted the amount of polymorphism in North American *A. fasciatus* and *A. circumscriptus*. Yet, this seems at odds with the fact that such effects apparently did not affect *A. silvaticus*, the rarest of the three species in North America. Moreover, in Europe *A. fasciatus* and *A. circumscriptus* are also considerably less polymorphic than *A. silvaticus*. So until compelling evidence indicates otherwise, we suggest that the previously reported lack of allozyme polymorphisms in North American and British *Carinarion* populations [6, 7] are most probably the result of sampling bias.

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