

PHYLOGEOGRAPHY OF ADDERS (*VIPERA BERUS*) FROM FENNOSCANDIA

MARTIN CARLSSON AND HÅKAN TEGELSTRÖM

ABSTRACT: Mitochondrial (mt) DNA variation evaluated in 135 Adders (*Vipera berus*) from 71 localities in Fennoscandia revealed low genetic diversity when compared to other sympatric taxa of animals. We found, however, that a clear geographic subdivision separates mtDNA lineages on eastern and western sides of the Baltic Sea. We suggest these two lineages descended from separate glacial refugia, as their level of sequence divergence corresponds to a time since divergence exceeding the duration of the last glaciation. The two lineages show a low level of within-lineage differentiation, exhibiting star phylogenies that indicate accumulation of de novo mutations in situ. The two populations meet at three areas within the surveyed region: in northern Finland and in the archipelagos of Umeå (Sweden) and Turku (Finland). Studies of other organisms in the region for which two mtDNA populations have been defined have identified a zone of contact in north-central Sweden. The present work illustrates a novel pattern. Two of the contact zones appear to result from across-water dispersal, which indicates a large potential for *V. berus* to cross open brackish water.

INTRODUCTION

The flora and fauna of present day northern Europe survived periods of glacial climate in retreats in the southern and central parts of Europe and Asia. The Weichselian glaciation, which started about 115,000 years before present (ybp) and reached its glacial maximum 22,000–18,000 ybp (Kleman, et al., 1997), covered much of northern Europe. Consequently, the fauna and flora of today have colonized the north only during the past 10,000–13,000 years (first suggested by Nilsson, 1847). A cold period, the Younger Dryas, which began 11,000 years ago and lasted for a few hundred years (Björck, 1995), effectively eliminated most early colonists (Lepiksaar, 1986). A second, less severe cold period 2,000 years later, also lasting about 200 years, had a dramatic effect on early colonists.

The various bodies of water preceding the present Baltic Sea left only two possible terrestrial routes for colonization into Fennoscandia. A land bridge connected Sweden to Europe proper for about 1,000 years until 9,200 ybp, thus allowing colonization from the south (Björck, 1995). Another colonization route was from the east, across Finland and north of the Gulf of Bothnia. Several vertebrate species used both terrestrial pathways, and for many of them a contact zone between the separate corridors has been identified in north-central Sweden (reviewed by Jaarola et al., 1999). This phenomenon, termed a suture zone (Remington, 1968), has been described for a number of species in different areas of Europe (Hewitt, 2000). Studies to date that have produced concordant patterns of colonization in this region have investigated mammals occurring far north, namely the Common Shrew (*Sorex araneus*; Fredga and Nawrin, 1977), Field Vole (*Microtus agrestis*; Jaarola and Tegelström,

1995), Brown Bear (*Ursus arctos*; Taberlet et al., 1995), and Bank Vole (*Clethrionomys glareolus*; Tegelström and Jaarola, 1998). As all these species have a high tolerance for cold climate, it seems reasonable to assume that they were early colonists.

An alternative colonization route is across water in several possible ways (i.e., swimming and moving across ice in winter, rafting, or through human-mediated transfer). For snakes, it is reasonable to rule out dispersal over ice, although the Adder (*Vipera berus* L.) may cross patches of snow (M. Carlsson, pers. observ.). On account of the multitude of island populations of *V. berus* in the Baltic, it is our contention that *V. berus* has a large potential for across-water dispersal, and should not necessarily depend upon historical land connections for the colonization of Fennoscandia. We also hypothesize that *V. berus* may have been one of the earliest vertebrates to colonize in wake of the glaciation, given its tolerance to cold climates and its present-day northern distribution.

Vipera berus is the northernmost occurring snake species, reaching north of the Arctic Circle at 68° N in Fennoscandia. It is also the most widespread terrestrial snake, with a longitudinal distribution reaching from Scotland to Pacific Russia. Across most of its range, there is little correlated variation in morphology or coloration based on geography, although frequencies of color morphs vary locally and some locally distinct morphs are known (Fog et al., 1997). Consequently, morphological characters may not resolve relationships among populations from different areas, as they have, for example, in Grass Snakes (*Natrix natrix*; Thorpe, 1984).

In the present study we examined geographically dependent genetic variation in *V. berus* from northern Europe, a species previously left unaccounted for in the reconstruction of the palaeoclimatological history of the region. Because a high concordance of

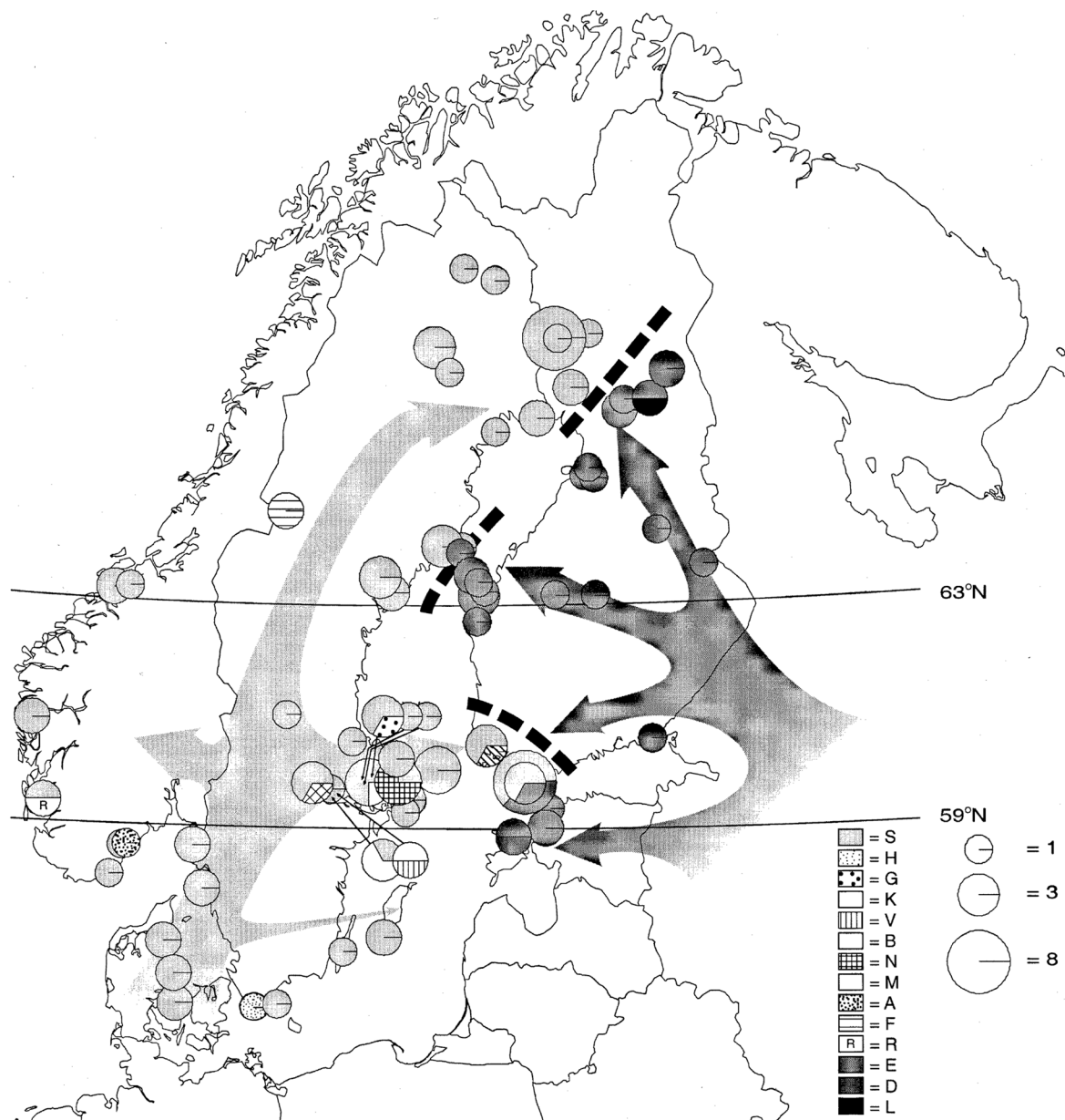


Fig. 1. Map showing collection localities and *Vipera berus* mtDNA haplotypes. Pie charts depict haplotype composition with the size of the chart corresponding to sample size for each locality. Pattern for each haplotype corresponds to those of Figure 2c. Shaded arrows indicate hypothesized colonization pathways, and thick hatched lines show zones of contact between the two mtDNA population lineages. Also shown are the latitudinal parallels used to divide the western population into three subsets, for diversity comparisons.

colonization patterns has been reported between reptiles and small mammals in North America (Avice, 2000), a similar comparison is warranted in northern Europe. Our analysis is based on restriction fragment length polymorphisms (RFLPs) of mitochondrial (mt) DNA. The latter has several characteristics making it a useful tool for phylogeographic inquiries, as has been shown and discussed elsewhere (Wilson et al., 1985; Avice, 1986; Moritz et al., 1987; Douglas et al., this volume).

MATERIALS AND METHODS

Sampling

A total of 135 *V. berus* were collected from 71 localities (Fig. 1). We opted to maximize the number of sampling localities at the expense of local sample sizes. This sampling approach is warranted in that we studied the geographic, rather than frequency distribution of matriline. The animals were sacrificed and stored at -70°C until DNA extraction. Mitochondria

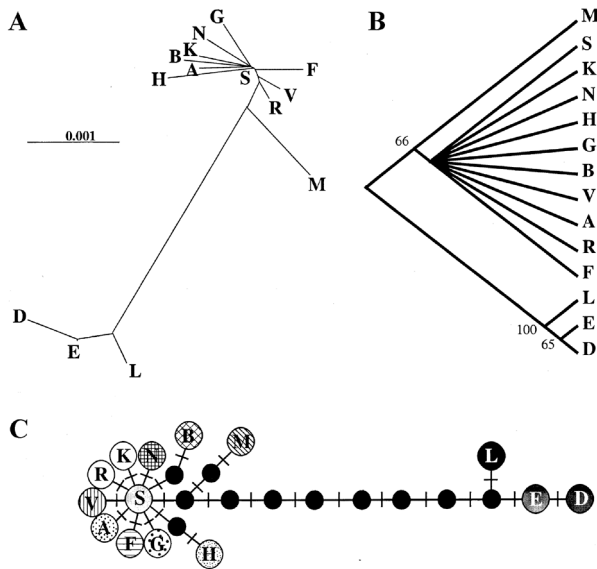


Fig. 2. (A) Phylogenetic tree produced by the neighbor-joining procedure for genetic distances (Nei and Li, 1979) from restriction enzyme fragment data of adder mtDNA. (B) A 23-step most-parsimonious tree from site data using PAUP. Numbers denote % bootstrap support for resolved bifurcations (1000 replicates, using Heuristic with TBR search option). (C) Combined mutation network (Avise et al., 1979) for the 14 haplotypes. Each bar represents one mutation step and small black circles denote hypothetical, intermediate, haplotypes not detected in the sample. Patterns are the same as in Figure 1.

from about 1 g of liver tissue were isolated according to Lansman et al. (1981). Purification of mtDNA was performed by the phenol/chloroform extraction method described by Powell and Zúñiga (1983) and modified by Jaarola and Tegelström (1995). Digestion of mtDNA samples was performed using eight separate tetranucleotide restriction enzymes (*Dde* I, *Hae* III, *Hinf* I, *Hpa* II, *Mbo* I, *Rsa* I, *Sau* 96 I and *Taq* I). Approximately 10–50 ng of mtDNA was digested for 1–2 hours at 37°C (60°C for *Taq* I) in a 10 µl reaction mixture containing 2.5 units of enzyme. Restriction fragments were separated in a 5% polyacrylamide gel for 130 minutes at 200 V and 25–50 mA. Gels were silver-stained and fragment sizes were scored visually with the aid of *Bgl* I digested lambda DNA as a size marker (Tegelström, 1992). This technique, although cumbersome, eliminates the risk of contamination from nuclear copies of mtDNA, and thus potential effects thereof.

Data Analysis

Restriction fragment patterns were analyzed, one enzyme at a time, and each unique fragment pattern was designated with a letter. To account for different fragment patterns, mutations in restriction sites were inferred by matching band sizes for each enzyme (Table 1). Fragments smaller than 100 bp generally did not appear on the gels, but were assumed to be present in some cases in order to explain observed variation. A mutation network (Avise et al., 1979) was

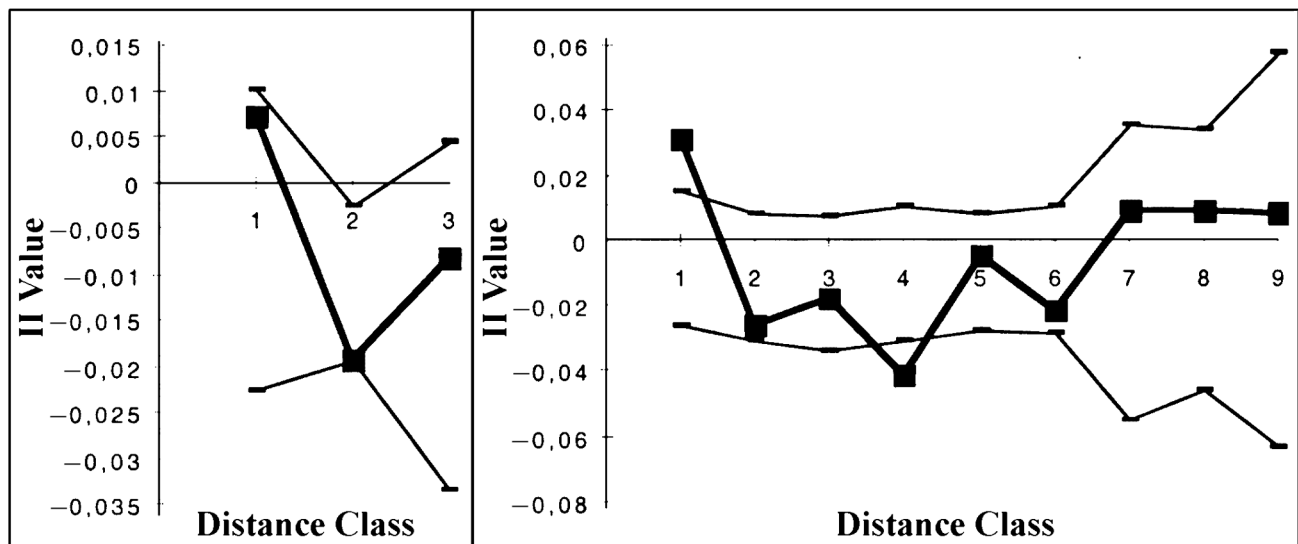


Fig. 3. Autocorrelation results. II values (joined with thick lines) with 99% confidence intervals (thin lines) for three and nine distance classes, respectively. Only two II values significantly correlate geographic with genetic distance, and this when the total data set is divided into nine groups. The most proximal localities tend to be more similar than expected, whereas intermediately spaced localities tend to be dissimilar.

Table 1. Haplotype definitions. Number of *Vipera berus* found with each respective mtDNA haplotype (N), number of fragments, and presence/absence of inferred restriction sites for the variable restriction enzymes for each of the identified haplotypes.

Hapl.	N	No. fragments	Dde I	Hinf I	Hpa II	Mbo I	Rsa I	Sau 96 I	Taq I
S	86	223	010	000	0	0000	0001110110	1	1
H	1	224	010	000	0	0000	1101110110	1	1
G	1	224	110	000	0	0000	0001110110	1	1
K	4	222	010	000	0	0000	0001110110	1	0
V	1	223	010	000	0	0000	0011110110	1	1
B	1	223	010	110	0	0000	0001110110	1	1
N	3	224	010	000	1	0000	0001110110	1	1
M	1	221	010	000	0	0000	0000000110	1	1
A	1	224	010	000	0	1000	0001110110	1	1
F	2	224	010	000	0	0100	0001110110	1	1
R	1	222	000	000	0	0000	0001110110	1	1
E	31	225	011	001	0	0010	0000111001	1	1
D	1	225	011	001	0	0010	0000111001	0	1
L	1	225	011	001	0	0001	0000111001	1	1

Table 2. Haplotype (h) and nucleotide (π) diversity estimates for the samples of *Vipera berus* presented herein. The western population was arbitrarily divided into three subpopulations along the 59th and the 63rd northern parallels (latitude) to elucidate the skewed distribution of haplotypes.

Location	N	No. haplotypes	h \pm SD	$\pi \pm$ SD
West < 59°N	19	3	0.2047 \pm 0.1192	0.000138 \pm 0.000000
West \geq 59°; < 63°N	47	8	0.4413 \pm 0.0884	0.000319 \pm 0.000000
West \geq 63°N	36	2	0.1079 \pm 0.0680	0.000061 \pm 0.000000
West total	102	11	0.2885 \pm 0.0592	0.000197 \pm 0.000000
East	33	3	0.1193 \pm 0.0756	0.000079 \pm 0.000000
Total	135	14	0.5434 \pm 0.0418	0.001573 \pm 0.000000

constructed by combining minimum mutation networks for each enzyme (Fig. 2c). Haplotype (h, Nei and Tajima, 1981; Nei, 1987) and nucleotide diversity values (π , Nei and Li, 1979; Nei, 1987) as well as genetic distance (d, Nei and Li, 1979) were calculated from a binary matrix of the mutation fragment data using REAP, version 4.0, computer program package (McElroy et al., 1992). A neighbor-joining analysis (Saitou and Nei, 1987) of the distance matrix was performed in Neighbor, a bundled application in PHYLIP, version 3.572 computer package (Felsenstein, 1995). A straight-forward parsimony analysis, i.e., heuristic search with tree-branching-reconnection branch-swapping (TBR), of the inferred restriction site data was performed in PAUP, version 3.1+3 (Swofford and Begle, 1993), and the robustness of the single shortest tree was tested using 1,000 bootstrap pseudo replicates.

To investigate whether there was any geographic structuring of the genetic variation not apparent from visual inspection of the data (Figs. 1–2), we per-

formed spatial autocorrelation analyses using the program AIDA (Bertorelle and Barbujani, 1995). The data were divided into three and nine distance classes, respectively, in two separate analyses. As an autocorrelation index for DNA analysis (AIDA), the calculated II coefficients, with confidence intervals estimated from 1,000 permutations, were employed for our interpretation. These II values are analogous to Moran's I coefficient for autocorrelation of geographic data with interval type data such as gene frequencies (Sokal and Oden, 1978), yet they differ by also taking genetic relatedness into account. Moreover, AIDA statistics can be calculated for small samples (Bertorelle and Barbujani, 1995).

RESULTS

A total of 14 different haplotypes were detected and the eight restriction enzymes, of which all but *Hae* III produced variable patterns, generated 221–225 fragments for the different haplotypes (mean 223.5 \pm 1.23 SD, Table 1). The genome size estimate of 17,749

bp \pm 1,314 (SD) is in accordance with squamate mtDNA sizes published for the entire sequenced mitochondrial genome: a colubrid, *Dinodon semicarinatus*, at 17,191 bp (Kumazawa et al., 1998), and 17,407 bp for a skink, *Eumeces egregius* (Kumazawa and Nishida, 1999). Approximately 5% of the mitochondrial genome was surveyed in the present study.

The minimum mutation network for the combined haplotypes (Fig. 2c) produced no ambiguities compared to the phylogenetic analyses. The two different phylogenetic approaches (Fig. 2a, b) yielded trees with the same topology, although bootstrapping of the site matrix yielded limited statistical support for two of the three resolved branches.

There was an obvious geographic structuring of the mtDNA haplotypes, with two genetically, and geographically separated mtDNA populations (sequence divergence $d = 0.00378$, Nei and Li, 1979) (Fig. 1). These two populations occur on either side of the Baltic and join in northern Finland. There are two areas, however, where they occur sympatrically: the Umeå Archipelago and at Tvärminne in the southern Finnish Archipelago. We consider this strong evidence for across-water dispersal.

Divergence of these two geographically separated mitochondrial populations was estimated at 95,000–380,000 ybp, assuming a mutation rate of 0.5–2%/lineage/million years. As Zamudio and Greene (1997) indicate, slower mutation rates may be most applicable to small ectotherms. Although a non-reliable range of estimates, it resonates well with a separation of the adder populations into two separate refugia at least during the last glacial period. As the total sample was clearly subdivided, the two populations were treated separately as eastern vs. western populations in the remaining analyses.

Haplotype and nucleotide diversity values proved to be low for both populations (Table 2). For the western sample, diversity levels were also estimated for three subsets of the population, defined by dividing the localities along the 59th and 63rd northern parallels. Most of the variation was found in the middle of the study area, as is evidenced by the diversity values of the middle subset (Table 2, Fig. 1).

Autocorrelation analyses were only performed on the western sample, as the eastern sample obviously had no variation to be correlated geographically (Fig. 1 and Table 1). Although the II values of the spatial autocorrelation are low, they do reveal a positive correlation ($II > 0$) between individuals from closely spaced localities and some divergence among inter-

mediately spaced localities (negative II values, Fig. 3). At larger distances there is no correlation. A closer examination of the data helps to explain this pattern. In the south there is very little diversity, whereas at mid-latitudes most of the variation is found, only to decrease again in the northernmost part of the western population. The only variant haplotype found in more than one locality was found in three places less than 60 km apart, while most other localities were separated by greater distances from one another. This will increase correlation for the smallest distance class, as there will be some comparisons of pairs of localities sharing two haplotypes instead of only the one present more or less everywhere (see haplotypes “K” and “S,” Fig. 1). Because of the congregation of variant haplotypes in the middle of the sampling area, the frequency of pairwise comparisons between dissimilar haplotypes can be expected to peak here, resulting in negative correlation for intermediate distance classes (see diversity values in Table 2 with the spatial correlation in Fig. 3). All these classes show negative correlation, although only one significantly so. Overall, a weak autocorrelation signal between genetic and geographic data was evident in the data set, possibly an artefact of sampling.

DISCUSSION

Our study shows a low level of genetic variation in mtDNA from northern *V. berus*. The phylogenetic analysis revealed a bifurcating tree with two mitochondrial clades, each present on either side of the Baltic Sea (Fig. 1). The three and 11 haplotypes of the eastern and the western clade, respectively, are closely related in typical star-shaped phylogenetic patterns, forming a “dumbbell” shape when put together (Avice, 2000). This is indicative of diversity that has arisen *in situ*, i.e., after the colonization event. The different phylogenetic approaches produced no ambiguities. The rationale for using both phenetic and cladistic approaches was that, although the fragment data contain interdependent information, the site data were inferred from these and assumed five fragments that were not detected on the gels (Table 1). The effect of these differences in data is evident in the star shapes of the western clade for the two trees, respectively (Fig. 2). Furthermore, with most OTUs being defined by single-mutation differences, the dataset is not well suited for statistical resampling assessments, such as bootstrapping. Hence, the importance of these values should not be overemphasized. Instead, comparing different methodological approaches can serve to

indicate the robustness of trees for shallow topologies such as the one presented here.

Vipera berus has a large-scale pattern of colonization of Fennoscandia, which is similar to other vertebrates studied (Fredga and Nawrin, 1977; Taberlet and Bouvet, 1994; Jaarola and Tegelström, 1995). Further, this taxon invaded this region from two directions, as previously reported by Carlsson et al. (1995). Presumably this pattern illustrates dispersal from separate glacial refugia. On a finer scale, however, the pattern found in *V. berus* differs to some degree. The main contact zone, for example, is not located within the suture zone of north-central Sweden as described for the above mentioned species (Jaarola et al., 1999). Instead, it is located in northern Finland.

The shift in location of the northern contact zone, when compared to mammals, may be viewed as consistent with the fact that *V. berus* is an ectotherm and colonized the area somewhat later. At that time, when the ice sheet was not present, large present-day landmasses to the north and east of the Gulf of Bothnia were under water. It is plausible that at this time *V. berus* could not advance at the same rate in eastern and western regions of Fennoscandia. Also, the area of northern Finland spanning the present contact zone around 6,000 ybp was covered with different vegetation from the surrounding areas, i.e., open boreal woodlands dominated by birch (Huntley and Birks, 1983), indicating wetter conditions than that of boreal forests covering the surrounding landscape. This area today is a flat, wet lowland, and after 10,000 years of landrise it remains largely devoid of suitable hibernation sites for *V. berus*.

More novel features revealed in our study are two additional areas of contact (Fig. 1), which may have been the result of across-water dispersal. The presence of *V. berus* on islands in outer areas of archipelagoes of both Sweden and Finland (Fog et al., 1997), is a strong indication of its ability to disperse across water. There are reports by fishermen of *V. berus* floating in coiled postures several kilometers from the coast (M. Höggren, pers. comm.). Our findings, coupled with the presence of *V. berus* in archipelagoes and such anecdotal reports, present a strong case for the potential of *V. berus* to colonize across vast stretches of open fresh or brackish water. Hence, *V. berus* was not necessarily dependent on a landbridge to the continent (southern Sweden) for their northward colonization, as is commonly assumed to have been the case in other taxa (Taberlet et al., 1998; Jaarola et al., 1999).

The level of genetic diversity among *V. berus* from Fennoscandia is intriguing. When compared to values reported for other organisms, the level of mtDNA diversity is low to extremely low in these populations. Nucleotide diversity values for the eastern and western mitochondrial populations ($\pi = 0.000079$ and $\pi = 0.000197$; Table 2), respectively, are one or two orders of magnitude lower than what is generally referred to as low (e.g., O'Corry-Crow et al., 1997; Slade and Moritz, 1998; Questiau et al., 1998, but see Kvist et al., 1999). Comparison of nucleotide diversities found for organisms in other studies may, however, be skewed based on the methods used. Sequencing of mtDNA apparently reveals more variation than RFLPs, depending on choice of sequence (Walker et al., 1995). RFLP-based nucleotide diversity values for 19 of 25 phylogeographic groups of turtles had higher to much higher π values than those we present here for *V. berus* (Walker and Avise, 1998), and for the remaining six turtle taxa, they may be of the same magnitude or lower than our π values. Thus, even when only comparing our RFLP data, *V. berus* from Fennoscandia show a low level of mtDNA variation.

Nonetheless, a small amount of genetic variation is present in *V. berus* from Fennoscandia, especially in the western mtDNA population. Although autocorrelation results revealed no geographic structuring of mtDNA diversity, it did serve to elucidate a perplexing skew in the distribution of haplotypes within the western lineage. This skew is most obvious when the western sample is divided into three subsets (Table 2). There is a clear over-representation of variant haplotypes in the central group as compared to southern or northern groups.

Classical population genetic theory predicts that a mosaic of small, isolated, subpopulations can sustain a larger overall genetic diversity than a single large population, although the subpopulations may lack variation (Wright, 1943; Maruyama, 1970, 1972). New mutations can survive to fixation through drift in each subpopulation, where stochastic factors have more drastic effects because of a smaller, effective population size. Using this rationale, we have discovered two separate areas within the central group warranting explanation, namely the eastern and western extremes, where the majority of variant haplotypes are found.

The highest number of variant haplotypes is found in east-central Sweden. Most of this area was below sea-level 6,000 years ago, not having recovered from the weight of the ice (Björck, 1995). As the land

rebounded through isostatic uplift, an ever-changing archipelago was created, which is an ongoing process in the region. Small populations of *V. berus* may have thrived on these islands, just as present day islands throughout the Baltic offer good habitat. However, for these subpopulations to retain more de novo variation than expected, they need to have been isolated for some time. This can be speculatively and paradoxically explained through isolation by water. First, we must assume that levels of salinity dictate a threshold for across water dispersal in *V. berus*. Although the present-day Baltic Sea is brackish, this has only been for the past 5,000 years. When the glacial cap began melting ca. 15,000 ybp, a freshwater lake (the Baltic Ice Lake) was formed roughly in the area occupied by the modern Baltic Sea. This freshwater lake later drained and formed a saltwater sea (Yoldia Sea) ca. 10,000–9,000 ybp. Similarly, another freshwater lake (Ancylus Lake), was replaced by a saltwater sea (Littorina Sea) ca. 7,500–5,000 ybp. After this period, the Baltic Sea has been brackish, with varying degrees of salinity (Andersen and Borns Jr., 1997). Assuming colonization of a proto-archipelago during the Ancylus Lake freshwater stage, there could have been isolated archipelagic populations of *V. berus* during the later saltwater Littorina Sea phase. This would allow for more diversity to be retained than if it had been one large panmictic population.

Three haplotypes are present among a sample of ten individuals from the westernmost localities along the Norwegian coast. The landscape of coastal Norway, with high mountain ranges separating valleys and saltwater fjords, arguably presents a challenge to a colonizing ectotherm. The climate has fluctuated drastically in the past; for example, a warm period 8,000–3,000 ybp (Andersen and Borns, 1997) would make traversing of mountains less difficult than today. Given a saltwater-induced isolation and a fluctuating climate, the colonization and subsequent isolation of Norwegian fjord populations of *V. berus* can be explained. Hence, the prerequisites for mosaics of temporally isolated subpopulations are met in both areas of concern.

One underlying assumption in the interpretation of our data is that the two separate mtDNA lineages represent different historical populations. An alternative explanation is that the pattern observed is merely the effect of lineage sorting from the original source population during the colonization process. Although this alternative view cannot be ruled out, it is unlikely that such strong purging of lineages would have

occurred simultaneously, yet separately, in both populations. Large areas of low genetic diversity are to be expected as an effect of long range, leptocurtic, dispersal (Nichols and Hewitt, 1994; Ibrahim et al., 1996). Likewise, a leading edge effect, where descendants of previous founder individuals colonize new land, could lead to homogenization of the genepool on a scale similar to that of Fennoscandian *V. berus*. In the present case, it would indicate that the variation resulting from glacial refugium/refugia would have been lost during the colonization of central Europe, i.e., before reaching the north. This explanation is attractive in explaining the low levels of variation in each of the two adder populations. We suggest, however, that the two areas of homogeneity are simply too large to be accounted for by lineage sorting, merely through either long-distance dispersal or leading edge effects. Hence, we invoke a separate refugia hypothesis to explain the two lineages, while favoring a leading edge effect when accounting for the low within lineage diversity values.

There are a number of uncertainties yielding large variances for estimates of times since divergence and any estimates should be treated with caution for a number of reasons. Time to coalescence estimates, which also take demographic factors into account and are based on more realistic population history models, suffer from larger variances. Still, as the two mtDNA populations of Fennoscandian *V. berus* differ on a scale matching isolation and divergence, at least during the last glaciation, we suggest that the range of times since divergence presented here support a hypothesis of different glacial refugia for the two lineages. Indeed, it is likely they represent populations that have been isolated during two or even three glacial cycles.

To summarize, we propose that *V. berus* utilized two separate colonization pathways into northern Europe, although it is not yet possible to deduce separate glacial origins for the two populations. Furthermore, the novel pattern of contact zones found, as well as the described potential for open water dispersal, make this species' phylogeographic history in northern Europe unique.

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