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Phylogeography of the Adder, *Vipera berus*

BY

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Abstract
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The phylogeography of a wide ranging temperate species, the adder, *Vipera berus*, was investigated using several genetic tools, with special emphasis on the post-glacial colonisation pattern of Fennoscandia. The area was colonised from two directions by adder populations representing different glacial refugia. The two populations meet in three places and the main contact zone is situated in Northern Finland. The two other contact zones are the result of dispersal across the Baltic Sea to the Umeå archepelago and South-Western Finland. Asymmetrically distributed nuclear genetic variation compared to mitochondrial DNA in the northern contact zone suggests a skewed gene flow from the east to the west across the zone. This pattern might reflect differences in dispersal among sexes and lineages, or may be accounted for by a selective advantage for nuclear variation of eastern origin among Fennoscandian adders.

The phylogeographic pattern for adders across the entire species range was addressed by sequencing part of the mitochondrial genome and scoring microsatellite markers. The adder can be divided into three major genetic groups. One group is confined to the Balkan peninsula harbouring the distribution range of *V. b. bosniensis*. A second, well differentiated group is restricted to the Southern Alps. These two areas have probably served as refugia for adders during a number of ice ages for the adders. The third group is distributed across the remainder of the species' range, from extreme Western Europe to Pacific Russia and can be further divided into one ancestral group inhabiting the Carpathians refugial area, and three more recent groups inhabiting areas west, north and east of the Alps. The adder provides an example of a species where the Mediterranean areas are housing endemic populations, rather than the sources for post-glacial continental colonisation. Continent-wide colonisation has instead occurred from up to three cryptic northern refugia.

Keywords: Phylogeography, Vipera, adders, mtDNA, microsatellites, RAPDs

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Introduction

During the Quaternary period, approximately the past 2.5 million years (Myr), the climate has fluctuated to a much greater extent than at any time during Tertiary (the past 65 Myr (Andersen & Borns Jr., 1997). The Quaternary geological period is characterised by numerous cycles of glacial climate. Until 0.9 Myr ago these cycles mainly occurred with a 41,000 year interval, corresponding to the wobbling tilt of the earth's axis. After this, the 100,000 year periodicity in orbital eccentricity of the earth has governed the main glacial fluctuations. Added to these two periodicities and further complicating the climatic pattern, is a 19,000-23,000 year quasi-periodic precession resulting from the earth's axial wobble (Hays *et al.*, 1976). In the Old World, the last three glaciations are considered to have been the most severe, with massive ice sheets covering all of northern Europe at the respective glacial maxima and with limited glaciers to the south and to the east on the Eurasian continent. Most of the continental areas, today experiencing a temperate climate regime, were barren lands of arctic dessert or tundra (Andersen & Borns Jr., 1997). Consequently, all of the present-day temperate biota had to find refuge in areas of hospitable climate further to the south (first suggested by Nilsson, 1847), or possibly in potential embedded pockets of benign climatic conditions (Stewart & Lister, 2001). The last glaciation, Weichsel, which started about 115,000 years before present (ybp), fluctuated greatly with warmer interstadials being replaced by colder periods until reaching a glacial maximum between 22,000-18,000 ybp and finally ending about 15,000 ybp (Kleman *et al.*, 1997). The glacier which had covered most, if not all, of northern Europe steadily melted away with an interruption at 11,000 ybp when a shorter cold period, Younger Dryas, led to a brief glacial advance. However, it was not until about 8,000 ybp that the glacial sheet disappeared from northern Sweden (Andersen & Borns Jr., 1997).

Traditionally, the peninsular areas south of the Alps have been regarded as having harboured refugial populations of northern biota during glacial times. Either some or all of the Iberian, Italian and Balkan peninsulas have been assumed to have housed the ancestors of extant populations north of the Alps. Further to the east, the Caucasus Mountains have also been invoked as a source area for European populations (Hewitt, 2000; Taberlet *et al.*, 1998).

This view has recently been challenged as the Mediterranean regions appear to be areas of endemism, rather than source areas for some plants and animals (Bilton *et al.*, 1998, and references therein).

The historical distribution of extant species has traditionally been traced by deducing relationships of present-day populations based on morphological comparisons and/or by studying the fossil record. With the advance of molecular genetic techniques during the past twenty years, the field of study has benefited immensely. Consequently, the amount of scientific publications has exploded in recent years concerning phylogeography, as the field has come to be known (Avise, 2000). To date, however, there has been a relative dearth of publications treating phylogeographies of Eurasian reptiles in general, and that of snakes in particular (but, see (Lenk *et al.*, 1999; Nilson & Andrén, 2001; Surget-Groba *et al.*, 2001; Thorpe, 1980). Most recent phylogeographic studies of snakes have been based on molecular data (e.g. Rodríguez-Robles, 1999 #60; (Burbrink, 2002; Douglas *et al.*, 2002), whereas traditional morphologically based studies are still being implemented (Nilson & Andrén, 2001). The fossil record has, unfortunately, thus far proven too poor to be of practical use in intraspecific phylogeographic enquiries of Eurasian snakes (but, see Szyndlar & Rage, 2002).

The adder

The adder (*Vipera berus*) is the northernmost and most widely distributed snake species of the world. It is member of a Palaearctic genus consisting of small to medium sized, ovoviviparous viper species. The family, Viperidae, is traditionally subdivided into Crotalinae, the pitvipers, and Viperinae, the true vipers of the Old World. In total, the family encompasses some 20-27 genera (depending on taxonomy applied) but only a few major lineages of the latter subfamily are still represented in the Palaearctic (Pough *et al.*, 2001). Today, 13 species of vipers occur within the geographical boundaries of Europe (Gasc *et al.*, 1997). Palaeontological data reveal a substantial prehistoric viper diversity in Europe indicating that the genus *Vipera* (*sensu lato*) evolved in the continent during Miocene, while the earliest fossil record of the *Vipera berus* complex dates back to late Pliocene/early Quaternary (≈ 2Myr ago) (Szyndlar & Rage, 2002).

The geographic range of the adder covers a huge territory from Scotland (6°W) in the west to the Pacific island of Sakhalin (143°E) in the Russian Far East, and from the vicinities of Inari, Finland, and Murmansk, Russia (69°N), to northernmost Albania (42°N) in the south (Gasc *et al.*, 1997). The adder is a true generalist with regards to biotope choice and altitudinal

distribution, from sea level in the north up to an elevation of 2600 m in the Swiss Alps (Gasc *et al.*, 1997). Suitable habitats may consist of hedgerows, forest edges, clearings, heaths, meadows, bogs and rocky slopes. Despite exhibiting a vast overall distribution the adder's actual occurrence in many regions is restricted to rather fragmented populations and isolated mountainous relicts (Schiemenz, 1995). Intense land-use continues to cause considerable declines in population numbers and densities, especially in fertile lowland habitats, across much of Europe. Hence, the adder follows the general trend of the continent's herpetofauna (Corbett, 1989). In several European countries, including Sweden, the adder is nowadays a protected species.

The adder exhibits a rather homogeneous external morphology across the northern and eastern parts of its range, with most geographically correlated morphological variation being documented in Central Europe and Balkan. Three subspecies are currently recognised, of which the nominate form covers most of the species distribution, *V. b. bosniensis* populates the Balkan peninsula and another subspecies, *V. b. sachalinensis*, inhabits the extreme eastern part of the distribution range (Schiemenz, 1995). It is sexually dimorphic both in size and colour, with females being the larger sex and reaching 85 cm total length in the extreme northern parts of the species' range. Males are typically grey with contrasting black dorsal markings, while females are brownish with a less pronounced pattern. Various degrees of melanism occur in both males and females with varying frequencies in different local populations. Other variant colour morphs include unicoloured rusty red, and greenish or blueish base colouration (Fog *et al.*, 1997; Schiemenz, 1995). Throughout its range, the adder hibernates during winter for up to eight months per year, starting in September to October, depending on altitude and latitude inhabited. Males normally emerge several weeks before females, allowing time for pre-mating spermiogenesis during spring. After a synchronised male ecdysis, a rather explosive mating period takes place in April or May. As a consequence of their high reproductive costs females normally have biennial or triennial breeding cycles, while males mate annually. Accordingly, the operational sex ratio is skewed towards males, which fight vigorously for the opportunity to mate. Between five and 15 offspring are born in August or September, and litters with mixed paternity have been recorded (Höggren & Tegelström, 1995).

The aim of the present thesis was to investigate the phylogeography of the adder across its entire range with a focus on post-glacial colonisation history of the Fennoscandian peninsula in the northern periphery of the species' geographical distribution. Furthermore, I aimed to elucidate the relationships of extant populations in order to identify potential refugia during past glaciations.

Material and methods

Adder samples used for this thesis were collected in the field, where a blood sample was drawn from their caudal vein (Höggren & Tegelström, 1995). Initially, however, adders were sacrificed to obtain liver tissue. Tissue from roadkills and up to 100 year old museum specimens was also utilised.

The present thesis is based on molecular datasets drawn from analyses of both mitochondrial and nuclear DNA. Pure mtDNA was isolated according to (Powell & Zúñiga, 1983) as modified by (Jaarola & Tegelström, 1995). Total genomic DNA was extracted using either a salt extraction technique (Paxton *et al.*, 1996)or Qiagen DNEasy Tissue Kit. A number of different techniques of studying DNA have been employed, and these will be briefly described below.

Mitochondrial DNA (mtDNA) analyses

The maternally inherited mitochondrial genome is a circular molecule of approximately 17,500 base pairs (bp) length in adders (Kumazawa *et al.*, 1998). One interesting feature of the mtDNA of so-called advanced snakes is that the roughly 1,000 bp long control region, where replication of the genome is initiated, is duplicated (Kumazawa *et al.*, 1996). The two control region sequences are nearly identical, save a single base pair or two, and subjected to concerted evolution, i.e. a mutation in one sequence will be copied in the other sequence, or reversed to its original state. It has been suggested that the reason for the duplication is that a double loop is formed during initiation of replication, where portions of the duplicated control regions (the D-loop like structures) pair up against each other. This pairing also explains the strong sequence similarity, i. e. the implied concerted evolution (Kumazawa *et al.*, 1996).

Restriction Fragment Length Polymorphisms (RFLP)

The first study presented herein is based on restriction enzyme fragment analysis of the mtDNA. Restriction enzymes, also known as restriction endonucleases, recognise a short specific sequence of nucleotides where the DNA double strand is cut. The position on the DNA molecule of this target

sequence is referred to as a restriction site. Restriction enzymes are found in prokaryotes and their function is to incapacitate foreign DNA and thereby protect the organism from, usually viral or plasmid, infection (Stryer, 1988). Purified DNA is digested by a restriction enzyme under benign enzymatic conditions until cleavage is complete. The enzyme is inactivated and the reaction mixture is loaded onto a gel for electrophoretic separation of the restriction fragments. The cleaved DNA, i.e. the restriction fragments, are then stained and visualised for the scoring of Restriction Fragment Length Polymorphisms (RFLP) (Lansman *et al.*, 1981; Tegelström, 1992). There are two ways of scoring the fragments. They can be scored as they are, i.e. as presence or absence of a each particular band for each sample. Alternatively, if variation and number of bands are low enough, the actual restriction sites can be inferred from the band patterns. Then, presence of a restriction site translates into the known DNA target sequence for the restriction enzyme used, and correspondingly, absence of a restriction site indicates a point mutation within that sequence. The probability of multiple point mutations within the same actual restriction site is usually negligible because of the low variation found, low mutation rates and the relatively low number of base pairs being surveyed (Nei & Tajima, 1981). Paper **I** is based on RFLPs from eight different restriction enzymes on purified mtDNA.

PCR

A segment of DNA is copied in large numbers via the polymerase chain reaction (PCR) in a cyclic reaction. First, the bonds are broken between the two strands of the double stranded DNA molecule and the DNA is denatured into two single strands. Small manufactured sequences of 10-30 base pairs (bp), called primer oligomers, bind to their complementary positions on the single stranded DNA. A specific enzyme, DNA polymerase, then synthesises a new DNA strand with the present one as template using a primer as starting position. The now again double stranded DNA is once more denatured and the cycle is repeated. If two primer positions are close enough to each other and on opposing DNA strands, the number of copies of the DNA sequence in between these two positions will be doubled for each cycle described above. The procedure is repeated 25-50 cycles eventually yielding more than a million times more copies of the target DNA sequence than were present at the start. In this way, enough of a particular DNA segment of interest can be produced even from minute quantities of sample (Palumbi, 1996). Different approaches based on PCR are explored in papers **II-V**.

Sequencing

In order to get the exact sequence of a DNA segment a similar approach to PCR can be applied. A primer, polymerase enzyme and a mix of the four bases are allowed to react. However, a proportion of one of the four bases is altered in such a way that the elongation reaction stops when one of these altered bases is incorporated. This will occur randomly and with enough reaction cycles, some sequence will be stopped at every position that particular base occurs on the sequence. A separate reaction is performed for each kind of base and the products of each of the four reactions are separated through gel or capillary electrophoresis. For every band on the gel, the last incorporated base is of the altered kind for that particular reaction mixture. With the four products run side by side, the DNA code can be read sequentially (Hillis *et al*., 1996). Papers **IV** and **V** involve sequencing of parts of the mitochondrial genome.

PCR-RFLP

This technique is merely a combination of the first two techniques described. First, a DNA segment is amplified through PCR. Then, a restriction enzyme with one or several known restriction site(s) within the given DNA segment can be used to diagnostically differentiate between DNA from different individuals. With many and large PCR products this technique can be a quick and relatively cheap way of estimating levels of genetic diversity, but as implemented in paper **III**, it is merely a tool for simple classification of samples into predefined categories.

Random Amplified Polymorphic DNA (RAPD)

A PCR as described above is conducted using very short primer oligomers of 10 bp length and random composition, one at a time. These primers will anneal to an unknown number of primer sites in the target DNA. Wherever in the genome two sites are close enough and on opposing DNA strands, the sequence in between will be amplified. For every primer used, a number of arbitrary PCR products will result. These PCR products are then separated by electrophoresis on an agarose gel and the PCR products in the gel are stained and visualised. A number of bands will appear on the gel, and if there is variation between PCR products from different individuals, scored as presence or absence of any particular band, then that band position is considered a locus. The method, known as Randomly Amplified Polymorphic DNAs (RAPDs) (Williams *et al.*, 1990), is relatively quick and inexpensive. One major drawback is that it is difficult to know what any particular band actually represents. It could be a composition of several PCR

products of similar size. Moreover, as the nuclear genome is diploid, one expects two copies, alleles, of each PCR product. It is relatively safe to assume that RAPDs exclusively scores nuclear polymorphisms as it is based on the random amplification of total DNA present and the nuclear genome is on the order of 10^9 bp compared to the $1.7x10^4$ bp mitochondrial genome. This method cannot, however, distinguish between an individual which is homozygous for the PCR product, i.e. has two amplified copies, or heterozygous, i.e. where only one allele is amplified. Further, there are potentially numerous reasons for non-amplification of any single polymorphic product. This creates difficulties during analysis of the genetic data. The worst problem with RAPDs, however, is erratic amplification during PCR, often resulting in low repeatability of band patterns when re-running the same samples under identical PCR conditions. Paper **II** compares the performance of RAPDs to that of using microsatellite markers, described below.

Microsatellites

The nuclear genome is littered with repetitive non-coding DNA sequences, mostly of unknown function, and considered to be selectively neutral (Kashi & Soller, 1999). Different lengths of the sequence motifs being repeated, tend to correspond to the rates of mutation for the loci, or positions in the genome. Mutations are usually in the form of insertion or deletion of one or more copies of the repeat motifs. The sequence repeats are, thus, classified according to the length of the DNA motif being repeated. The analysis of microsatellite repeats has become common practice in population studies. A microsatellite is a one to six base pair sequence with up to 250 repeats in tandem.

Data analysis

For details concerning the computational analyses of the molecular raw data the reader is referred to papers **I**-**V**. Here, a few generalisations will be made in order to convey general principles for the analyses performed.

Geographic structure - AMOVA/Spatial autocorrelation

Most analyses in the papers of the present thesis aim at investigating geographic structure of the genetic variation encountered using the different methods and samples employed. Spatial autocorrelation is a way of correlating genetic variation to geographic distance. Some measure of

genetic distance between samples from different locations is correlated to some measure of the physical distance between the sampling locations. In paper **I**, the geographic distances between samples are grouped into first three and then nine distance classes in order to appreciate any effect on level of resolution. No assumptions concerning topography are superimposed on the classification of distances.

In papers **II**-**V** Analyses of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) are performed. With this approach, a hierarchical relationship between population samples is predefined and the level of genetic variation on different hierarchical levels is evaluated. Population samples that are believed to be more related to each other than to other samples in the study are grouped, and the proportion of the total genetic variation is calculated for what can be found within populations, between populations but within the same groups and finally between the different predefined groups.

Estimating phylogenies

Phylogenetic analyses aim at investigating the evolutionary relationships between different samples and similar techniques can be applied to a vast range of data sets and different hierarchical levels (Nei & Kumar, 2000). The different phylogenetic approaches can be lumped into two principally different groups. Either relationships are inferred from some distance value, which is basically a kind of sum of differences revealed by pair-wise comparisons of the populations/taxa. Or else, characters, or loci, are treated as discrete entities and the phylogenetic relationships between taxa are built around minimising the total number of state changes, i.e. inferred historical mutations, required to explain the data matrix, while still resolving a bifurcating phylogeny (e.g. Li, 1997).

Assignment tests

The gene pool of a sample of individuals can be used to calculate the probability of an individual genotype belonging to that sample. In its simplest form, and assignment test multiplies the allele frequencies for a particular population sample corresponding to the alleles present in the individual being tested. In this way a cumulative genotype frequency, or probability is obtained for the population the individual is tested against. Repeating this for several population samples will yield one most likely population of origin for the individual (Pritchard *et al.*, 2000).

Results and Discussion

Paper I: Variation in adder mtDNA across Fennoscandia

This paper was based on a sample of 135 adders from 71 localities in northern Europe (Fig. 1). Using RFLPs of eight tetranucleotide restriction enzymes, approximately 900 bp of the mitochondrial genome was investigated. The data was analysed for haplotype- and nucleotide diversities (Nei, 1987), genetic distance (Nei & Li, 1979), and genetic relationships through three separate phylogeny reconstructions. Geographic structure of the genetic variation was analysed through spatial autocorrelation using the program AIDA (Bertorelle & Barbujani, 1995), by dividing the data into three and nine distance classes, respectively, in two separate analyses.

In all, 14 haplotypes were identified in the total sample. There was an obvious geographic structuring of the mtDNA haplotypes, with two genetically, and geographically separated mtDNA populations (sequence divergence d = 0.00378, Nei & Li, 1979) (Fig. 1). These two populations occur on either side of the Baltic and meet in northern Finland. There are two more areas, however, where they meet. In the Umeå Archipelago and at Tvärminne, in the southern Finnish Archipelago, haplotypes representing both major lineages were detected. There was very little haplotype diversity, with two main haplotypes found in 122 adders. Variant haplotypes differed by only one or two mutations from the two most common ones, and this low level of variation is evident from the low haplotype and nucleotide diversity estimates of the data (Table 1). The samples belonging to the western mtDNA population, which housed 11 of the 14 mtDNA haplotypes, were subdivided into three arbitrary groups along the 59th and 63rd parallel (Fig. 1) in order to investigate geographic structuring of the data, and diversity estimates for these groups are also presented in Table 1.

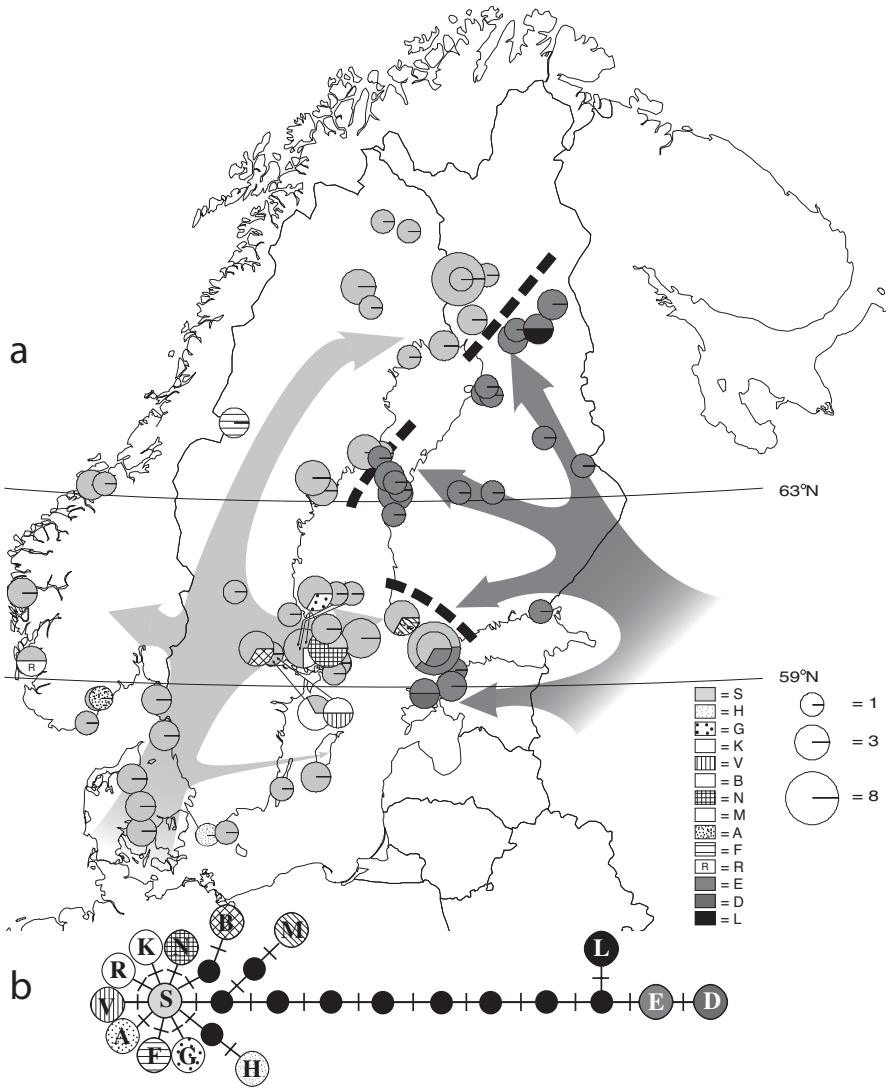

Fig. 1**.** a) Map showing collection localities and *Vipera berus* mtDNA haplotypes. Pie charts depict haplotype composition with the size of the chart corresponding to sample size for each locality and haplotype pattern corresponding to those of Figure 1b. Shaded arrows indicate hypothesised colonisation 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, see Table 1. b) Minimum mutation network of the 14 haplotypes identified.

As Fig. 1 shows, most of the haplotypic variation can be found in the central group. This apparent geographical skew in genetic variation was somewhat verified when the data was investigated by autocorrelation of the western

data using AIDA (Bertorelle & Barbujani, 1995) although no real geographic structuring of the mtDNA diversity could be proven. At short distance intervals there was a positive correlation, a result of one variant haplotype being found in three places less than 60 km apart (haplotype K, Fig. 1). At intermediate distances there was a negative correlation. This could be explained as follows: most haplotypic variation is present in the central group, with both the northern and the southern being close to fixed for the common western haplotype (haplotype S, Fig. 1) and, thus comparisons over intermediate geographic distances will more often yield high pair-wise differentiation.

Table 1. Haplotype (h) and nucleotide (π) diversity estimates from RFLPs of the mtDNA for 135 samples of *Vipera berus*. 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. N = number of samples, NH = number of haplotypes.

Location	N	NH	h ± SD	π ± SD
West < 59°N	19	3	0.2047 ± 0.1192	0.000138 ± 0.000000
West ≥ 59°;< 63°N	47	8	0.4413 ± 0.0884	0.000319 ± 0.000000
West ≥ 63°N	36	2	0.1079 ± 0.0680	0.000061 ± 0.000000
West total	102	11	0.2885 ± 0.0592	0.000197 ± 0.000000
East	33	3	0.1193 ± 0.0756	0.000079 ± 0.000000
Total	135	14	0.5434 ± 0.0418	0.001573 ± 0.000000

The overall pattern of colonisation of the Fennoscandian peninsula by the adder is similar to that previously reported for a number of vertebrates with a bi-directional immigration pattern to the region (Fredga & Nawrin, 1977; Jaarola & Tegelström, 1995; Taberlet & Bouvet, 1994). However, the detailed pattern is rather different. Whereas, the two separate population lineages of each of the other species studied meet in north-central Sweden, the two mtDNA populations of the adder meet to form a contact zone in the north of Finland (Fig. 1). A large portion of northern Finland was left inundated for thousands of years longer than most of northern Sweden, while slowly rising and recovering from the weight of the glacial ice sheet. This part of northern Finland is predominantly flat and still mainly composed of wet lowlands. It is speculated that these areas of northern Finland have become suitable for adders more recently than corresponding areas of northern Sweden, and that the advance of colonisation has been allowed to progress at a higher pace in the west than in the east, where colonisation may

have stalled. Moreover, there are two additional zones of contact, in the Umeå archipelago off the north central Swedish coast, and in the southwest of Finland (Fig. 1). Both of these areas of introgression must, arguably, be the results of across-water dispersal. Thus, the present study shows that the adder may not have been dependent on a landbridge from the continent in order to colonise Fennoscandia from the southwest, which has been the accepted explanation of colonisation by terrestrial vertebrates into the area in the past (Jaarola *et al.*, 1999; Taberlet *et al.*, 1998).

An assumption based on the results of the RFLP investigation of variation in Fennoscandia, is that the two mtDNA lineages of North European adders represent two populations from separate glacial refugia. This assumption is addressed in papers **IV** and **V**.

Paper II: Comparison of genetic variation in Fennoscandian adders between two nuclear datasets

With a similar, although not identical, sampling design as in paper **I,** the present study was based on 122 adder samples from 71 localities (Fig. 2). A RAPD dataset of 42 variable, binary, loci was compared to microsatellite data from six polymorphic loci, with a total of 3-91 alleles per locus, for the same individual samples. The geographic structure of the genetic variation is investigated through AMOVA analysis (Excoffier *et al.*, 1992), and especially using assignment testing. A novel approach to compensate for different population sample sizes is presented and utilised. A large difference in sample size can have a dramatic effect on the outcome of the assignment tests. Individuals from larger population samples will tend to assign to smaller population samples for two reasons: If the allele of a particular locus in the tested individual is present in the small population, there is a high probability that it is fixed because of the small sample size (Pamilo, *pers. comm*.). Additionally, if an allele present in the tested individual is relatively rare in the correct population sample, the allele frequency may actually become higher in some other, smaller, population even if the particular allele is not present because of the null-frequency allele compensation necessitated by the assignment test. Hence, in order to compensate for size differences among population samples a jack-knife resampling of the data was performed with standardised population sample sizes corresponding to the smallest population sample size. By randomly drawing individuals from each respective sub-sample without replacement, new data matrices (n = 10,000) were generated and assignment tested. The mean assignment values for all replicates were then scored.

Both sets of data were differentiated into an eastern and a western population in accordance with the population lineages defined by mtDNA (**I**). The level of genetic differentiation between the two population lineagaes was, however, rather different for the two different kinds of nuclear genetic markers. The AMOVA analyses, based on arbitrarily dividing the data into eight geographical population samples, revealed that 29% of the total genetic variation in the RAPD dataset was portioned between the eastern and the western lineages, whereas only 1% of the total variation in the microsatellite data differentiated the two lineages. Investigating the most likely population subdivision of both kinds of nuclear data using the assignment test program Structure (Pritchard *et al.*, 2000) also resulted in dividing the data into an eastern and a western population, with no internal structure within the lineages being detected for the present data.

In order to further investigate any potential geographical structure within each of the eastern and western population lineages, which both the AMOVA and Structure analyses failed to detect, assignment tests with the calculator Doh (Brzustowski, 2002) were performed using the same arbitrary population subdivisions as for the AMOVA. When compensated for unequal sample sizes in a manner described above, a bias of assignment towards the smallest samples was eliminated. The overall performance was rather poor, but for both kinds of data, the population of origin tended to get the highest assignment values. Thus, even with arbitrary population definitions, there appeared to be some geographic structure in the data. There was also a difference in the level of resolution between the two datasets. In the total sample of 122 adders, there were three small samples (n = 5, 5 and 8) from single sample localities. These samples thus represented true population samples. When these were included as three separate population samples in an additional assignment run, they got very high assignment values for the microsatellite data, but not for the RAPD data. This indicates that on a finer geographic scale, using real population samples, the microsatellite markers outperformed the RAPD markers.

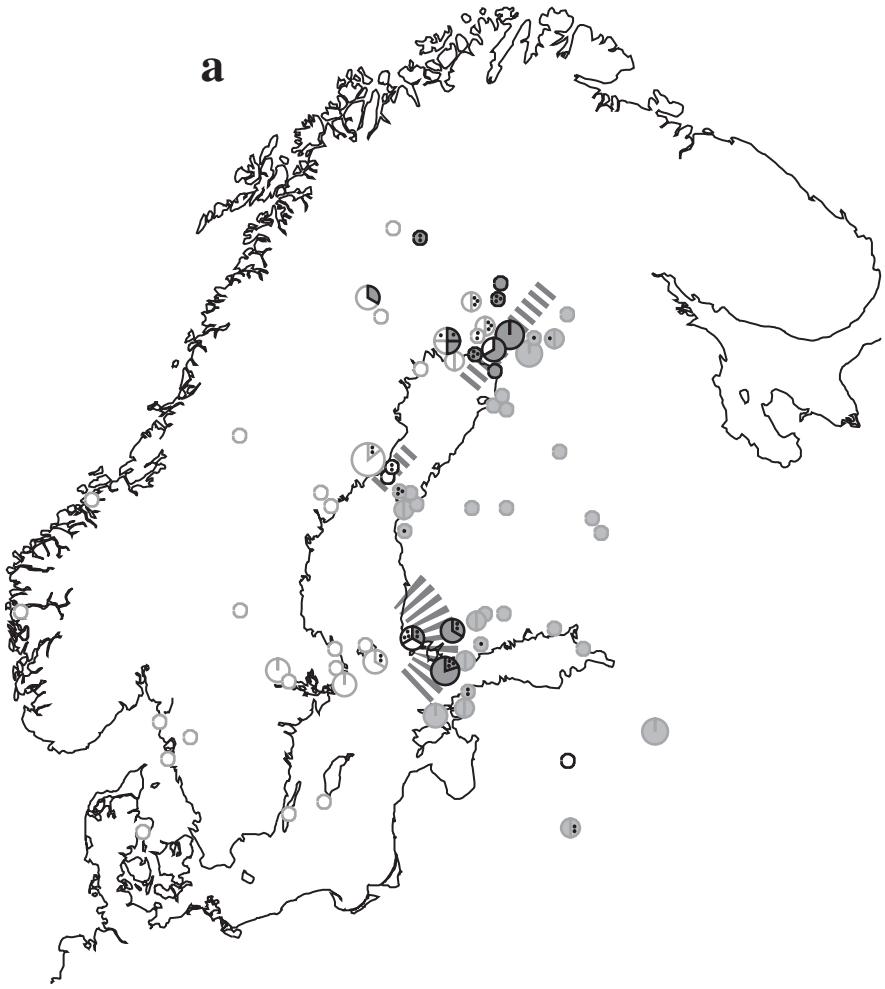

Figure 2. Maps showing collection localities and assignment results, where grey samples have assigned to the eastern group and non-coloured samples have assiogned to the western group. 24 individuals that were omitted from the group definitions, located at or close to mt DNA contact zones (hatched areas, **I**) and individuals that assigned contary to expectations are shown with black circles. Individuals with more or less equal assignment probabilities are marked with 1, 2 or 3 dots corresponding to assignment scores within a factor 10, 5 or 2 of each other. **a**) Assignments from RAPD genotypes.

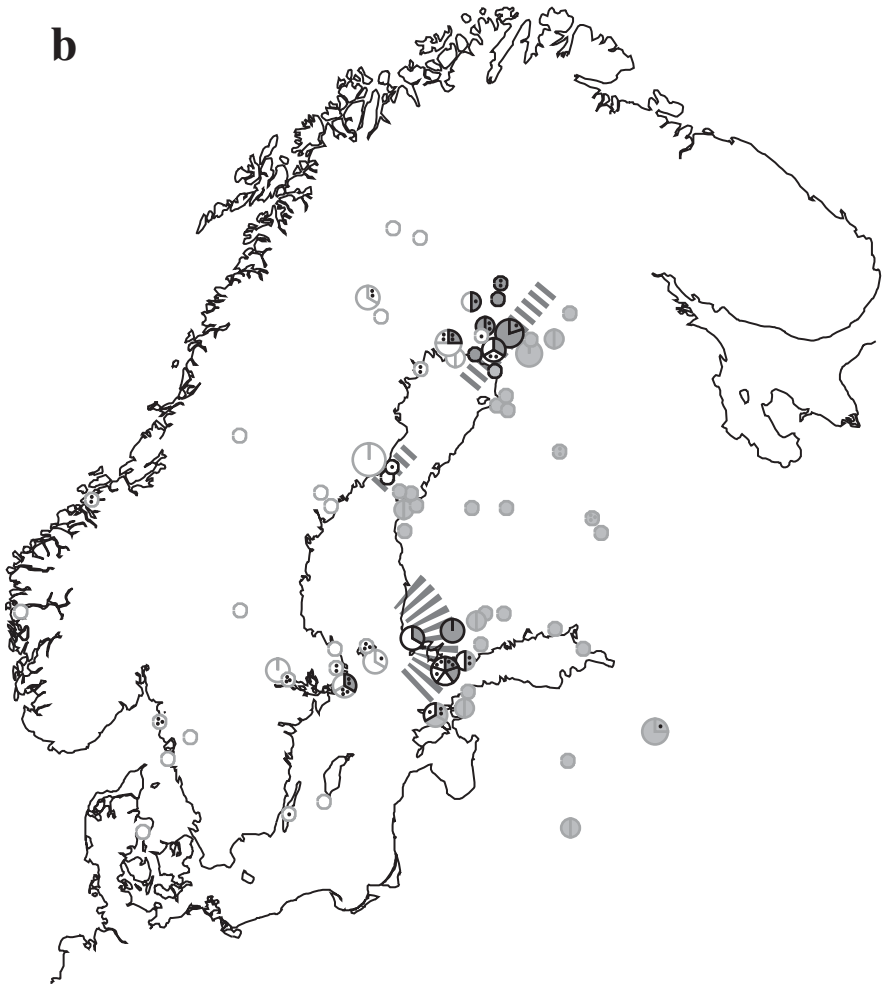

Figure 2. b) Assignments from microsatellite genotypes.

The zones of contact that had been identified with the mtDNA data (**I**) were also detected with the two nuclear data sets, using the assignment test calculator Doh (Brzustowski, 2002). Both sets of nuclear data indicated gene flow across all three contact zones, although there was only a large discrepancy between the different data for the northern zone. In fact, west of the northern contact zone there appeared to be a substantial influence of nuclear DNA of eastern origin reaching quite far into northern Sweden (Fig. 2ab). This pattern was evident from a number of erroneously assigned individuals in this region. Also, by comparing the actual probabilities for each individual to assign either to the east or the west, individuals with almost equal probabilities were also marked on the map (Fig. 2). Arguably, these individuals, although correctly assigned, might be considered to have

mixed ancestry. Thus, it can serve as a crude approach to identifying individuals of hybrid origin, an approach similar to that of Campton (1985). In the present case, the mere distribution of such individuals of presumed dubious origin, with a majority being found at or near contact zones, lends support to the approach while also indicating gene flow across the contact zones (Fig. 2).

The comparison between genetic variation found in RAPD and microsatellite markers among Fennoscandian adders, thus corroborated the findings from mtDNA regarding a bi-directional colonisation of northern Europe by the adder, yielding further support to the assumption that the two lineages represent populations from different glacial refugia. The contact zones found for mtDNA were the same for both nuclear markers, but substantial nuclear gene flow from the east across the northern contact zone was apparent in both kinds of data compared to the pattern found for mtDNA.

Paper III: Investigation of gene flow across a contact zone

In order to investigate the genetic structure around the northern contact zone, 129 adders collected from eleven local populations and an additional 14 adders from ten scattered localities were analysed for microsatellite variation and typed for mtDNA descent using PCR-RFLP. An AMOVA was performed by grouping the population samples into three groups, one western (n = 3 population samples), one group of presumed mixed origin, defined by the mtDNA PCR-RFLP assessment (n = 6 population samples), and one eastern group (n = 2 population samples). Assignment tests using the calculator Doh were performed with jack-knife resampling compensation for different population sample sizes, and a straight forward assignment was also made in order to trace affinities of the 14 stray samples.

The mtDNA data indicated a narrow zone of contact between the two population lineages of less than 30 km in the studied region. The microsatellite data, however, showed a larger but skewed zone of introgression, of eastern nuclear DNA across the zone to the west.

The AMOVA analysis revealed no geographic structuring of the genetic variation. Assignment testing with the calculator Doh, did reveal some structure, however. On average 46.2% of the samples assigned correctly. Moreover, the samples that did not assign to their population sample of origin tended to assign to eastern populations. That is, individuals from the western population samples tended to miss-assign either to other western samples, or to population samples of presumed mixed origin, whereas those

of presumed mixed origin tended to miss-assign to other population samples of mixed origin or to eastern population samples and, finally, individuals from eastern population samples tended to mainly miss-assign to the other eastern population sample. Moreover, the stray individuals also showed a tendency to assign to the east. Although, caution should be applied when interpreting the present data, as the above stated tendencies are just that, they do abide by *a priori* expectations based on previous results (**II**) regarding gene flow from the east to the west.

There could be several explanations to the asymmetric distribution of genetic variation encountered across the contact zone. Male adders of eastern origin could be better dispersers than their western counterparts. Alternatively, selection on the nuclear genome is benefiting DNA of eastern origin. Recently, there have been several tests proposed to genetically investigate sex-biased dispersal (Goudet *et al.*, 2002). These tests, however, are sensitive to varying sample sizes and low migration rates. Reports of migration in adders are scarce and the species is generally regarded as highly philopatric (Viitanen, 1967; Madsen & Shine, 1992; Luiselli, 1993). The present data suffers from low assignment accuracy, with a mean correct assignment of 46.2% per population sample. Using additional markers should increase assignment accuracy, and increasing the local sample sizes would make testing for sex-biased dispersal an interesting prospect.

Paper IV: Phylogeography of adder mtDNA across the entire geographic range

This study aimed at reconstructing the colonisation history of the adder across its entire geographic distribution range using mtDNA sequence data. In all, control region II (CRII) sequences from 80 adders and 60 localities were obtained. In addition, 50 of these adders were also examined for cytochrome b (cyt. b) sequence variation. A further three samples representing the closely related species *V. dinniki*, *V. seoanei* and *V. ursinii* were also analysed for comparison. A phylogenetic analysis employing maximum parsimony (MP) criteria was performed in PAUP*, version 4.0b10 (Swofford, 2002) for the combined data set of 1981 bp.

There were 45 different haplotypes among the 80 *Vipera berus* CR sequences and there were 59 (6.4%) variable sites (12.0% including outgroup taxa), of which 39 (4.3%, 7.9% including outgroup) were phylogenetically informative under MP criteria. Four indels occurred within the *V. berus* sequences, with another three indels among outgroup taxa. For the CR and cyt. b combined analysis, comprising 1981 bp from 50 samples of *Vipera berus*, 143 sites (7.2%) were variable among the ingroup taxa

(13.9% including outgroups), with 85 sites (4.3%) being parsimony informative (7.6% including outgroups). Uncorrected distance divergence (p) ranged between 0% to 2.6% within *V. berus* and 5.4%, 5.5% and 5.7% respectively between *V. berus* and *V. seoanei*, *V. dinniki* and *V. ursinii*. Hence, approximately the same amount of phylogenetically informative information was contributed by each sequence data set. The two data sets were tested for substitution saturation, and both showed the same trend. There was no, or at least a very low level of, saturation among *V. berus* sequences, but a considerable degree of saturation when including the three outgroup taxa. Therefore, a parsimony analysis was performed to find the most basal clade of *V. berus* to use as root after having removed the sequences from the other three species. The reason for removing those sequences was to improve resolution of the analysis by decreasing the amount of homoplasies among inferred branches, which could obscure some of the true phylogenetic signal in the data.

The phylogenetic analysis consisted of a MP analysis, which recovered 18,360 shortest trees. Although, a large number of trees, they collapsed into a strict consensus tree with considerable resolution retained, and the topology of the consensus tree was checked for statistical bootstrap support (Fig. 3).

There was a clear phylogeographic signal in the data with good resolution for the three major phylogenetic lineages. The most divergent clade, also used to root the tree, was one containing all samples of *V. b. bosniensis*, which included all adder samples from the Balkan peninsula. The second clade included samples from the Southern Alps (Italy, northern Slovenia, Austria and South-Eastern Switzerland) The third clade included all adders from Europe east, north and west of the Alps as well as all adders from the Asian distribution range. The third clade could be subdivided into a basal Carpathian subclade, containing adders from Romania, eastern Slovakia and southern Poland. The remaining haplotypes formed a separate, northern group with high bootstrap support, which contained three monophyletic subclades. All adders from Russia and Finland formed one distinct subclade, as did adders from France, Switzerland and the western extreme of Austria. All other haplotypes within the northern clade formed a subclade with low bootstrap support, and most haplotypes within this subclade were phylogenetically unresolved, with variation only at terminal branches. However, they all originated from North-Western Europe.

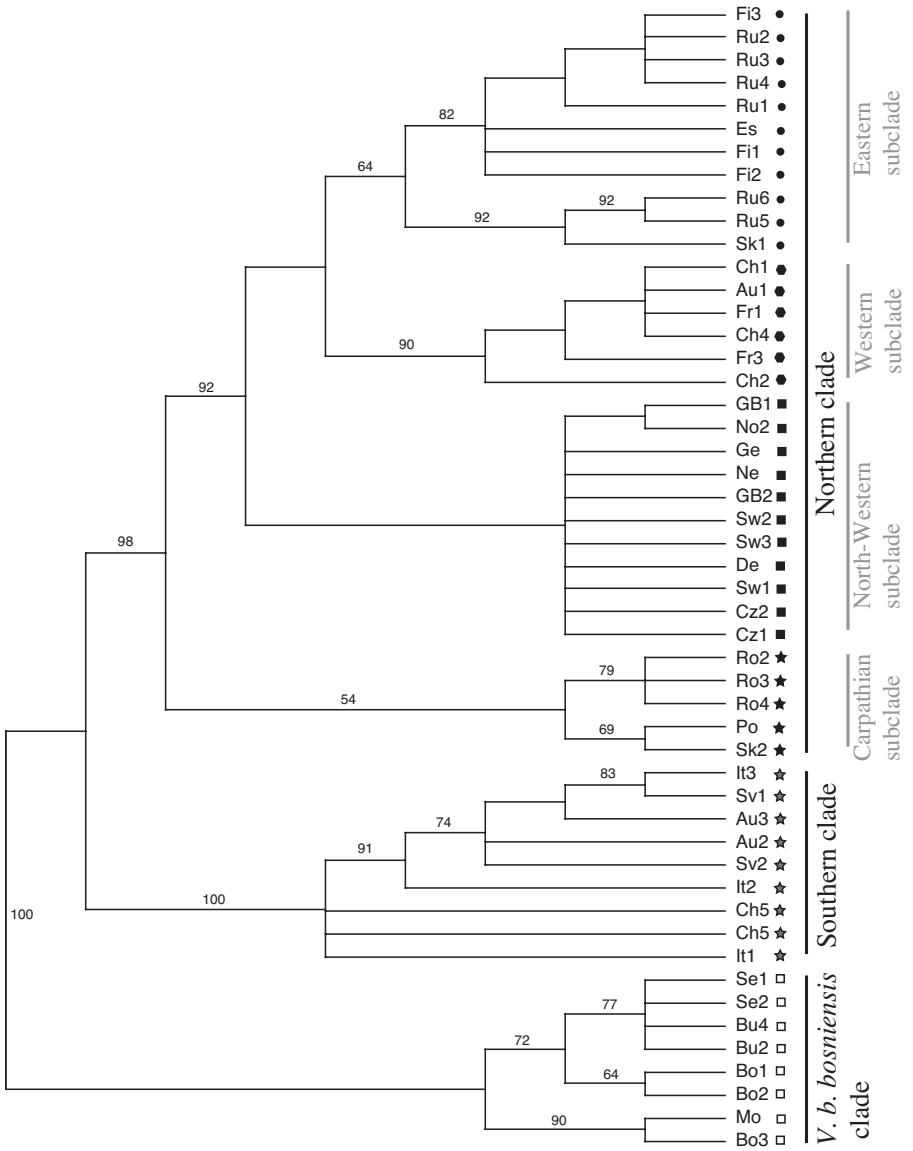

Fig 3. Strict consensus tree from maximum parsimony analysis of 50 adders mtDNA combined sequences. Figures on branches represent support from 10,000 bootstrap replicates. Clade definitions as delineated in Fig. 4 (Individual ID and symbol refer to Fig. 1, **I**).

Several studies of European species have provided evidence of glacial refugia in the three peninsular areas of Southern Europe, namely, Iberia,

Italy and the Balkans and one or several of these refugia have been shown or postulated to have housed the source for all present–day populations inhabiting the previously inhospitable parts of the continent to the north (reviewed in Taberlet *et al*. (1998) and Hewitt (2000)). The results for adders, however, indicate that the Mediterranean refugial areas are inhabited by adder populations that are genetically divergent from their northern conspecifics. Both the Balkan and Italian peninsulas house adder populations that, arguably, have been separated from the populations containing the mtDNA lineages found to the east, west and north of the Alps for a considerable duration of time, encompassing several clacial cycles. The idea of the Mediterranean areas not being source areas for northward colonisation, but rather areas of endemism has been suggested based on evidence from small mammals (Bilton *et al.*, 1998). This pattern, also appears to describe the geographic distribution of adder mtDNA variation in the southern parts of the species range. Further to the north, the phylogeographic pattern inferred from adder mtDNA is rather novel. The adder populations in the region of the Carpathian Mountains form a basal clade to all other northern adders, and it is hypothesised that all northern lineages can trace their origins to this region, probably several ice ages ago.

During the most recent ice age(s) it appears that adders have utilised one refugium in the west, possibly around Massif Central in France. All adders north and northeast of the Carpathian Mountains, including the pacific subspecies, share a recent history. Possibly, these adders have survived in areas close to the Carpathians or further east, south of the Ural Mountains.

A third population refugium is, however, also evident in the data, which must be situated somewhere on the European continent. All adders in between the eastern and western lineages described above, share a recent ancestry, according to the mtDNA variation. As all traditionally inferred refugial areas in the south and southeast appear populated by other adder populations, and even a not so commonly implied refugium in the vicinity of South-Central France (but, see Guillaume *et al.*, 2000) appears to house genetically differentiated adder populations, it is argued, that there could have been another refugial area somewhere in central Europe, north of the Alps. The boundaries for the respective mtDNA populations described above can be seen in Fig.4, although data refer to Paper **V**.

The mtDNA haplotypes of the two specimens of *V. b. sachalinensis* analysed in this study were genetically related to the mtDNA of a Slovakian specimen, although this lineage of three samples was divergent from all other eastern lineages (Fig. 3). Hence, accepting the subspecific status of the pacific adders, makes the remaining group of eastern adders paraphyletic with respect to their mtDNA origin.

Paper V: Corroborating the mtDNA phylogeography of adders using nuclear genetic markers

In order to verify the phylogeographic pattern obtained from adder mtDNA with data from nuclear markers, a study of population structure across Europe based on microsatellites was performed. Fourteen population samples and some stray individual samples from Scandinavia (n = 232 adders) were analysed for microsatellite variation and the geographic structure was assessed by performing an AMOVA (Excoffier *et al.*, 1992) and assignment tests using Structure (Pritchard *et al.*, 2000). To obtain a better resolution of mtDNA variation, 50 new sequences of 563 bp of the duplicated control region (CRII) were analysed together with 20 sequences from Paper **IV**. Five of the 14 population samples analysed for microsatellite variation were also scored for within population mtDNA variation, by sequencing five to seven individuals per population sample. Haplotype and nucleotide diversities (Nei, 1987) were calculated for the mtDNA of the respective phylogenetic lineages outlined previously (**IV**). A phylogenetic analysis of the mtDNA sequences was performed in PAUP*, version 4.0b10 (Swofford, 2002), based on neighbour-joining (Saitou & Nei, 1987) of uncorrected pairwise distances (Fig. 5).

The results of the AMOVA revealed that 4.76% of the total genetic variation was structured among groups, 4.12% of the variation differentiated population samples within groups and the remaining 91.12% of the total variation could be accounted for by genetic variation at the population level. The analysis using Structure yielded a highest probability of the data given 12 subpopulations, but with an almost equal log likelihood value for 14 subpopulations. Individuals from the Finnish population samples (E1-E3, Fig. 4) from the eastern phylogenetic lineage and Swedish population samples, representing the north-western lineage (NW1-NW5, Fig. 4) assigned arbitrarily to three constructed populations each. Thus, there was little genetic structure evident in the north. The two population samples (Z1 and Z2, Fig. 4) from the northern contact zones (described in papers **I-III**) had a high level of structural identity, as did the Russian (E4, Fig. 4) and southern population sample (S1, Fig. 4). The two western population samples (W1 and W2, Fig. 4) clustered into one constructed population, which included all individuals from the two population samples. Interestingly, the Swedish population sample NW1 was comprised of 6 specimens collected in 1991 and 14 specimens collected in 1992. The six individuals from 1991 were grouped into a unique constructed population in all Structure replicate runs. The other 14 individuals assign in the same arbitrary way as the rest of the Swedish populations. Thus, there was a great difference in allelic composition and frequency in the population sample

among years, indicating a temporal variation in the genetic variation found in that particular adder population. This is not a complete surprise, given the biennual reproduction pattern in female adders and low number of reproductive seasons on average per female (Schiemenz, 1995). The analysis in Structure using predefined population samples yielded a very high assignment accuracy for all population samples, (78.9% to 99.9% correct assignment accuracy per population sample), suggesting more genetic identity within the eastern and north-western population samples than was first indicated.

A geographically correlated distribution of nuclear genetic variation can be seen for locus 37 (Fig. 4). The alleles of this highly polymorphic locus were grouped into five size classes according to the natural distribution of allele sizes, which was discontinuously pentamodal with a 10-80 bp gap between size classes where no alleles were detected. Several of the five allele classes are regionally confined to phylogenetically distinguished groups of adders, and thus the geographic distribution of allele size classes in locus 37 lends support to the phylogeography of adders that has been suggested based on mtDNA variation (**IV**).

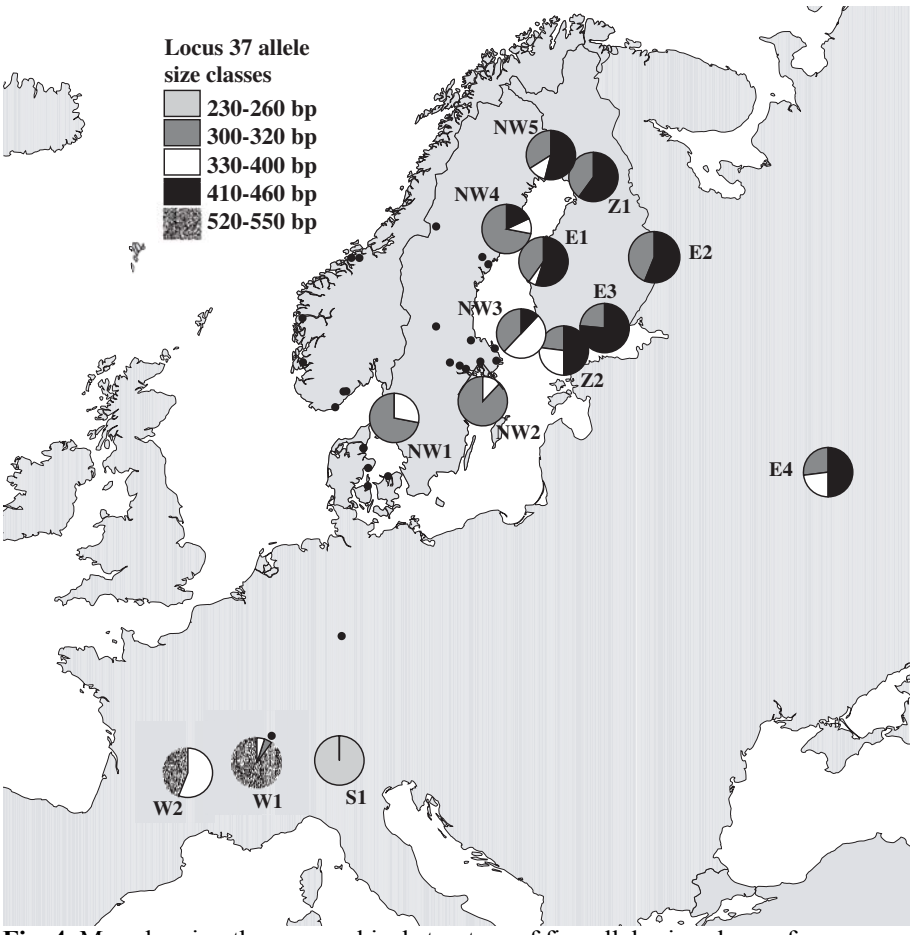

Fig. 4. Map showing the geographical structure of five allele size classes for microsatellite locus 37. Delineated on the map are also the areas populated by the different mtDNA lineages.

While no population samples were available from the *V. b. bosniensis* and Romanian phylogenetic groups with which to compare microsatellite variation, these lineages were excluded also in the mtDNA analysis. Instead, the southern population sample (S1) was used as outgroup to study the genetic variation among the northern adder lineages. The phylogenetic analysis, using neighbour-joining, corroborated the results from paper **IV.** Based on only a 563 bp sequence, the analysis resolved the major lineages with little ambiguities. These ambiguities consisted of one Danish and one Norwegian sample, which appeared to retain one ancestral character state each (Fig. 5).

The five population samples represented in the neighbour-joining tree differed in appearance (Fig.5). While there was no within population

variation in the NW1 and E3 samples, there was a high level of diversity in the western population samples, with three different haplotypes among five individual W1 samples and five different haplotypes among seven samples from W2. The southern population sample, S1, comprised two haplotypes with equal frequencies (Fig. 5)**.** Add to this pattern the low level of differentiation among samples from the north-western lineage, especially those from Sweden, and among eastern samples, mainly the Finnish, and a trend of loss of variation in the presumed direction of the post-glacial colonisation is suggested in the data.

In summary, this study confirmed the presence of three separate population lineages of adders west, north and east of the Alps and Carpathians using a combination of nuclear and mitochondrial genetic information. The exact locations of the three glacial refugia inferred for the genetically separated populations could, however, not be established with the present set of data and remains an open issue.

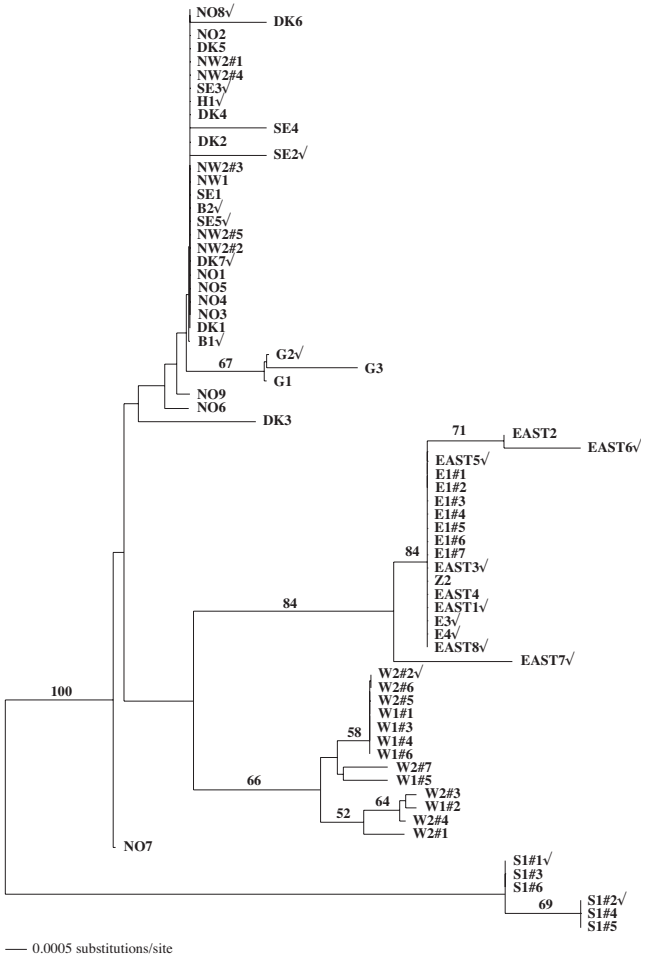

Fig. 5. Neighbour-Joining tree based on uncorrected pairwise distances among 50 control region II sequences from adders. Figures on branches are bootstrap support values from 1,000 replicates.

Conclusions

The results of my studies reveal several patterns of genetic differentiation in the adder, *Vipera berus*, that have not been recorded before. On a regional scale, Fennoscandia has been colonised from two directions, as has been the case for several terrestrial vertebrates studied previously. However, the two adder lineages meet in Northern Finland instead of North-Central Sweden, which is novel and perplexing considereing the different kinds of vertebrates (brown bear, field vole, common shrew) that have displayed the same pattern of colonisation.

The adders of Fennoscandia are shown to have traversed vast stretches of water and to have formed two additional contact zones as a direct result of dispersal across the Baltic and Bothnian Seas. Hence, this species was not dependent on a post-glacial land-bridge from the continent during its colonisation phase.

In the northern contact zone, there is an asymmetry in the distribution of genetic variation. Nuclear genetic variation from the eastern population lineage has introgressed a large distance into the western population lineage, whereas there is no evidence of nuclear gene flow from the west to the east across the zone. Furthermore, the zone of introgression of mtDNA is rather narrow. Thus, it appears that there is a selective bias in favour of the eastern nuclear DNA, or male adders from the east may be much better dispersers.

On a species wide distribution level, from Scotland to Sakhalin, the adder is morphologically homogeneous. The investigations presented do, however reveal three major and well differentiated geographic population lineages. Two inhabit the geographically small Mediterranean peninsular areas of the species' distribution range, the Northern Italian and the Balkan peninsulas. The third group is distributed across most of the species range. This group can be further, subdivided into four distinct population lineages representing separate glacial refugia. First, and ancestral to the others is a genetically diverse population lineage, which is located around the Carpathian mountains. North and east of this group, the eastern lineage, which is encountered in Finland, has colonised the entire Asian part of the distribution range, as well as eastern and northeastern Europe. Representatives of this phylogenetic group include the Pacific form of the adder, currently recognised as *V. b. sachalinensis*. The wehereabouts of the

former refugial population(s) of this group is not well studied, but it can be speculated that the area south of the Ural mountains may have housed benign conditions for the adder during the Weichsel glaciation. West of the Alps, another genetically distinct group of adders occur, which probably survived glacial times around the Massif Central region of France. The fourth group is found in central and North-Western Europe, including all of Scandinavia.

The ice age refugia populated by the ancestral adder lineages in the south and south-east neccesitate invoking alternative refugial areas for all the northern adder lineages. I suggest that European adders have survived the Weichselian glaciation in at least three separate cryptic northern refugia, although no hard evidence for their whereabouts are presented herein.

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I dedicate this thesis to the unfortunate subjects of my studies, with the intent that it was not for nothing, and that they remain ever-prescent at EBC to encourage the practice of noninvasive sampling techniques in the future.

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