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Allozyme diversity in slugs of the *Carinarion* complex (Mollusca, Pulmonata)

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Previous allozyme analyses of the hermaphroditic terrestrial slugs *Arion fasciatus*, *A. circumscriptus* and *A. silvaticus* (subgenus *Carinarion*) have suggested that in North America these species are each single monomorphic strains. However, new data on 18 putative enzyme loci show that in western Europe the three taxa, respectively, consist of at least three, two and 12 homozygous multilocus genotypes (strains), which regularly co-occur. The current opinion that American and European *Carinarion* populations are similarly structured, and that colonization events did not affect the population genetics of North American *Carinarion*, should therefore be readdressed. The present data also provide the first indication of heterozygosity and possible outcrossing in *Carinarion*. Nevertheless, uniparental reproduction is confirmed as the main breeding system in West European *Carinarion*, although the high incidence of multistrain populations in *A. silvaticus* and *A. fasciatus* appears at variance with the current model of population genetic structuring in selfing terrestrial pulmonates. Finally, the systematic status of the three *Carinarion* spp. is tentatively questioned.

Keywords: allozymes, hermaphroditic slugs, homozygous strains, population genetics, uniparental reproduction.

Introduction

Arion fasciatus (Nilsson, 1823), *A. circumscriptus* Johnston, 1828 and *A. silvaticus* Lohmander, 1937 are morphologically highly similar, hermaphroditic terrestrial slug species, which belong to the arionid subgenus *Carinarion* Hesse, 1926. The three species are widely distributed in Europe and North America (Chichester & Getz, 1969; Kerney *et al.*, 1983) and their population genetics has been dealt with in two major allozyme surveys. McCracken & Selander (1980) reported that in North America each species consists of a single homozygous strain, whereas in Ireland Foltz *et al.* (1982) observed one homozygous strain of *A. circumscriptus* and two of *A. silvaticus*. These data were interpreted as suggesting that: (i) *Carinarion* spp. are selfers; (ii) the single strain structure of American populations is not the result of a loss of genetic variation (e.g. founder effects) caused by their introduction from more variable European populations (Selander & Ochman, 1983; Foltz *et al.*, 1984); and (iii) the three taxa involved deserve species rank because of their low mean genetic identities, respectively $I = 0.65$ between the three species (McCracken & Selander, 1980) and $I = 0.74$ between *A. circumscriptus* and *A. silvaticus* (Foltz *et al.*, 1982).

However, Foltz *et al.* (1982) did not consider *A. fasciatus* (which was the most intensively studied species in North America) and did not investigate continental populations of both other species. Hence, to assess whether the studies of McCracken & Selander (1980) and Foltz *et al.* (1982) were sufficiently representative for *Carinarion* as a whole, we extended the survey by including European *A. fasciatus* and by screening the two other species in the European mainland.

Materials and methods

Vertical polyacrylamide gel electrophoresis (PAGE) was used to screen 16 enzymes in 53 *A. fasciatus*, 71 *A. circumscriptus* and 115 *A. silvaticus* (Table 1). Species identifications were based on Lohmander

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Table 1 Origins, numbers of specimens and numbers of strains (in parentheses) of *Arion fasciatus* (*fasc.*), *A. silvaticus* (*silv.*) and *A. circumscriptus* (*circ.*) sampled for this study

Locality	*fasc.*	*silv.*	*circ.*	Total
Austria				
Rottenegg	1(1)	1(1)	—	2(2)
Belgium				
Antwerpen, 't Half Maantje	—	12(3)	14(2)	26(5)
Antwerpen, Hobokense Polder	—	4(4)	—	4(4)
Antwerpen, RUCA campus	—	14(1)	—	14(1)
Antwerpen, RUCA Plein	—	6(2)	3(1)	9(3)
Balen	—	17(2)	—	17(2)
Dinant, Bouvignes	—	1(1)	3(1)	4(2)
Dourbes	—	—	2(1)	2(1)
Eben-Emael	—	—	2(1)	2(1)
Eprave	—	2(2)	—	2(2)
Hoogstraten	—	16(3)	—	16(3)
Koninksem	—	1(1)	2(1)	3(2)
Leopoldsburg	—	1(1)	6(1)	7(2)
Loenhout	—	—	6(1)	6(1)
Olloy-sur-Viroin	—	3(1)	—	3(1)
St.-Pieters Voeren	—	4(2)	—	4(2)
Turnhout	—	11(1)	—	11(1)
Vielsalm	—	5(1)	3(*)	8(2)
Great Britain				
Bolton, W. Yorkshire	—	2(1)	1(1)	3(2)
Catrigg Force, Standforth	8(2)	1(1)	—	9(3)
Muker, River Swale	—	3(2)	—	3(2)
South Croydon, Surrey	—	1(1)	14(1)	15(2)
Norway				
Fleslandsvika, Bergen	7(1)	—	—	7(1)
Sweden				
Göteborg, NHM	18(3)	—	—	18(3)
Göteborg, Annedal church	2(1)	—	—	2(1)
Göteborg, Vitsippsdalen	7(1)	—	12(1)	19(2)
Hven	9(1)	—	—	9(1)
The Netherlands				
Sirjansland	—	2(2)	—	2(2)

Localities at which only single specimens were collected: *A. fasciatus*: **Great Britain** — Gisburn, W. Yorkshire; *A. silvaticus*: **Austria** — Öischevtormäuer; **Belgium** — Blanden; Dinant, Celles; Rijkhoven; Schilde; Schoten; Stekene; Wellin; *A. circumscriptus*: **Belgium** — Erezée; Lomprez; **The Netherlands** — Vlieland.

A. circumscriptus from Vielsalm comprised two *Mdh* heterozygotes and was not interpreted in terms of strains, as indicated by (*).

(1937) and Waldén (1955). In case of doubt the esterase and albumen gland protein zymograms described by Backeljau *et al.* (1987) were considered decisive. Specimens were collected at the localities listed in Table 1. In each case sampling sites consisted of only a few square metres.

PAGE procedures and sample preparation of individual digestive gland homogenates followed Backeljau (1987). A continuous Tris/Citric acid (pH 8.0) buffer was used to resolve malate dehydrogenase (*Mdh*, EC 1.1.1.37), isocitrate dehydrogenase (*Idh*, EC 1.1.1.42), phosphogluconate dehydrogenase (*Pgdh*, EC 1.1.1.44), alanine aminotransferase (*Alat*, EC 2.6.1.2), esterase Q (*EsQ*, EC 3.1.1.1 see Backeljau *et al.*, 1987), fumarate hydratase (*Fumh*, EC 4.2.1.2) and glucose-6-phosphate isomerase (*Gpi*,

EC 5.3.1.9). A discontinuous buffer combination consisting of Tris/HCl (pH 9.0) in the gel and Tris/Glycine (pH 9.0) in the tray, was used to resolve glycerol-3-phosphate dehydrogenase (*G3pdh*, EC 1.1.1.8), lactate dehydrogenase (*Ldh*, EC 1.1.1.27), glucose 1-dehydrogenase (*Gcdh*, EC 1.1.1.47), dihydrolipoamide dehydrogenase (*Ddh*, EC 1.8.1.4), superoxide dismutase (*Sod*, EC 1.15.1.1), aspartate aminotransferase (*Aat*, EC 2.6.1.1), phosphoglucomutase (*Pgm*, EC 5.4.2.2), α-amylase (*Amy*, EC 3.2.1.1.) and leucylalanine aminopeptidase (*Pep*, EC 3.4.11). Staining recipes were adapted from Harris & Hopkinson (1976).

Electromorph terminology was based on the mobility (r_m) of the gene products relative to the fastest electromorph ($r_m = 100$). We assumed an allelic basis for the observed enzyme profiles and used the BIOSYS package (Swofford & Selander, 1981) for estimating allele frequencies, percentages of polymorphic loci (P, 0.99 criterion), Nei's (1978) unbiased expected heterozygosities (H_e) and Nei's (1972) genetic identities (I) and distances (D). Observed heterozygosities (H_o) were determined by direct count. Heterozygote deviations (D_H) were calculated as $D_H = (H_o - H_e)/H_e$ and averaged over loci. Genetic distances were subjected to UPGMA clustering with the DISPAN (v. 1.1) package (Ota, 1993), which was also used to test the stability of the UPGMA tree by bootstrapping over 1000 replicates.

Results

Eighteen putative enzyme loci were resolved, 10 of which were fixed for the same allele in the three species, whereas eight were polymorphic over *Carinarion* as a whole ($P = 0.44$; Table 2). Some of these latter loci were also polymorphic within species (and populations), yielding positive H_e values (Table 2). No heterozygotes were observed, however, except for two specimens of *A. circumscriptus* from Vielsalm, Belgium, which were heterozygous at *Mdh*. Hence, overall heterozygote deviations were extremely negative (Table 2).

In the near absence of heterozygotes, 17 homozygous multilocus genotypes (strains) could be distinguished: three in *A. fasciatus* (A–C), 12 in *A. silvaticus* (D–O) and two in *A. circumscriptus* (P–Q) (Table 2). Obviously, more strains would be expected if additional loci were screened, i.e. probably every strain defined in this study is in turn a complex of monomorphic lineages.

Considering only populations with at least two specimens, nine out of 14 *A. silvaticus* (64 per cent), two out of six *A. fasciatus* (33 per cent), but only one out of 11 *A. circumscriptus* (9 per cent) yielded at least two different strains (Table 1).

Strains of different species co-occurred at 11 sites (Table 1). Thus, 19 of the 28 localities where more than one *Carinarion* specimen was collected (68 per cent), yielded two or more strains (irrespective of the species) (Table 1). At most sites with mixed species (seven of 11), we found one strain per species. But, in two localities ('Antwerpen, RUCA Plein' and 'Catrigg Force, Standforth'), one species was represented by two strains, and in a third locality ('Antwerpen, 't Half Maantje') we observed three *A. silvaticus* and two *A. circumscriptus* strains (Table 1). The remaining site with mixed species ('Vielsalm') was unique because of two *A. circumscriptus* specimens with presumed heterozygous Mdh^{100}/Mdh^{86} genotypes.

Table 3 summarizes the genetic identities and distances between strains, resulting in a mean interstrain identity of $I = 0.81$. The UPGMA tree of the 17 strains clustered conspecific strains together, even though bootstrap values were very low (Fig. 1). Nevertheless, there was a strong support for a sister group relationship between *A. fasciatus* and *A. silvaticus*.

Discussion

Contrary to previous studies by McCracken & Selander (1980), Foltz *et al.* (1982), Selander & Ochman (1983) and Dolan & Fleming (1988), our work clearly shows that the three *Carinarion* spp. are polymorphic, multistrain complexes, instead of single monomorphic lines. This is surprising for the literature data on *A. fasciatus* and *A. circumscriptus* were based on considerably larger numbers of specimens and comparable numbers of populations. McCracken & Selander (1980), for example, surveyed 10 populations of *A. fasciatus* in North America (814 specimens) and the same authors, together with Foltz *et al.* (1982) and Dolan & Fleming (1988), investigated 362 specimens (respectively, 312, 46 and four) from 10 populations (respectively, four, five and one) of *A. circumscriptus*. Only for *A. silvaticus* were the earlier data of McCracken & Selander (1980) and Foltz *et al.* (1982) based on a smaller total sample size, i.e. 77 specimens (29 and 48) from seven populations (respectively, three and four). However, as we found no significant correlation between the number of strains detected and the number of specimens screened per population in *A. silvaticus* ($r = 0.124$, $N_{obs.} = 14$, $P = 0.67$), we assume that sampling bias may be of little, if any, importance.

Table 2 Allelic composition of strains (on the right: 1, present; 0, absent), overall allele frequencies, percentages of polymorphic loci (*P*, 0.99 criterion), Nei's (1978) unbiased heterozygosities (H_e), observed heterozygosities (H_o) and heterozygote deviations (D_H) at 18 enzyme loci in *Arion fasciatus* (*fasc.*), *A. silvaticus* (*silv.*) and *A. circumscriptus* (*circ.*)

Locus/allele		*fasc.*	*silv.*	*circ.*	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
EsQ	100	—	—	1.00	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	88	1.00	1.00	—	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
	(*N*)	(40)	(45)	(34)																	
Pgm-1	100	—	0.83	—	0	0	0	1	1	1	1	1	1	0	1	0	0	1	1	0	0
	93	—	0.17	1.00	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	1
	85	1.00	—	—	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	(*N*)	(36)	(102)	(53)																	
Alat	100	—	1.00	—	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0
	88	1.00	—	1.00	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	(*N*)	(29)	(16)	(16)																	
Fumh	100	—	—	1.00	0	0	0	0	0	—	—	0	0	0	0	0	—	0	—	1	1
	93	1.00	0.39	—	1	1	1	1	0	—	—	1	1	1	0	0	—	0	—	0	0
	86	—	0.61	—	0	0	0	0	1	—	—	0	0	0	1	1	—	1	—	0	0
	(*N*)	(51)	(44)	(22)																	
Mdh	100	1.00	1.00	0.03	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0
	86	—	—	0.97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
	(*N*)	(27)	(35)	(38)																	
Ldh	100	0.52	0.74	1.00	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0	1	1
	83	0.48	0.26	—	0	0	1	0	1	1	1	1	0	0	0	1	0	0	1	0	0
	(*N*)	(25)	(90)	(34)																	
Pep-2	100	0.32	0.44	0.18	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	0
	91	0.68	0.56	0.82	0	1	0	0	0	0	1	0	1	1	0	0	0	1	1	0	1
	(*N*)	(37)	(88)	(45)																	
Pep-3	100	1.00	0.87	1.00	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1
	92	—	0.13	—	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	(*N*)	(45)	(107)	(62)																	
P		0.110	0.280	0.110		*fasc.*							*silv.*							*circ.*	
H_e		0.053	0.104	0.019																	
H_o		0.000	0.000	0.003																	
D_H		−1.000	−1.000	−0.950																	

The *Fumh* alleles in strains F, G, M and O were not scored. Monomorphic loci: *Aat*, *Amy*, *Sod*, *Pgdh*, *G3pdh*, *Idh*, *Gcdh*, *Gpi*, *Ddh* and *Pep-1*.

Table 3 Mean Nei's (1972) genetic identities (I_{av}) and distances (D_{av}), between 17 *Carinarion* strains at different levels of comparison

Comparisons	$D_{av} \pm SD$	Min.	Max.	$I_{av} \pm SD$	Min.	Max.
All strains	0.22 ± 0.12	0.06	0.59	0.81 ± 0.10	0.56	0.94
Intra *A. fasciatus* (f)	0.08 ± 0.04	0.06	0.12	0.93 ± 0.03	0.89	0.94
Intra *A. silvaticus* (s)	0.14 ± 0.06	0.06	0.33	0.87 ± 0.05	0.72	0.94
Intra *A. circumscriptus* (c)	0.06	—	—	0.94	—	—
Inter f/s	0.24 ± 0.08	0.12	0.41	0.79 ± 0.06	0.67	0.89
Inter f/c	0.31 ± 0.06	0.25	0.41	0.73 ± 0.04	0.67	0.78
Inter c/s	0.40 ± 0.09	0.25	0.59	0.67 ± 0.06	0.56	0.78

Values for the strains F, G, M and O were calculated by assuming that they were fixed for *Fumh*^{86}; *Mdh* heterozygotes were not considered.

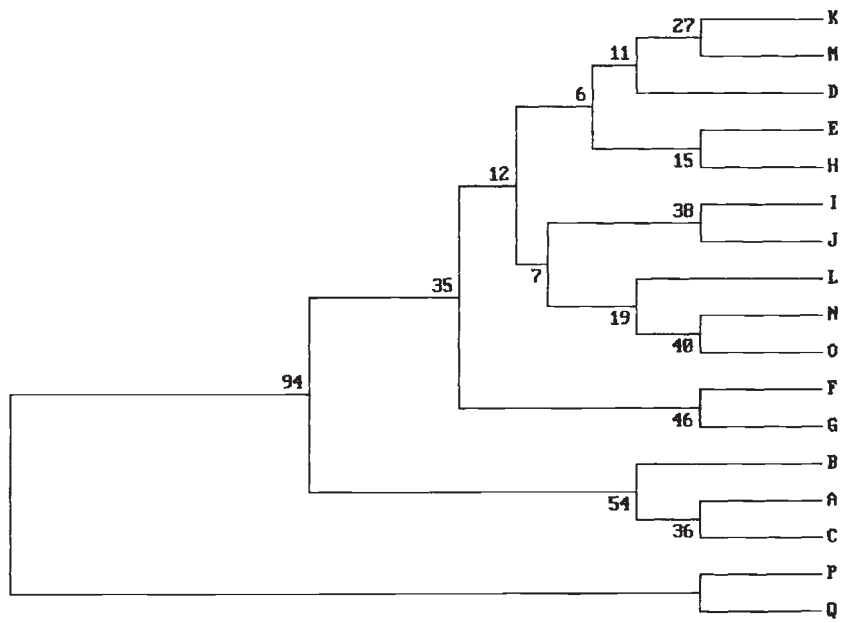

Fig. 1 UPGMA tree of Nei's (1972) genetic distances between 17 *Carinarion* strains: *A. fasciatus* (A–C), *A. silvaticus* (D–O) and *A. circumscriptus* (P–Q). Numbers at the nodes are bootstrap percentages based on 1000 replicates.

Two other artefacts might explain why previous studies did not detect variation: bias in the choice of enzymes and differences in the resolving power of electrophoretic techniques (e.g. Coyne *et al.*, 1979). However, we included four variable enzymes that were also screened by previous authors (*EsQ*, *Pgm*, *Mdh* and *Pep*) and these enzymes still allow the detection of ten strains (two in *A. fasciatus*, two in *A. circumscriptus* and six in *A. silvaticus*). Nevertheless, McCracken & Selander (1980) and Foltz *et al.* (1982) found with starch gel electrophoresis (SGE) only one monomorphic locus for leucylalanine aminopeptidase (their *Pep-1*; R.K. Selander *in litt.*, 1986), whereas with PAGE this enzyme yielded at least two polymorphic loci (Table 2). Similarly, using SGE Dolan & Fleming (1988) were unable to separate the *Pgm* and *Sod* alleles of *A. circumscriptus* from those of other arionids, whereas Backeljau & De Winter (1987) showed with PAGE that these alleles are not shared between *Carinarion* and other arionids. On the other hand, SGE detected the Pgm^b allele (Foltz *et al.*, 1982), which cannot be homologized with the *Pgm* alleles we found, even if we assume that Pgm^a is our $Pgm\text{-}1^{100}$ and Pgm^c is our $Pgm\text{-}1^{93}$ (compare our Table 2 with table 5 of Foltz *et al.*, 1982).

Without further experimentation we cannot decide whether the lack of genetic variation in north American *Carinarion* (McCracken & Selander, 1980) is artifactual. Nevertheless, our data unequivocally show that at least in Europe the three species are polymorphic and may produce heterozygotes. The generally accepted idea that the single strain structure of American *Carinarion* populations is not the

result of a loss of genetic variation caused by their introduction from more variable European populations (Selander & Ochman, 1983; Foltz *et al.*, 1984), should therefore be readdressed. In this context it is noteworthy also that of the selfing Mediterranean land snail, *Rumina decollata*, only one out of many European strains was introduced into the U.S.A., where it is now widespread (Selander & Kaufman, 1973).

Our data further confirm that *Carinarion* mainly reproduces uniparentally (e.g. McCracken & Selander, 1980; Foltz *et al.*, 1982, 1984; Backeljau *et al.*, 1987), for this seems the most parsimonious explanation for the rarity of heterozygotes in widely distributed, polymorphic populations (e.g. Foltz *et al.*, 1982, 1984; Selander & Ochman, 1983). Yet, whether this uniparental breeding system involves autogamy, automixis or apomixis (sensu Mogie, 1986), remains to be decided (e.g. Nicklas & Hoffmann, 1981; Hoffmann, 1983; Selander & Ochman, 1983; Foltz *et al.*, 1984; Tompa, 1984; Backeljau & De Bruyn, 1991). Anyhow, the two *Mdh*-heterozygotes in *A. circumscriptus* suggest that outcrossing is possible in natural populations, even though it is unclear how often and under what conditions it may occur. This issue, and the estimation of selfing rates, can only be addressed appropriately with breeding experiments using known genetic markers (Jarne & Charlesworth, 1993; Jarne & Städler, 1995). Indeed, it may be questioned whether uniparental reproduction is the main breeding system over the entire range of *Carinarion*, for preliminary data from East European populations suggest that in these regions outcrossing may be more common (K. Jordaens, T. Backeljau and H. De Wolf, unpublished data).

The high incidence of multistrain populations in *A. silvaticus* (64 per cent) and *A. fasciatus* (33 per cent) seems at variance with the predictions of Selander & Ochman (1983), who modelled interactions between self-compatible strains of terrestrial pulmonates. This model states that slight fecundity differences between strains may lead to the eventual elimination of the less fecund ones. Therefore, most local populations of mainly, but not exclusively, selfing multistrain species would consist of single strains (Selander & Ochman, 1983). Consequently, this model does not seem directly applicable to *A. silvaticus* and *A. fasciatus*. Nevertheless, it remains possible that co-occurring *Carinarion* strains also interact competitively or are eco-ethologically differentiated in other traits. Furthermore, it is unknown whether multistrain populations are stable in time.

In conclusion, our data suggest that earlier population genetic surveys of *Carinarion* were not sufficiently representative either because of artifactual issues or (and?) because of a true lack of variation in American populations. This implies that the conclusions of McCracken & Selander (1980) as to the species status of *A. fasciatus*, *A. circumscriptus* and *A. silvaticus* also need to be re-evaluated. Although it is beyond the scope of this paper to expand on this issue, we find it suggestive that: (i) many other terrestrial pulmonates, such as *Rumina decollata*, *Chondrina clienta*, *A. intermedius*, *A. subfuscus* and the *Cochlicopa* complex are multistrain taxa that often consist of several 'discrete' morphotypes based on colour, size and shell form (e.g. Selander & Kaufman, 1973; Selander & Hudson, 1976; McCracken & Selander, 1980; Baur & Klemm, 1989; Backeljau & De Bruyn, 1991; Backeljau *et al.*, 1992; Armbruster & Schlegel, 1994); (ii) the mean genetic identity between *Carinarion* strains ($I = 0.81$) is comparable to that of conspecific strains in *Chondrina clienta* ($I = 0.81$; Baur & Klemm, 1989) or *A. intermedius* ($I = 0.87$; McCracken & Selander, 1980); and (iii) the separation of *A. silvaticus* and *A. fasciatus* in the tree of Fig. 1 disappears when East European material is included (K. Jordaens, T. Backeljau & H. De Wolf, unpublished data). Therefore, we currently speculate that *Carinarion* might very well represent a single 'species', which because of its mainly uniparental breeding system is divided into a series of morphotypes and genetic strains.

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