

Postglacial recolonization in a cold climate specialist in western Europe: patterns of genetic diversity in the adder (*Vipera berus*) support the central–marginal hypothesis

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Abstract

Understanding the impact of postglacial recolonization on genetic diversity is essential in explaining current patterns of genetic variation. The central–marginal hypothesis (CMH) predicts a reduction in genetic diversity from the core of the distribution to peripheral populations, as well as reduced connectivity between peripheral populations. While the CMH has received considerable empirical support, its broad applicability is still debated and alternative hypotheses predict different spatial patterns of genetic diversity. Using microsatellite markers, we analysed the genetic diversity of the adder (*Vipera berus*) in western Europe to reconstruct postglacial recolonization. Approximate Bayesian Computation (ABC) analyses suggested a postglacial recolonization from two routes: a western route from the Atlantic Coast up to Belgium and a central route from the Massif Central to the Alps. This cold-adapted species likely used two isolated glacial refugia in southern France, in permafrost-free areas during the last glacial maximum. Adder populations further from putative glacial refugia had lower genetic diversity and reduced connectivity; therefore, our results support the predictions of the CMH. Our study also illustrates the utility of highly variable nuclear markers, such as microsatellites, and ABC to test competing recolonization hypotheses.

Keywords: Approximate Bayesian Computation, glacial refugia, microsatellite, population genetic structure, snake

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Introduction

Current genetic characteristics of populations are related to intrinsic population parameters, such as population size, but are also strongly influenced by historical events. The various combinations of genetic drift, gene flow and natural selection can lead to population divergence, and these processes are strongly dependent upon population size and spatial distribution (Allendorf & Luikart 2007).

According to the central–marginal hypothesis (CMH) (Carson 1959; Sagarin & Gaines 2002), within-species genetic diversity should be higher in past glacial refugia compared to more recently established peripheral populations. The loss of genetic diversity in peripheral populations could be due to progressive loss at the colonization front following subsequent colonization events or could be due to demographic effects (Robuchon *et al.* 2014). Moreover, according to the CMH, effective population size and gene flow should be lower in peripheral populations compared to populations located close to the glacial refugium.

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Numerous studies have examined the CMH in plants (Comps *et al.* 2001; Lonn & Prentice 2002; Pfeifer *et al.* 2009; Jadwiszczak *et al.* 2011; Pouget *et al.* 2013), mammals (Heckel *et al.* 2005), reptiles (Böhme *et al.* 2007; Howes & Loughheed 2008), amphibians (Palo *et al.* 2003; Garner *et al.* 2004) and invertebrates (Cassel & Tammaru 2003). Eckert *et al.* (2008) compiled 134 publications on plants and animals that compared genetic diversity in core and peripheral populations and found a decline in diversity towards the periphery in 64% of them. Given this mixed support, the broad applicability of the CMH is debated (Soulé 1973; Brussard 1984; Saffriel *et al.* 1994; Lesica & Allendorf 1995; Barton 2001; Guo 2012) and alternative hypotheses have been proposed. For instance, Fisher (1930) suggested that the environmental conditions are more stable in the distribution core while peripheral populations occur mainly in suboptimal habitats where they face more variable conditions (Gullberg *et al.* 1998). Fisher's hypothesis predicts that genetic diversity should increase from core to peripheral populations. Kark *et al.* (2008) suggested a nonlinear association between genetic diversity and distance from the core of the distribution, with the highest genetic diversity predicted to occur in populations situated between the core and the periphery of the range. Kark *et al.* (2008) also suggested that apparent support for the CMH may arise because of a common failure to sample intermediate populations.

Understanding how genetic diversity is distributed between peripheral and core populations also has clear implications for conservation (Parmesan & Yohe 2003; Hampe & Petit 2005). In the context of climate change, for instance, populations located at the expanding margin of the range ('the leading edge') are of prime interest because they provide individuals capable of colonizing new, favourable habitats (Hewitt 2000; Gibson *et al.* 2009; Cassel-Lundhagen 2010; Diekmann & Serrão 2012). Meanwhile, rear-edge populations are not always given appropriate consideration (Hampe & Petit 2005) despite their potentially high conservation value (Diekmann & Serrão 2012). Indeed, rear-edge populations should harbour high genetic diversity, but they are imperiled due to climate change (Hampe & Petit 2005).

The effect of climate change on populations should be especially acute in ectotherms, such as squamates, because their biology is much more tightly linked to temperature than that of endotherms (Buckley *et al.* 2012). In Europe, vipers of the genus *Vipera* have parapatric distributions (Saint Girons 1980; Brito & Crespo 2002; Scali *et al.* 2011; Tarroso *et al.* 2014), probably reflecting different climatic niches. Parapatry appears to be a result of interspecific differences in thermoregulatory behaviour and physiology, rather than a consequence of competition (Guillon 2012; Lourdaïs *et al.*

2013; Guillon *et al.* 2014) notably between the common adder (*Vipera berus*, L. 1758) and the asp viper (*Vipera aspis*, L. 1758).

The common adder is an excellent model to study recolonization events and to test current hypotheses about genetic diversity in peripheral populations. This species has particularly low dispersal abilities, and populations separated by only a few kilometres are significantly differentiated (Ursenbacher *et al.* 2009). The common adder is a cold-adapted species with a large boreal distribution (probably the largest for a terrestrial snake; Saint Girons 1980) reaching high latitude (>60°N) and the permafrost seems to limit its distribution (Nilson *et al.* 2005; Levavasasseur *et al.* 2011). The range-wide mitochondrial DNA phylogeography of the adder indicated three major clades: one in the Balkan peninsula, one in the Italian peninsula and one with a large distribution from France to Sakhalin Island (Kalyabina-Hauf *et al.* 2004; Ursenbacher *et al.* 2006). The latter clade shows marked genetic structure, with several subclades for which the location of glacial refugia remains unclear. Westernmost adders belong to the western subclade (Ursenbacher *et al.* 2006), which is limited to France, western and central Switzerland, Belgium, westernmost Austria and southwestern Germany. During the LGM (26–200 kya), however, the current distribution of this clade was covered by ice sheets (the Alps, the Jura Mountains and the top of the Massif Central; Van Vliet-Lanoë & Hallegouët 2001) or with permafrost (Van Vliet-Lanoë & Hallegouët 2001; Clark *et al.* 2009). During the LGM, only two areas free of ice or permafrost remained in France: (i) south of the Alps and the Massif Central (near Montpellier and Marseille) and (ii) the southern part of the Atlantic Coast (near Bordeaux; Fig. 1).

Here, we use species-specific highly variable markers (microsatellites; Ursenbacher *et al.* 2009) to describe patterns of genetic diversity within the western subclade of *V. berus*. First, we determined whether the genetic data are consistent with the use of one or both of the putative glacial refugia identified based on geological evidence. Then, we used the genetic data to reconstruct postglacial recolonization routes and to test the predictions of the CMH. Specifically, we tested (i) for a decrease in genetic diversity from populations located close to glacial refugia to peripheral populations and (ii) for a decrease in genetic connectivity from populations located close to glacial refugia to peripheral populations.

Materials and methods

Sampling and DNA extraction

We estimated genetic diversity in 32 populations (602 adders, between 5 and 30 individuals per population)

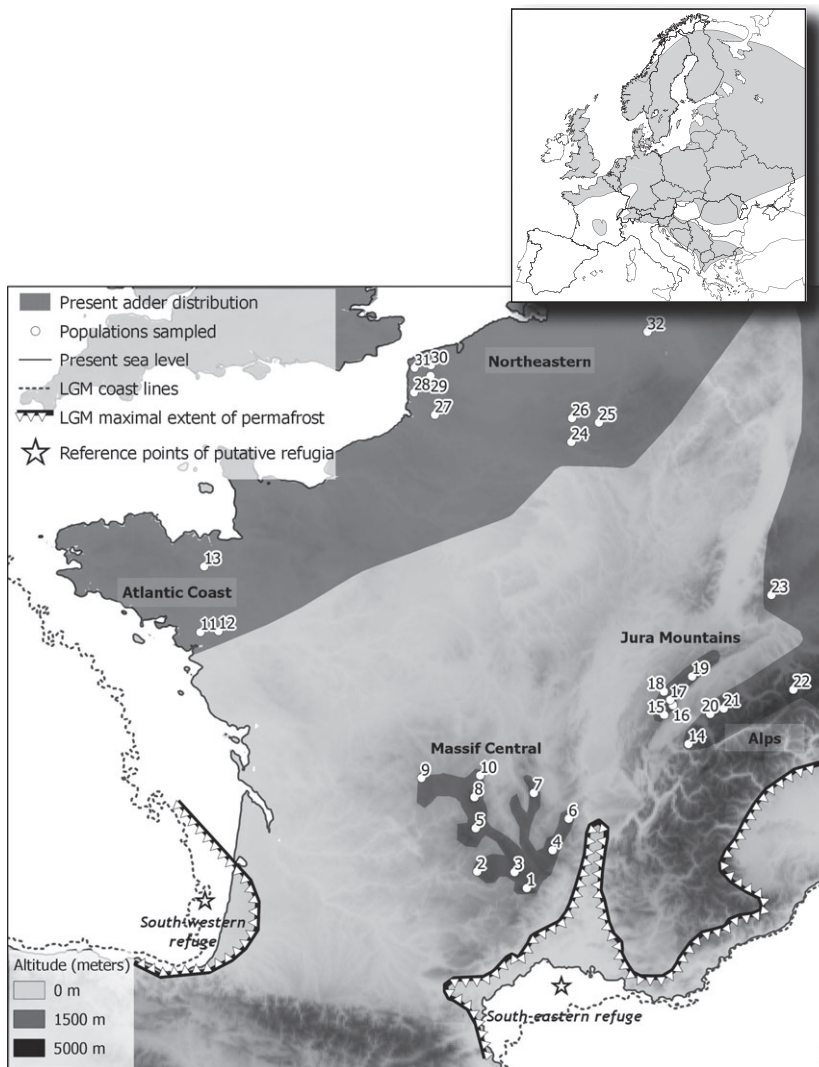


Fig. 1 Distribution of the sampled populations of *Vipera berus*. Population codes correspond to Table 1. Current adder distribution (dark area), last glacial maximum (LGM) extent of permafrost (lines with triangles) and last glacial maximum coast lines (dotted lines) are also displayed following Van Vliet-Lanoë *et al.* (2004). The two stars represent the reference locations used for both putative glacial refugia.

originating from France, Switzerland, Germany, Belgium and the Netherlands (see Table 1 and Fig. 1). Populations were grouped in five regions (Massif Central, Atlantic Coast, Jura Mountains, Alps, Northeastern) corresponding to geographic affinities. DNA was obtained from samples of blood, scales, skin or from buccal swabs. Total DNA was extracted using QIAamp DNA Mini Kits (Qiagen).

Microsatellite amplification

We estimated genetic diversity using nine microsatellite makers (Vb-A8, Vb-A11, Vb-B'2, Vb-B'9, Vb-B10, Vb-B'10, Vb-B18, Vb-D'10 and Vb-D17; see Ursenbacher *et al.* 2009) specifically developed for the adder. For populations 13 and 15–22, most individuals were already genotyped as part of a previous study (Ursenbacher *et al.* 2009). The additional samples were amplified in 10 μ L reactions following Ursenbacher *et al.*

(2009), microsatellite PCR products were run on an ABI 3130xl automated sequencer, and allele sizes were determined using the program PEAKSCANNER 3.1 (Applied Biosystems). Because genotyping methodologies were not exactly the same among all samples, several individuals were analysed using both methodologies, and conversion tables were produced for each primer.

Genetic analyses

To avoid analytical problems due to null alleles, we selected only the markers for which the absence of null alleles was confirmed by Ursenbacher *et al.* (2009). We nevertheless tested for the presence of null alleles in each population using MICROCHECKER 2.2.3 (Van Oosterhout *et al.* 2004). In addition, we tested for linkage disequilibrium among loci with FSTAT 2.9.3 (Goudet 1995). Allele frequency, allelic richness (A_r) and expected heterozygosity (H_E) were estimated using FSTAT. The het-

Table 1 Genetic diversity for 32 *Vipera berus* populations based on six microsatellite markers with standard error; n = sample size; H_E = expected heterozygosity; Ar_r = allelic richness (based on five diploid individuals); Mean F_{ST} = mean fixation index. Populations with particular history (see text) that were not used to evaluate the regional genetic parameters are indicated in *italic*. The regional allelic richness (based on 49 diploid individuals) is presented in the last column. All metrics have been calculated using FSTAT (Goudet 1995)

Population number	Region	Country	n	Ar	H_E	Mean F_{ST}	Regional Ar
1	Massif Central	France	17	3.36	0.63	0.302	
2	Massif Central	France	9	4.02	0.63	0.230	
3	Massif Central	France	17	4.14	0.68	0.232	
4	Massif Central	France	16	3.83	0.68	0.252	
5	Massif Central	France	21	3.74	0.62	0.244	
6	Massif Central	France	19	2.94	0.46	0.339	
7	Massif Central	France	15	3.82	0.69	0.252	
8	Massif Central	France	18	3.44	0.60	0.255	
9	Massif Central	France	18	3.89	0.61	0.239	
10	Massif Central	France	16	3.03	0.55	0.252	
	Massif Central			3.62 ± 0.13	0.61 ± 0.02	0.260 ± 0.011	10.03 ± 2.51
11	Atlantic Coast	France	17	3.96	0.68	0.252	
12	Atlantic Coast	France	18	4.23	0.68	0.228	
13	Atlantic Coast	France	20	3.99	0.65	0.223	
	Atlantic Coast			4.06 ± 0.08	0.67 ± 0.01	0.234 ± 0.008	8.76 ± 2.95
15	Jura Mountains	Switzerland	25	2.78	0.39	0.367	
16	Jura Mountains	Switzerland	30	3.11	0.54	0.309	
17	Jura Mountains	Switzerland	14	2.43	0.44	0.371	
18	Jura Mountains	Switzerland	24	3.32	0.54	0.293	
19	Jura Mountains	Switzerland	28	1.85	0.29	0.434	
23	Jura Mountains	Germany	21	1.35	0.09	0.605	
	Jura Mountains			2.70 ± 0.26	0.44 ± 0.05	0.355 ± 0.025	6.81 ± 2.48
14	Alps	France	8	1.10	0.02	0.534	
20	Alps	Switzerland	30	2.39	0.40	0.410	
21	Alps	Switzerland	23	2.59	0.44	0.371	
22	Alps	Switzerland	20	2.73	0.45	0.330	
	Alps			2.57 ± 0.10	0.43 ± 0.02	0.371 ± 0.023	6.46 ± 1.80
24	Northeastern	France	5	2.33	0.35	0.398	
25	Northeastern	Belgium	18	2.84	0.49	0.342	
26	Northeastern	Belgium	11	1.80	0.17	0.504	
27	Northeastern	France	16	2.47	0.42	0.397	
28	Northeastern	France	17	3.43	0.61	0.276	
29	Northeastern	France	18	2.57	0.46	0.351	
30	Northeastern	France	21	2.37	0.36	0.414	
31	Northeastern	France	23	1.82	0.26	0.486	
32	Northeastern	the Netherlands	29	2.51	0.35	0.435	
	Northeastern			2.46 ± 0.19	0.39 ± 0.05	0.396 ± 0.027	7.12 ± 1.95

erozygote deficit within populations (F_{IS}) and genetic differentiation between populations (F_{ST}) were calculated and tested for significant departure from zero by permutation (1000 replicates) with FSTAT. We tested for possible bottlenecks within populations with restricted genetic diversity using BOTTLENECK (Cornuet & Luikart 1996; Piry *et al.* 1999) with settings (TPM model, with 95% of SMM; variance = 12) suggested by Piry *et al.* (1999). Regional genetic diversity (Ar) was also evaluated for the five geographical regions using FSTAT. Proportions of the total genetic variation present within and among regions were calculated using AMOVA in GEN-

ALEX 6.4 (Peakall & Smouse 2006) with 999 replications. Genetic distances between pairs of populations were calculated using Cavalli-Sforza and Edwards D_c distance (Cavalli-Sforza & Edwards 1967), and phylogenetic reconstruction was conducted using the program POPULATIONS 1.2.28 (Langella 1999). According to Takezaki & Nei (1996), the D_c measure of genetic distance is one of the most appropriate to reconstruct phylogenetic relationships among populations. Population structure was inferred using model-based clustering methods with multilocus genotype data with STRUCTURE 2.3.3 (Pritchard *et al.* 2000). A nonadmixture model was used

because our study populations are isolated and 10 independent runs for K between 1 and 32 with 600 000 iterations (200 000 removed as burnin) were performed. To determine whether the geographical distance between current populations and putative glacial refugia has an impact on genetic diversity (Ar), we used a linear regression in SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Testing recolonization scenarios

We used approximate Bayesian computation (ABC) to test for different recolonization scenarios. The principle of ABC is to obtain the posterior distribution of the parameters using simulation under a defined demographic model (Beaumont *et al.* 2002; Beaumont 2010). ABC methods avoid the exact likelihood calculations by evaluating summary statistics (Csillery *et al.* 2010). Genetic data sets were simulated from defined priors, such as effective population size distributions, and microsatellite mutation rate and timing distributions. We first conducted a rejection step, which consisted in accepting only the simulations for which the summary statistics were close to the ones calculated from the data set. As summary statistics, we retained the mean number of alleles, the mean gene diversity, the mean size variance, the mean M index (Garza & Williamson 2001) across loci, and, between two samples, the F_{ST} , the mean index of classification, the $(d\mu)^2$ distance, the mean number of alleles, the mean gene diversity, the mean allele size variance and the shared allele distance. All the computations were carried out using DIYABC 1.0.4.46 (Cornuet *et al.* 2010), and the calculations for the different indices are explained in the manual. After selection of the most appropriate priors, posterior probabilities of scenarios were obtained with a logistic regression. Test simulations were run with 10^6 iterations, whereas 4×10^6 iterations were conducted for the final simulation. Four scenarios were tested (Fig. 2): a simultaneous split except for the Alpine and Jura Mountains regions (scenario 1); a northern colonization route (grouping the Atlantic Coast and the Northeastern regions) and a southern colonization route with the Massif Central as the first isolated (source) region (scenario 2); a simultaneous split among the Atlantic Coast, the Northeastern and the southern (Massif Central, Alps and Jura Mountains) regions (scenario 3); and a simultaneous split among the five regions (scenario 4). The total size of the adder population was considered constant for all the analyses. The population sizes for each population were assumed to equal the effective population sizes (N_e). We used a generation time of 8 years based on the equation $T = \alpha + [s/1(1 - s)]$ defined by Lande *et al.* (2003) where α is the age at maturity (4 years: Nilson *et al.* 2005) and s is the annual survival

rate (0.80; the mean from Prestt 1971; Forsman 1993 and Phelps 2004). It is however possible that the generation time was longer during the LGM as is currently the case for high altitude populations (Nilson *et al.* 2005).

Results

Null alleles and genetic diversity

No loci were in linkage disequilibrium. Null alleles were detected in several populations of the Massif Central and of the Atlantic Coast regions for the loci Vb-B10 and Vb-B18 and for Vb-B'2 in the Massif Central region only. We consequently removed these three loci from further analyses.

Genetic diversity for each population is presented in Table 1. Differences in allelic richness (based on five diploid individuals) were found among populations on the Atlantic Coast (mean: 4.06 ± 0.08), in the Massif Central (3.62 ± 0.13), in the Jura Mountains (2.70 ± 0.26 —without population 23—see below), in the Alps (2.57 ± 0.10 —without population 14—see below) and in the Northeastern region (France and Belgium: 2.46 ± 0.19 —without population 32—see below) (ANOVA_{4,181} = 10.09; $P < 0.001$). Post hoc Tukey tests indicated that Atlantic Coast and Massif Central populations had significantly higher allelic richness than the other three regions. We found similar results for the expected heterozygosity (ANOVA_{4, 181} = 11.74; $P < 0.001$). Very low genetic diversity was found in populations 14 and 23 ($Ar = 1.10$ and 1.35 , respectively). Regional genetic diversity was not significantly different among the different geographical regions (ANOVA_{4,25} = 0.40; $P = 0.81$). Significant intrapopulation structure (F_{IS}) was only found in population 1. Recent bottlenecks were detected in population 23 (Wilcoxon test, $P = 0.031$), whereas no information was available for population 14 due to the presence of a single polymorphic locus.

Population differentiation and affinities

We found high genetic differentiation among most populations (mean F_{ST} value: 0.34 ± 0.16 ; min = 0.02; max = 0.87), most (94%) being significant. Most nonsignificant F_{ST} values involved populations 2 and 24 because of the limited number of samples from these populations ($N = 9$ and $N = 5$, respectively). Mean F_{ST} values were smaller for the Massif Central (0.260) and the Atlantic Coast regions (0.234), compared to the Jura Mountains (0.355), the Alps (0.371) and the Northwestern region (0.401; Table 1). The AMOVA indicated that 57% of the genetic variation was distributed within individuals, 10% among individuals, 25% among populations and 8% among geographical regions.

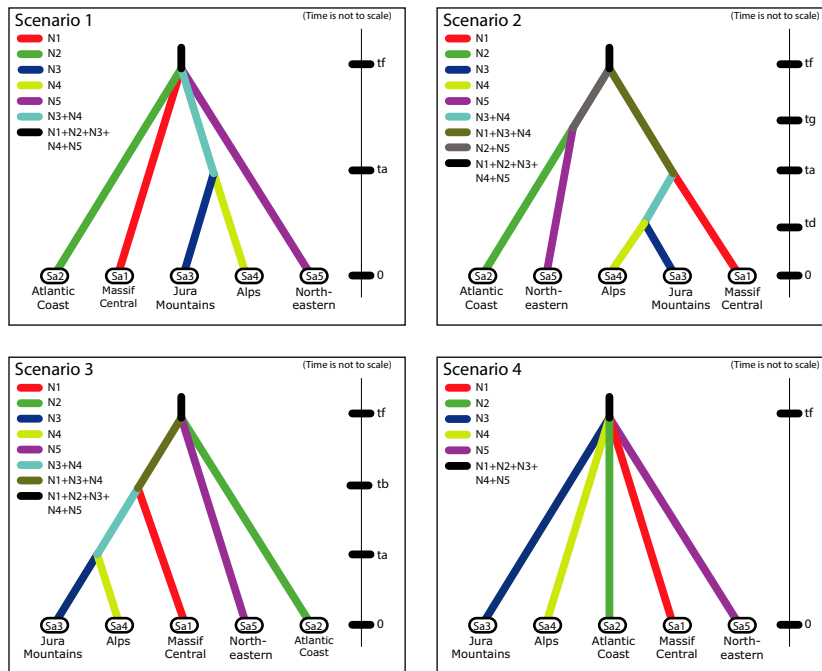


Fig. 2 Four demographic scenarios tested using approximate Bayesian computations (using DIYABC, Cornuet *et al.* 2010). Each region was considered as one unit, and the total population size was kept constant.

We were unable to determine an ideal number of population clusters based on the structure analyses, the posterior probabilities $L(K)$ being maximal for $K = 22$. Most populations belong to a single cluster (with a mean assignment value > 0.70), but populations 14 and 22 (Alps) grouped together and populations 24, 25 and 26 (Northeastern region: French and Belgian Ardennes) grouped together. Using the methodology developed by Evanno *et al.* (2005), the second-order difference (ΔK) suggested the occurrence of two groups, one comprising all populations from the Northeastern region and the other comprising all populations from the Jura region. Populations from others regions were split between both groups.

Population relationships

The genetic distances among all populations demonstrated the central position of the populations from the Massif Central (Fig. 3). Two groups were observed in this region, one in the southeastern part of this region (populations 1, 4, and 6, and part of population 3) and the other in the central, northern and western parts of this region. Populations of the Northeastern region, on the one hand, and from the Alps and Jura Mountains, on the other hand, formed two separate groups. Atlantic Coast populations appeared intermediate between Massif Central and Northeastern populations. Surprisingly, population 32 (from the Netherlands) is unrelated to the populations from the Northeastern region, but is more related to the populations from the Alps and Jura

Mountains. Suspicions of human introduction of this population, confirmed by our genetic data, lead us to remove this population from further analyses.

ABC analyses

Eight test simulations were run until we found priors yielding similar values between observed and simulated summary statistics. We ran an additional four simulations with slight modifications of a few priors, but these additional simulations failed to improve the results. The most likely scenario is scenario 2 (Fig. 2), with a posterior probability of 0.72 (95% CI: 0.59–0.85). The effective population sizes of the different regions were estimated between about 5000 individuals (Alps and Jura Mountains) to over 30 000 (Massif Central) (Table 2). The split between the central and northern colonization routes was estimated to have occurred 3720 generations ago, or 30 kya considering a generation time of 8 years. The split between the Massif Central populations and the Alps and Jura Mountains populations was estimated to have occurred about 2000 generations ago, or 15 kya. The most recent split between the Alps and the Jura Mountains populations occurred < 4 kya (Table 2).

Genetic diversity and distance to glacial refugia

As demonstrated by the ABC analyses, populations from Northeastern regions are more related to the Atlantic Coast populations (Northern group) and populations from the Alps and the Jura regions likely origi-

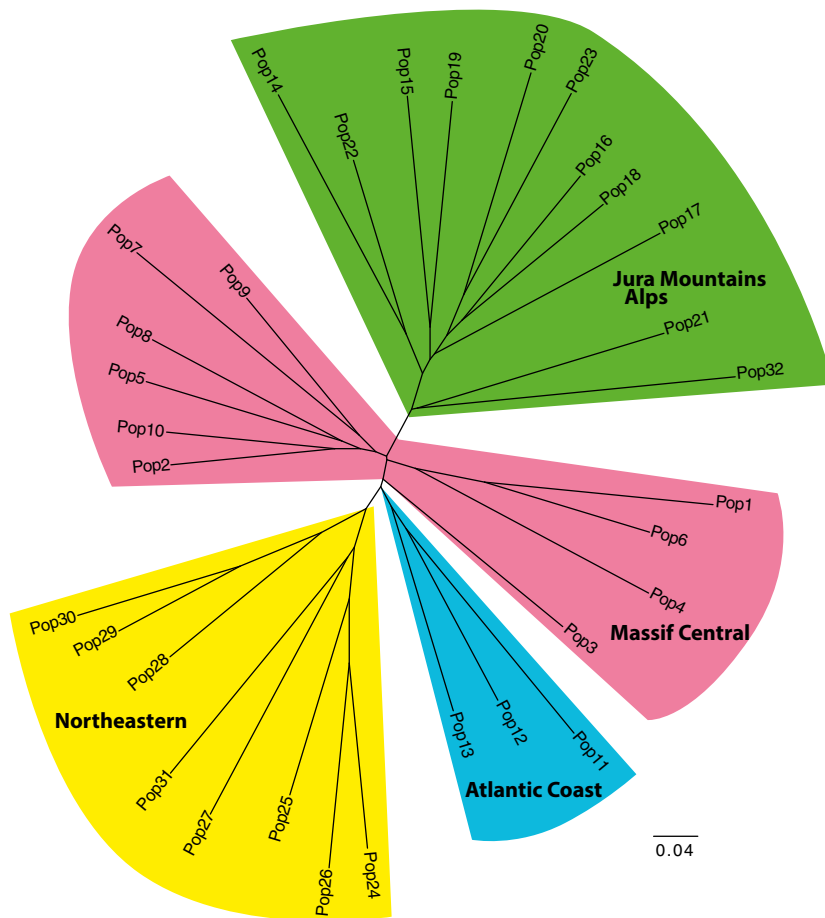


Fig. 3 Neighbour-joining tree calculated using the D_c genetic distance (Cavalli-Sforza & Edwards 1967) among the different populations. Each colour corresponds to one region, except for the Alps and the Jura Mountains that are mixed. Population codes correspond to Table 1.

nated from the Massif Central area (Central group). It appears that these two groups used unconnected glacial refugia in southwestern (Northern group) and southeastern (Central group) France (Fig. 1). We found strong negative relationships between allelic richness (A_r) and distance from each putative glacial refugium (Northern group: $r = -0.727$; $P < 0.001$; Central group: $r = -0.778$; $P < 0.001$; Fig. 4).

Discussion

We studied the genetic diversity of a boreal viper in western Europe using six microsatellite markers that did not show evidence of null alleles. We identified two distinct postglacial recolonization routes originating from two isolated glacial refugia. Adder populations closest to putative glacial refugia had the highest genetic diversity and, for both refugia, the distance to the refugium explained a large portion of the variation in genetic diversity observed in current populations. Overall, our results provide support for the CMH and illustrate the complexity of species distribution patterns during the last glacial maximum. Our study also sup-

ports the presence of 'cryptic' northern refugia for cold climate species, in addition to the main southern refugia in the three European peninsulas (Stewart & Lister 2001).

Glacial refugia and postglacial recolonization routes

Geological evidence suggests that two permafrost-free areas offered possible refugia in southwestern and in southeastern France during the LGM (Van Vliet-Lanoë & Hallegouët 2001). Even if several plants and invertebrates were able to survive in ice-covered regions using well-exposed rocky emergences during the LGM (nunataks; Stehlik *et al.* 2002; Haase *et al.* 2003; Holderegger & Thiel-Egenter 2009; Ursenbacher *et al.* 2010), several lines of evidence suggest that it was not the case for the adder. First, the current adder distribution clearly appears restricted in the north by ice cover and permafrost. Second, previous phylogeographic investigations failed to locate possible refugia in areas covered by ice or by permafrost during the LGM (Ursenbacher *et al.* 2006). Lastly, none of the small vertebrate prey consumed by the adder (i.e. small lizards, rodents see

Table 2 Estimations (with 95% confidence intervals) of the effective population size (N_e), the divergence time among the different regions and the mean mutation rate calculated using DIYABC 1.0.4.46 (Cornuet *et al.* 2010) based on 602 samples grouped in five geographical regions. The divergence times were calculated from the number of generations assuming a generation time of 8 years. Regions refer to Table 1 and split label to Fig. 2

	N_e	95% CI		
Massif Central	31 500	18 000–45 400		
Atlantic Coast	16 100	5600–36 100		
Jura Mountains	5090	1920–8600		
Alps	5140	2000–8640		
Northeastern	8030	5760–9700		
	Generations	95% CI	Years	95% CI
tf	3720	2130–4880	29 760	17 040–39 040
td	443	89–961	3544	712–7688
ta	1930	892–3160	15 440	7136–25 280
tg	2330	1060–3930	18 640	8480–31 440
Mean mutation rate	8.02×10^{-5}	3.46×10^{-5} – 1.56×10^{-4}		

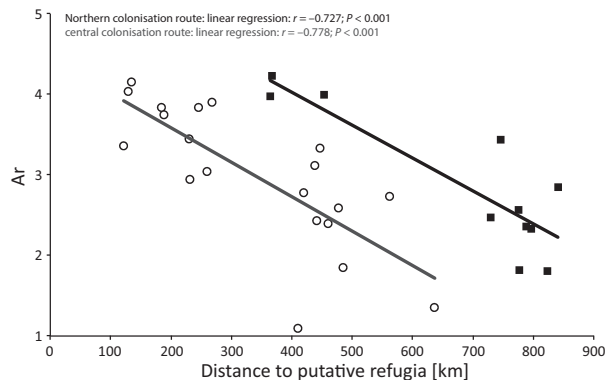


Fig. 4 Linear regressions between the allelic richness of each population (A_r) and the distance to the putative glacial refugia. The black line and plain squares are the northern colonization route (Atlantic Coast and Northeastern regions), whereas the grey line and the open circles correspond to the central colonization route (Massif Central, Alps and Jura Mountain regions). Linear regression: Northern group: A_r /distance to putative refugia: $r = -0.727$; $P < 0.001$; Central group: A_r /distance to putative refugia: $r = -0.778$; $P < 0.001$.

Luiselli *et al.* 1995; Monney 1995) seem to have survived in nunataks (Surget-Groba *et al.* 2001; Jaarola & Searle 2002). It is consequently more likely that the adder took refuge in permafrost-free areas during the LGM.

We observed significant differences in genetic diversity among our five study regions. Even if some populations, probably owing to unknown historical events, presented very low genetic diversity, most populations within a region exhibited similar genetic diversity. Similar patterns were detected for the mean F_{ST} values. Genetic diversity, genetic differentiation and ABC all strongly support the hypothesis that adders recolonized

western Europe from two distinct routes: one western route reaching first the French Atlantic Coast and later northern France and Belgium, and one eastern route reaching first the Massif Central and later the Alps and the Jura Mountains. The two recolonization routes started from two distinct refugial areas: the 'Atlantic Coast' for the western route and the 'edges of Massif Central' for the eastern route (Fig. 1).

Temperature has a crucial influence on the biology of ectotherms (Angilletta *et al.* 2002; Kearney & Porter 2009), and shifts in distribution are likely related to changes in temperatures. Our analyses indicated that the split between the two recolonization routes occurred about 30 kya, thus before the LGM. The timing of this split further supports the presence of two glacial refugia isolated during the LGM. On the other hand, the separation between the two glacial refugia (the Atlantic Coast and the edges of the Massif Central) and the peripheral populations (the Northeastern region and the Alps and Jura Mountains regions) occurred just after the LGM (about 15 kya). The timing of this last split suggests a rapid recolonization of the deglaciated areas by the adder, following the fast warming period that occurred at the end of the LGM (Clark *et al.* 2009). Finally, the differentiation between the Alps and Jura Mountains regions is more recent (4 kya), probably due to the more recent release of Alpine and Jura valleys by ice sheets (melting started approximately 16 kya, and this region was free of ice by about 13 kya, Blavoux 1988) and the short geographical distance between these two regions. At that time, the adder was consequently able to reach the lowland between the Alps and the Jura Mountains, and these two mountain ranges later. The Alps and Jura

Mountains populations were then likely separated following the continued temperature increase and possibly the arrival of the asp viper (Monney 1996). The timing of these splits is based on a generation time of 8 years. The age at maturity and the survival rate we used to calculate the generation time were derived from current populations occupying optimal habitat. It is possible that generation time was longer for populations in less optimal habitat, such as edge populations during recolonization. It is thus possible that we underestimated the timing of population splits.

Spatial patterns of genetic diversity support the central–marginal hypothesis

We found that genetic diversity in the adder decreased with increasing distance to the putative glacial refugia, which supports the prediction of the CMH (Carson 1959; Sagarin & Gaines 2002). Additionally, mean F_{ST} values were also higher in peripheral populations, indicating a reduced genetic connectivity in peripheral populations compared to populations located closer to the glacial refugia, which supports another prediction of the CMH. The literature review conducted by Eckert *et al.* (2008) revealed that 64% of the studies documented a reduction in genetic diversity between populations at the core of the distribution (which in our case corresponds to the populations located closer to the glacial refugia) and populations at the periphery, but the review also uncovered important variation among taxa. An alternative hypothesis developed by Kark *et al.* (2008) predicts the occurrence of a unimodal pattern of within-species genetic diversity. This latter hypothesis is very unlikely to be supported by our genetic data on the adder. Indeed, in our study, the relationships between genetic diversity and distance to the putative glacial refugia were linear and highly significant, even if we do not have complete historical geographical coverage because the adder moved northward and eastward for thermal reasons (Lourdais *et al.* 2013). More recently, Guo (2012) demonstrated that latitude is frequently responsible for some deviations from the predictions of the CMH, a possibility raised in the review by Eckert *et al.* (2008). In the case of the adder, the effect of the geographical distance to putative refugia could not be disentangled from the effect of latitude because the two were highly correlated ($r > 0.90$).

Peculiar results for some populations

Three populations (14, 23 and 32) had unexpected genetic diversities or affinities. Population 32 (from the Netherlands) is geographically close to other Northeastern populations, but the genetic distance suggests that

this population is more closely related to Alpine and Jura Mountains populations. It is likely that this population was introduced and some historical information supports this possibility (P. Janssen & A. J. W. Lenders, personal communication).

The lack of genetic diversity within the only known adder population in the French Alps (population 14) is surprising because this population is geographically the closest to glacial refugia. However, the ice cover disappeared only recently from the area occupied by this population (Darnault *et al.* 2012). The lack of diversity observed at most microsatellite markers in this population strongly suggests a considerable bottleneck (Hampe & Petit 2005) or a recent founder event, but we could not use statistical methods implemented in BOTTLENECK (Cornuet & Luikart 1996; Piry *et al.* 1999) or in MSVAR (Beaumont 1999) to distinguish between these possibilities due to the lack of genetic diversity (only one locus is not fixed). The location occupied by this population being particularly remote, a recent introduction seems unlikely. Additionally, other cold specialist species (*Salamandra atra* and *Picoides tridactylus*) have been found recently in the same area (J. F. Desmet, personal communication). Therefore, this population appears relictual and is likely vulnerable due to its restricted genetic diversity (see a similar example with the adder: Madsen *et al.* 1996, 1999). Population 23 from the Black Forest also exhibited limited genetic diversity, especially when compared to other Jura Mountains populations. A bottleneck signal was detected in this population, suggesting a strong reduction in population size, or a founder event, in the recent past.

Benefits of highly variable markers

The use of highly variable nuclear markers for phylogeographic studies is less developed than that of mtDNA, mainly due to the high polymorphism characteristic of nuclear markers and the corresponding need for large sample sizes. Nevertheless, nuclear markers have successfully been used to study phylogeography in plants (e.g. Alvarez *et al.* 2012; Garcia *et al.* 2012). Carlsson *et al.* (2004) identified the contact zone and hybridization in Fennoscandian adders using nuclear markers. In the current study, we demonstrated that, within a mitochondrial clade, the use of highly variable nuclear markers can provide precious information on postglacial recolonization and can improve phylogeographic resolution. Additionally, the recent development of ABC for phylogeographic studies allows to confront possible scenarios (especially for 'recent' events) and provides accurate timing of the splits. ABC analysis thus provides a refined way to test between competing phylogeographic hypotheses.

Conclusion

The adder is a cold-adapted species apparently limited by permafrost. In western Europe, the adder likely persisted in two isolated glacial refugia from which originated two postglacial recolonization routes. Consistent with the CMH, we found reduced genetic diversity and reduced genetic connectivity in populations located further from the putative glacial refugia. Further investigations should confront our phylogeographic results with the potential distribution of the species during the LGM. A complete reconstruction of postglacial recolonization by the adder could be obtained by combining genetic data (for instance, using SPLATCHE; Currat *et al.* 2004), even if the migration rate of the species is not known, and climatic niche modelling (given several scenarios of postglacial warming). Finally, our results underline the conservation importance of rear-edge adder populations (Massif Central and Atlantic Coast) that harbour significantly higher levels of genetic diversity and thus require specific conservation measures (Hampe & Petit 2005; Gibson *et al.* 2009; Zimmerman *et al.* 2010).

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S.U., M.G. and O.L. designed the experiment. M.G., H.C. and A.D. performed the field sampling, whereas A.D. and S.U. conducted the genetic analyses. S.U., M.G. and O.L. analysed the data and wrote the paper, and all authors contributed substantially to revisions.

Data accessibility

Complete microsatellite data: Dryad access: <http://dx.doi.org/10.5061/dryad.6rh72>. Population distance tree: Dryad access: <http://dx.doi.org/10.5061/dryad.6rh72>. Full data for the relationship between the

allelic richness and the distance between the current location of the populations and the distance to the putative refugia (in km): Dryad access: <http://dx.doi.org/10.5061/dryad.6rh72>.