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Large scale patterns of genetic differentiation at enzyme loci in the land snails *Cepaea nemoralis* and *Cepaea hortensis*

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Samples of 231 populations of the land snails *Cepaea nemoralis* and *C. hortensis* from Britain, France, Switzerland and Spain were analysed for genetic polymorphism in six enzyme systems. These sibling species show similar levels of variation detected by electrophoresis, and have a generally similar degree of local divergence over homologous enzyme loci. As well as extensive local and regional differentiation, both species show large-scale changes in allele frequency across Europe. In *C. hortensis* there is a continuous gradient in allele frequency from northern Britain to northern Spain, while in *C. nemoralis* north–south clines on the continent of Europe are reversed in direction in Britain. There are few obvious correlations of allele frequency change at single loci with components of the environment. Patterns of allele frequency variation in 24 sympatric populations of the two species are generally independent of each other, although there is a positive association of the frequencies of alleles at a leucine aminopeptidase locus in the two species. The statistical significance of this association depends on a single locality, and there is little indication of shared patterns of allele frequency change which might reflect a common response to natural selection. In addition, there is no evidence that the extensive geographical change in *C. nemoralis* and *C. hortensis* is a precursor of speciation.

INTRODUCTION

The land snails *Cepaea nemoralis* and *C. hortensis* are sibling species whose conspicuous shell polymorphisms have been the subject of extensive ecological and genetic research (Jones *et al.*, 1977). The frequencies of these characters respond to various agents of natural selection (e.g., Cain and Sheppard, 1954; Arnold, 1968) but analysis of their associations with the environment is complicated by the fact that other evolutionary forces, such as migration and fluctuations in population size, can also influence the distribution of genes (Nei, 1975; Selander, 1975).

In many places, both species of *Cepaea* show patterns of extreme local differentiation ("area effects"; Cain and Currey, 1963), in which certain shell morphs predominate over large and ecologically diverse regions separated by steep clines from adjacent areas with quite different morph frequency. This microgeographic structuring exists within a context of large-scale gradients of allele frequency for shell characters across Europe, some of which are associated with climate (Jones *et al.*, 1977). Some populations of *C. nemoralis* also show marked geographical differentiation in allele frequencies at structural gene loci detected by enzyme electrophoresis (Johnson, 1979; Jones *et al.*, 1980; Caugant *et al.*, 1982; Valdez-Forsans, 1983). In the Pyrenees, in particular, enzyme polymorphisms show patterns of strong local divergence which are concordant over loci (Ochman *et al.*, 1983). These regions of relative genetic uniformity (or "molecular area effects") are analogous to area effects for shell characters but exist on a much larger scale.

There are several models for the origin of area effects, including microclimatic selection (Cain and Currey, 1963; Arnold, 1968), local coadaptation (Clarke, 1966; Slatkin, 1982) and allopatric divergence followed by secondary contact (Goodhart, 1962; Cameron *et al.*, 1980). In the absence of evidence for selection, we have suggested that molecular area effects arise from

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stochastic factors related to founder effects and genetic drift in populations isolated by Pyrenean glaciations.

Comparisons of patterns of geographic variation of shell polymorphism in the two species of *Cepaea* have provided some useful insights into their evolution (Clarke, 1962). It has often been suggested that parallel variation at homologous enzyme loci can be taken as evidence for natural selection even when the causative agents are not known (Clarke, 1975; Borowsky, 1977; Varvio-Aho and Pamilo, 1982), and this approach has been used to infer the action of selection on enzyme loci in sympatric species of fish (Johnson, 1974), butterflies (McKechnie *et al.*, 1975), estuarine molluscs (Koehn and Mitton, 1972; Newkirk and Doyle, 1979), field crickets (Harrison, 1977), grasshoppers (Gill, 1981) and *Drosophila* (Borowsky, 1977; Anderson and Oakeshott, 1984).

Here we examine patterns of geographic differentiation at enzyme loci in *Cepaea nemoralis* and *C. hortensis* across western Europe to determine the scale of organization of enzyme polymorphism in these species. This may make it possible to identify common selective or demographic forces which act upon them. As both species are highly polymorphic and share alleles at many enzyme loci (Levan and Fredga, 1972; Johnson, 1979; Selander and Ochman, 1983) we pay particular attention to correlations among enzyme loci in sympatric populations of these snails which might give evidence on the action of selection.

MATERIALS AND METHODS

One hundred and thirty four populations of *C. nemoralis* and 92 populations of *C. hortensis* were sampled in Britain and Europe between 1978 and 1982 (fig. 1). The collections were separated by at least 25 km and were taken from a wide range of habitats. The mean sample size is approximately 30, and all snails collected were scored for shell characters. Ten individuals were taken from each sample for enzyme electrophoresis (Selander *et al.*, 1971). Six enzyme systems known to be polymorphic in *nemoralis* were assayed in each species: phosphoglucose isomerase (*Pgi*), indophenol oxidase (*Ipo*), leucine aminopeptidase (*Lap*), malate dehydrogenase (*Mdh*), phosphoglucomutase

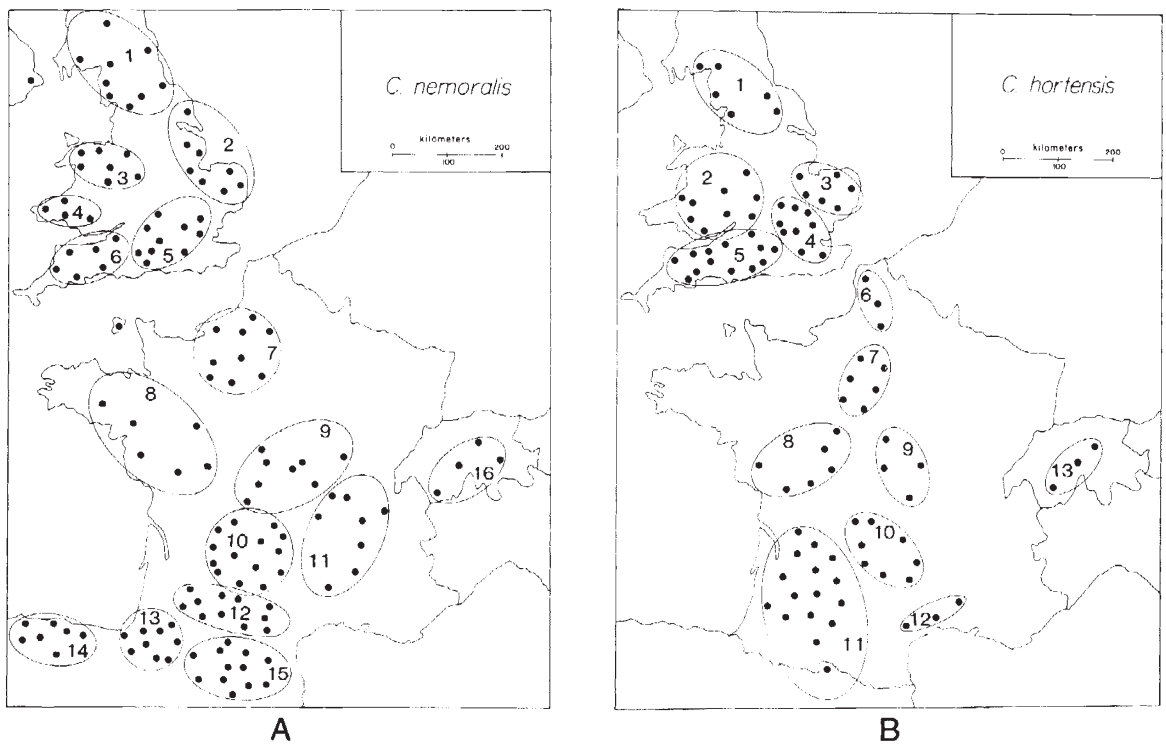


Figure 1 Collection localities for *Cepaea nemoralis* and *C. hortensis*. Ellipses define geographic regions used in the analysis of population structure.

(*Pgm*) and isocitrate dehydrogenase (*Idh*). This produced eight putative polymorphic loci in *hortensis* (which shows variation in at least some populations at two *Lap* and two *Idh* loci) and seven in *nemoralis* (which has only one polymorphic *Idh* locus). Mendelian inheritance of allozymes at many of these loci has been established by Johnson (1979). Six of them (*Pgi, Ipo, Lap-2, Mdh, Pgm,* and *Idh-1*) were polymorphic in most populations and were used to establish patterns of geographic structure; the other two are only sporadically variable and were utilised only in comparisons of interspecific patterns of genetic change.

Hierarchical *F*-statistics (Wright, 1978) were used to estimate standardised variances in allele frequency at several levels of population structure: F_{DR}, among demes (sampling sites) within regions (16 regions for *C. nemoralis* and 13 for *C. hortensis*, as indicated by ellipses in fig. 1); F_{RS}, among regions within subdivisions (Britain and continental Europe); and F_{ST}, between subdivisions within the total area sampled. All estimates were adjusted for sampling error. A principal components analysis (Sneath and Sokal, 1973) was used to examine spatial patterns of association of variables among loci. Large-scale patterns of allele frequency were examined utilising maps similar to those used by Menozzi *et al.* (1978) and Piazza *et al.* (1981a, b) for human populations. In these maps, factor scores are interpolated at the nodes of 65 km square grid over the whole area sampled. At each node, the unweighted average of factor scores (scaled from zero to one) within a circle with a radius equal to the distance between each node is taken, and the range of the averaged factor scores divided into uniform intervals which can be represented as different intensities of shading on a map.

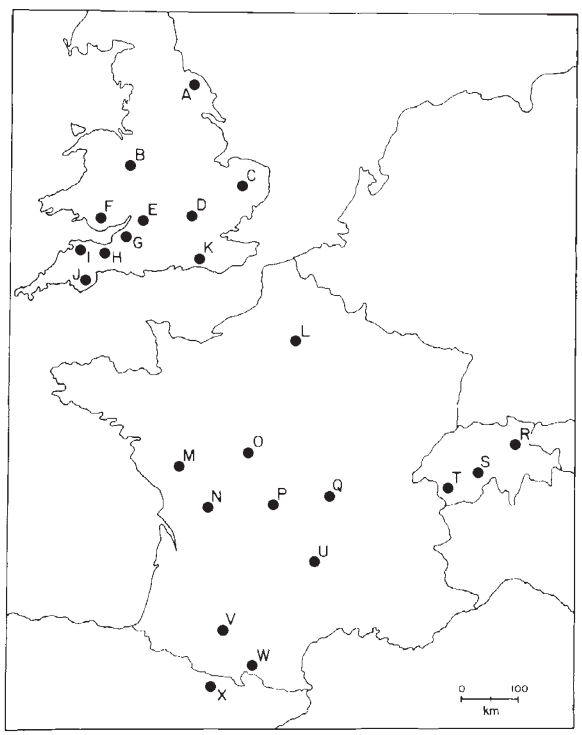


Figure 2 Locations of sympatric populations of *C. nemoralis* and *C. hortensis*.

Parallel variation in allele frequencies at homologous loci were analysed in sympatric populations of *nemoralis* and *hortensis* from 24 sites in Britain, France, Switzerland and Spain (fig. 2). Only those enzyme loci which were polymorphic in more than five of these populations were used in the analysis. For each locus, arcsine-transformed frequencies of the most common alleles in *nemoralis* were plotted against those in *hortensis* from the same place. Not all samples could be used in the analysis of *Lap-2* because of the presence of a null allele in some populations.

RESULTS

Analysis of genetic diversity and variance

Allele frequencies at enzyme loci and at those controlling shell characters are listed in Ochman *et al.* (1986). We will not consider shell polymorphism in detail here, as this is the subject of a separate study based on much more extensive information. Although *C. hortensis* is generally less polymorphic for shell characters than is *C. nemoralis*, genetic diversity over the polymorphic enzyme loci studied was the same in the two species, with a mean heterozygosity of 0·34 for each.

Geographic variation in allele frequency at three levels of population structure is summarised in table 1. The relatively high scores for F_{DR} indicate that both species show large components of components of diversity within geographic regions. Although there is no apparent relationship between values of this statistic at homologous enzyme loci in the two species ($r = -0{\cdot}32$), the mean F_{DR} over all enzyme loci is similar in the two species (F_{DR}, *nemoralis* = 0·187; *hortensis* = 0·197).

Average regional differentiation within subdivisions (F_{RS}) for enzyme loci in *nemoralis* (0·132) is about twice that estimated for *hortensis* (0·06). This difference might arise in part because *nemoralis* was sampled over a larger area than was *hortensis*, and includes samples from the Pyrenees,

where there are areas of marked differentiation at enzyme loci (Ochman *et al.*, 1983). If the Pyrenees are omitted from the analysis, F_{RS} for *nemoralis* is reduced to 0·096. However, when only British samples of the two species are considered, regional differentiation of *nemoralis* is about four times greater than is that for *hortensis*. *C. nemoralis* does appear to be more regionally subdivided than is its sibling species.

Mean standardised variances in allele frequencies between samples from Britain and those from the European continent (F_{ST}) are, at 0·02, the same in both species. This component of geographical variance is small compared with that at the demic or regional level.

Patterns of differentiation at enzyme loci

To study the concordance of patterns of geographic variation at all loci simultaneously, we subjected arcsine-transformed allele frequencies at the six enzyme loci to a principal components analysis. Figs. 3 and 4 show component scores, scaled from zero to one, on the first two principal axes for each collection locality. Much of the genetic variation at enzyme loci in these populations is associated with geographic position, and the scores for factors 1 and 3 in *nemoralis* and factors 1 and 2 in *hortensis* show significant north–south gradients (table 2).

Although such correlations of factor score with latitude could be interpreted as evidence for climatic selection, closer examination of the spatial patterns described here suggests that this is not likely to be the case, as there are marked differences in the patterns of spatial variation at particular loci and for principal component scores within each species. In *hortensis* there is a consistent change in allele frequencies at loci correlated with the first principal axis which extends across Britain and Europe (fig. 3 and inset). Despite an overall correlation with latitude, the situation in *nemoralis* is rather different; British and European samples show trends in opposite directions, with factor scores increasing as to the North in Britain and to the South in Europe south of the English Channel (inset to fig. 3(a): points within the ellipse represent the 44 British samples; $r = -0{\cdot}459$, $p < 0{\cdot}001$ for these samples).

Two of the three loci (*Pgm* and *Lap*) with high loadings on the first principal component are the

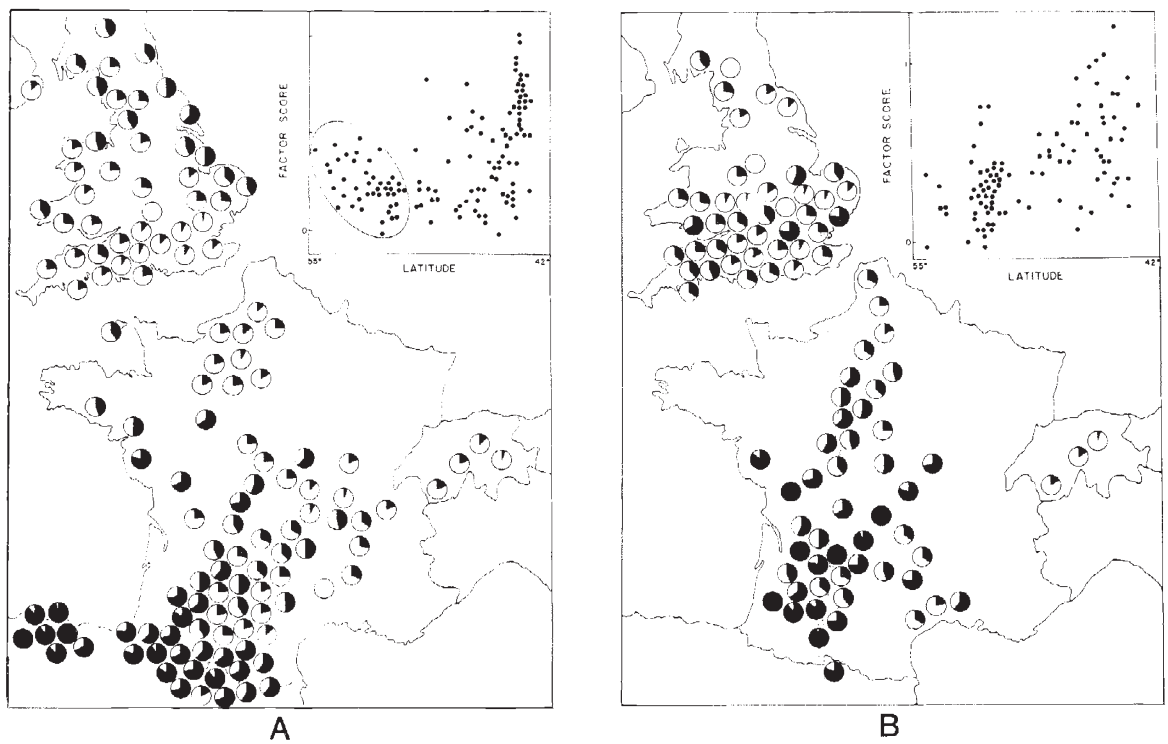


Figure 3 Scores, scaled from zero to one, for Factor 1 (shaded), based on allele frequencies at six polymorphic enzyme loci in *C. nemoralis* (a) and *C. hortensis* (b). Insets show the relationship between factor score and latitude. Points within the ellipse in fig. 3(a) represent collections from Britain.

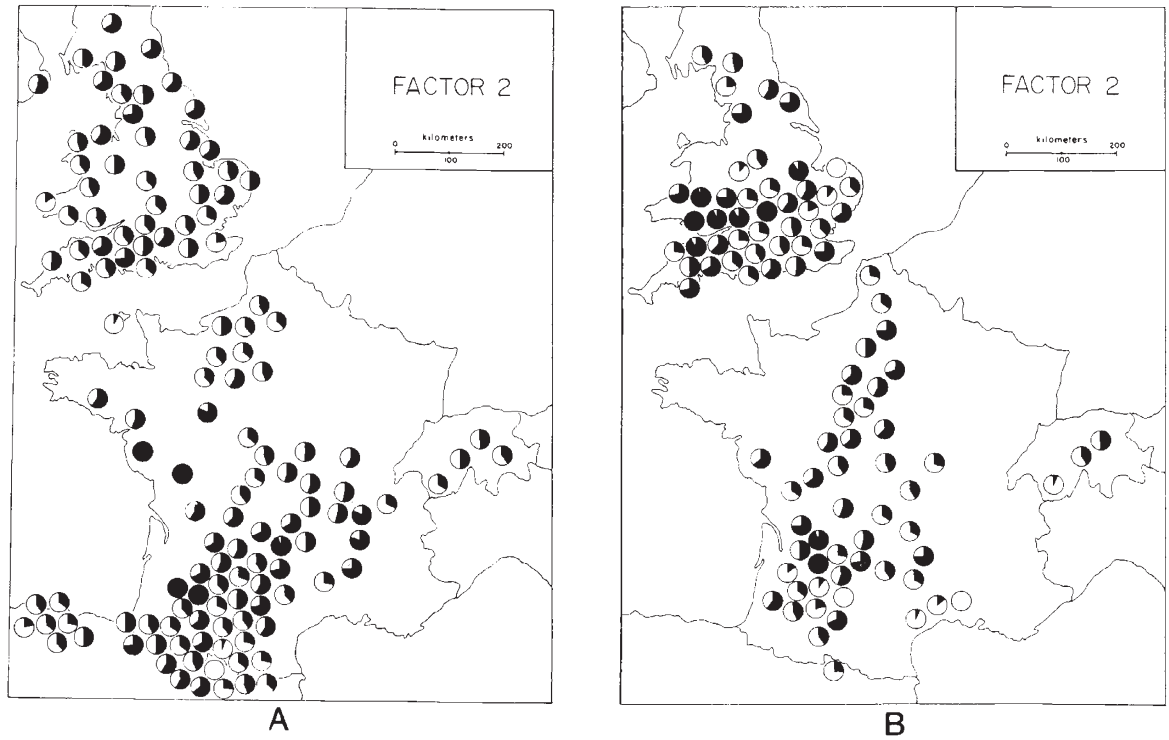


Figure 4 Scores, scaled from zero to one, for Factor 2 (shaded) in *C. nemoralis* (a) and *C. hortensis* (b).

same in the two species. The probability of this occurring by chance is 0·3.

The clines in allele frequency (as reflected by scores on the first principal component) are shown in figs. 5(a) and 5(b) which use a "running average" to eliminate much of the local variation seen in the previous figures. North-south clines are still apparent in British populations of both species, but the major patterns of variation which emerge in Europe are clines from east to west, with eastern populations being more similar to those in Britain for both *nemoralis* and *hortensis*.

Correlations among loci in sympatric populations

At 24 localities, both species of *Cepaea* were collected. Only 3 of 40 comparisons of allele frequencies in *nemoralis* and *hortensis* gave statistically significant correlations (fig. 6). One of these involved comparisons of indistinguishable electromorphs at the *Lap-1* locus. In general, northern populations of both species are monomorphic at this locus, but there is no clear-cut gradient in the degree of polymorphism. However, *Lap-1* is not a very polymorphic locus, and by eliminating the

Table 1 Estimates of variance from hierarchical F-statistics analysis for *Cepaea nemoralis* and *C. hortensis* in western Europe

	*Cepaea nemoralis**			*Cepaea hortensis*†		
Locus	F_{DR}	F_{RS}	F_{ST}	F_{DR}	F_{RS}	F_{ST}
Pgi	0·264	0·222	0	0·163	0·016	0·016
Ipo	0·176	0·089	0·049	0·203	0·091	0·040
Lap	0·186	0·070	0·008	0·214	0·139	0·040
Mdh	0·231	0·152	0·016	0·155	0·022	0
Pgm	0·141	0·197	0·036	0·252	0·056	0·016
Idh	0·120	0·064	0·088	0·197	0·039	0·012
$\bar{X}_{(\text{enzyme loci})}$	0·187	0·132	0·020	0·197	0·060	0·020

* 134 demes in 16 regions in two subdivisions
† 92 demes in 13 regions in two subdivisions

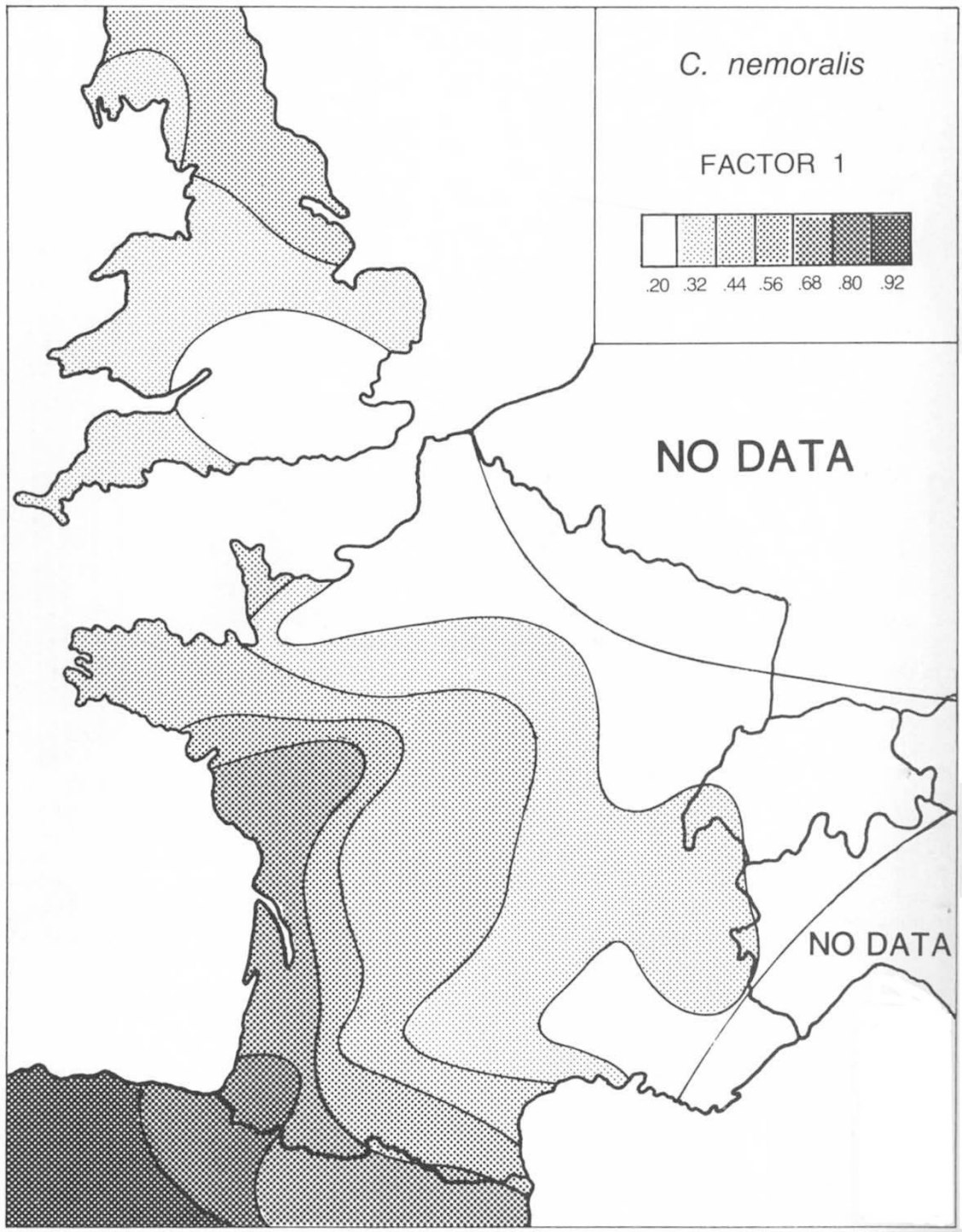


Figure 5 First principal component of allele frequencies at six polymorphic enzyme loci, with intensity of shading indicating the magnitude of factor scores (see text).

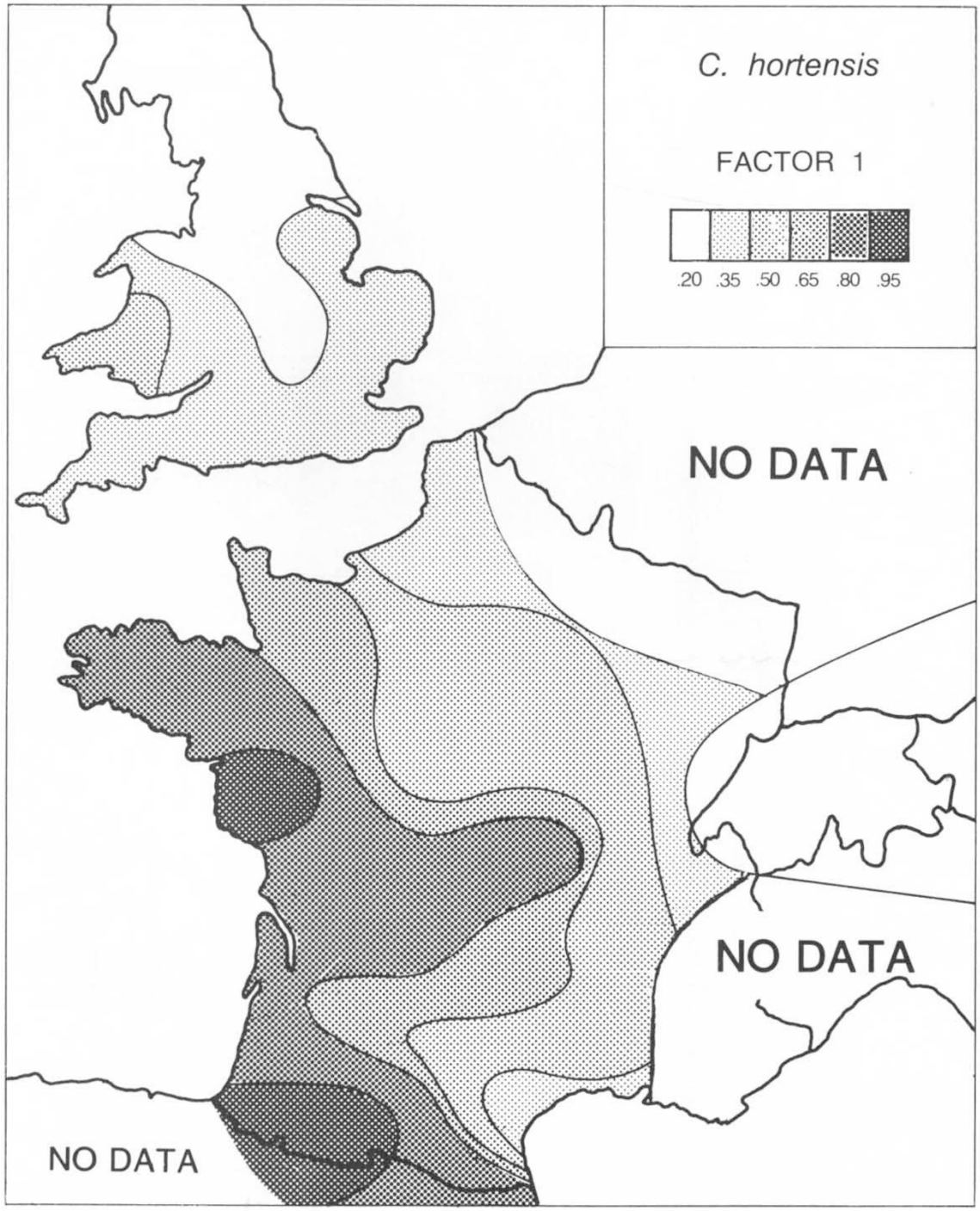
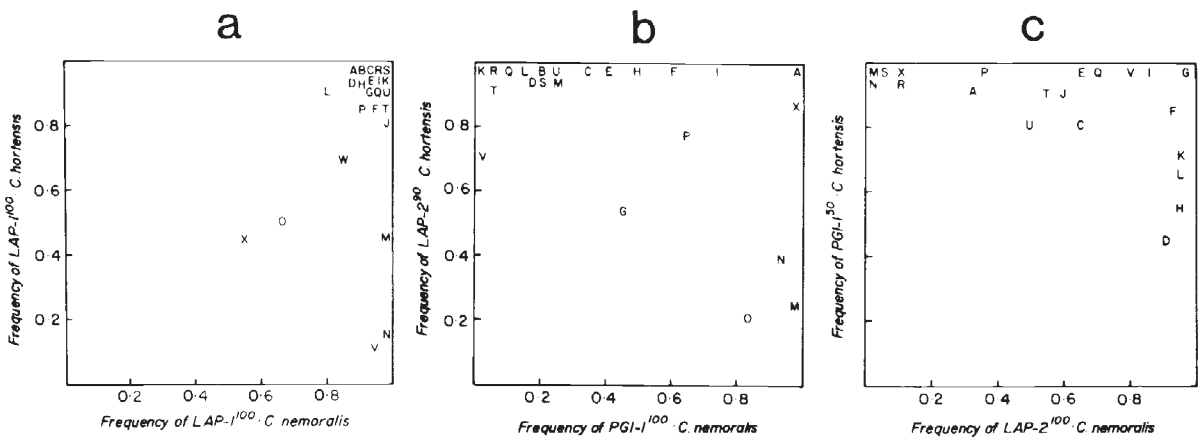

C. hortensis
FACTOR 1
.20 .35 .50 .65 .80 .95
NO DATA
NO DATA
NO DATA

Table 2 Summary of principal components analysis

	Cepaea nemoralis ($n = 132$)	*Cepaea hortensis* ($n = 94$)
Factor 1		
Loci correlated with factor*	*Pgi, Pgm, Lap*	*Ipo, Lap, Pgm*
Variance explained	25·1%	20·8%
Correlation with latitude	$r = 0{\cdot}481$ ($p < 0{\cdot}001$)	$r = 0{\cdot}546$ ($p < 0{\cdot}001$)
Factor 2		
Loci correlated with factor	*Idh, Ipo, Lap*	*Pgi, Idh*
Variance explained	17·0%	14·4%
Correlation with latitude	$r = 0{\cdot}022$ ($p < 0{\cdot}5$)	$r = 0{\cdot}235$ ($p < 0{\cdot}05$)
Factor 3		
Loci correlated with factor	*Mdh, Pgm*	*Mdh, Pgi*
Variance explained	8·7%	11·7%
Correlation with latitude	$r = -0{\cdot}383$ ($p < 0{\cdot}001$)	$r = -0{\cdot}058$ ($p < 0{\cdot}5$)

* Includes loci with loadings greater than ±0·5 for one or more alleles; loci listed in order of decreasing factor loadings



Type of comparison	Number of Comparisons	Number of Significant correlations	Loci involved	Level of significance
Homologous enzyme loci	5	1	Lap-1_{nem} - Lap-1_{hort}	< .05
Non-homologous enzyme loci	35	2	Pgi-1_{nem} - Lap-2_{hort}	< .01
			Lap-2_{nem} - Pgi-1_{hort}	< .05

Figure 6 Plots of allele frequencies in *C. nemoralis* and *C. hortensis* which have significant ($p < 0{\cdot}05$) correlation coefficients. Letters indicate the collection localities shown on fig. 2. The table includes all comparisons which reach statistical significance.

most variable collection (X) the correlation coefficient between the two species drops to 0·385 and is no longer significant ($0{\cdot}10 > p > 0{\cdot}05$). The two significant correlations among the 35 comparisons of non-homologous enzymes involve the same loci in each species (figs. 6(b) and 6(c)). However, for neither *Pgi* nor *Lap-2* considered alone is there a significant parallel variation between species (for *Pgi* $r = 0{\cdot}225$, df = 23; and for *Lap* $r = 0{\cdot}264$, df = 19), so that these correlations are likely to arise from chance alone.

DISCUSSION

Much of the genetic differentiation at enzyme loci in *Cepaea* is in the form of clines which extend across Britain and Europe. These are superimposed on patterns of extensive local differentiation at enzyme loci which produce marked genetic subdivision in some places. For example, populations of *nemoralis* in the Pyrenees (Caugant *et al.*, 1982; Ochman *et al.*, 1983) have differentiated to a degree which is equivalent to that found among races, subspecies or even species in other genera (Nei, 1975; Avise and Aquadro, 1982). On the European scale there is also considerable genetic differentiation at each of the loci studied here. In *C. nemoralis* different populations are fixed for alternative alleles at four of the six loci, and in *C. hortensis* two of these loci are monomorphic for different variants in geographically separated populations. This pattern of local geographic variation within a context of large-scale clines is analogous to the patterning of many of the loci controlling shell polymorphism in both species of *Cepaea* (Jones *et al.*, 1977).

C. nemoralis and *C. hortensis* differ in the direction of geographic change in allele frequency and in the alleles involved in clinal change. In *nemoralis*, populations with relatively high frequencies of particular alleles at *Pgi*, *Pgm* and *Lap* (and to some extent *Mdh* and *Ipo*, which have loadings of 0·45 and 0·44 on principal component I) occur near the English Channel and decrease in frequency to the north and south of this. In contrast, *hortensis* shows continuous gradients in allele frequency at loci correlated with Factor I (*Ipo*, *Lap*, *Pgm*) from northern England to Spain.

Geographical patterns of gene frequency within species are not very helpful in determining the relative importance of selection and drift. Although there is little consistency in the genetic patterning of these species on a small scale, there are some rather striking large-scale geographical patterns of some principal components which could in principle be ascribed to an inter-regional selective differential partly obscured by interdemic drift. However, no single selective force can easily be invoked to account for the patterns of genetic differentiation found within either of the species studied here. In *nemoralis*, latitudinal changes in allele frequency in British populations are reversed in France and Spain. Although there are some consistent trends in *hortensis*, these usually involve different loci from those which show large-scale patterning in *nemoralis* and it is not easy to see why any geographic trend in selection should act in only one of a pair of sibling species. Several detailed surveys of allele frequency distribution at enzyme loci in *C. nemoralis* give no evidence of association of genetic structuring with identifiable components of the environment (Johnson, 1979; Jones *et al.*, 1980; Ochman *et al.*, 1983).

It is in principle possible to detect the action of selection by identifying correlated patterns of geographic change in sympatric populations of closely related species. Comparison of allele frequencies in *nemoralis* and *hortensis* at the 24 sites in which both species were present gave three statistically significant correlations. The correlation between electromorphs at the *Lap-1* locus depends on a single locality, and is not compelling evidence of a common agent of natural selection. There is, however, some evidence at selection acting at an *Lap* locus in the mussel *Mytilus* (Koehn *et al.*, 1976, 1980) and Foltz *et al.* (1982) describe an unusually high level of geographic differentiation for *Lap* in four species of slugs. The reciprocal correlation which we have found between *Pgi* and *Lap-2* in *C. nemoralis* and *C. hortensis*, although significant, is somewhat enigmatic. As these loci are not functionally or structurally related it is difficult to suggest a meaningful explanation for this association.

Correlated patterns of geographic change in closely related species have often been claimed to result from the action of selection. There are two potential problems in evaluating such claims. First, there may be bias as negative results are rarely reported. Second, there is sometimes a problem of multiple testing; as such studies usually involve a considerable number of loci, allele frequency associations among loci will often arise by chance (Varvio-Aho and Pamilo, 1982). For example, if all the observed variation were neutral, the probability of a single significant result in as few as 10 interspecies comparison is 0·4 by chance alone, with the probability increasing as a function of the

number of loci (n) examined by $1-0{\cdot}95^n$ (Tukey, 1977). There is hence a danger of erroneously accepting the action of selection unless all interspecific comparisons of allele frequency are considered during such analyses.

Although there are some parallels in the patterns of large scale genetic change at enzyme loci in the two species studied here these are not strong evidence for the action of selection; such patterns are equally likely to arise from a shared demographic history. Only shared mosaic patterns of parallel change can confidently be ascribed to selection, and no information of this kind is yet available for enzyme loci in *Cepaea*. The scale and pattern of genetic differentiation at the various loci which he have studied shows a general similarity within each of the two species. Such similarities among loci of very different function are perhaps more likely to result from a shared history of population subdivision (and to a common pattern of genetic change arising from drift and migration which may influence many loci simultaneously) than to the joint action of selection on a diverse group of enzyme loci. The probable importance of random change in controlling the distribution of alleles at enzyme loci in *Cepaea* is supported by the extensive local differentiation—sufficient to lead to the fixation of alternative alleles in populations separated by a few kilometres—which exists in Pyrenean populations of *C. nemoralis*. These extensive changes, which are concordant over loci, are not easy to relate to an obvious ecological factor, and may have arisen from population bottlenecks occurring during periods of glaciation. Although the scale of sampling used here is too coarse to reveal details of population structure, concordant local differences as extensive as those found in the Pyrenees do not appear to be a general feature of the genetic structure of either species. However, both *nemoralis* and *hortensis* show relatively high population differentiation in the region north of the Pyrenees, perhaps because of a common demography during recent glaciations.

Geographic patterning of enzyme polymorphism in both species of *Cepaea* is most manifest among demes within geographic regions. F_{DR} values (which reflect this) are 0·187 for *nemoralis* and 0·197 for *hortensis*. These figures are close to the mean F_{DR} of 0·198 for a number of enzyme loci in populations of the recently introduced snail *Helix aspersa* in regions of California of about the same area as that of the present survey (Selander and Whittam, 1983). The rapid spread and extensive population subdivision known to exist in *H. aspersa* suggests that most of its spatial variation arises from drift, a view supported by the similarity of variance estimates over its enzyme loci. The extent of genetic subdivision is less homogeneous over loci in *Cepaea* than in Californian *Helix*, perhaps because *Cepaea* populations have undergone more complex patterns of local fission and fusion during their evolutionary history. The local distribution and abundance of *C. nemoralis* in southern England has undergone considerable fluctuations over periods of a few years (Williamson *et al.*, 1977; Cameron *et al.*, 1980), and subfossil samples show that the ranges of *nemoralis* and *hortensis* have altered greatly since the most recent European glaciations (Cain, 1971). The demographic history of these snails supports the view that genetic changes at enzyme loci reflect the action of genetic drift, but the action of as yet unidentified local forces of selection cannot be excluded.

The extent of such genetic changes within each of the species studied here is far greater than that which exists among many pairs of sibling species in other groups. Indeed, within *nemoralis* or *hortensis* it is possible to identify populations which differ from each other at enzyme loci to an extent considerably greater than the mean genetic differences between the two species. However, in spite of their extensive genetic divergence, there is little evidence of reproductive isolation among *C. nemoralis* collected from Britain, France, Spain and North America (Cain *et al.*, 1960; Johnson *et al.*, 1984) and no indication that its populations are in the early stage of speciation. The existence of extensive genetic change at the molecular level in freely interbreeding populations of *Cepaea* provides little support for the view (White, 1978*a*, *b*) that new species are likely to arise as a product of genetic differentiation in continuous populations of organisms with low mobility.

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