High Genetic Differentiation Among French Populations of the Orsini's Viper (*Vipera ursinii ursinii*) Based on Mitochondrial and Microsatellite Data: Implications for Conservation Management

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Abstract

The Orsini's viper (*Vipera ursinii*) is one of the most threatened snakes in Europe due to its highly fragmented distribution and specific open environment (steppic habitat) requirement. French populations are isolated on top of mountain massifs of the southern Prealps/Alps. Mitochondrial sequences (cytochrome *b*) and 6 microsatellite loci have been used to estimate the levels of genetic diversity and isolation within and among 11 French fragmented populations (a total of 157 individuals). Eleven cytochrome *b* haplotypes with a limited divergence were observed (mean divergence between haplotypes: 0.31%). However, we detected considerable genetic differentiation among populations (global $F_{ST} = 0.76$ and 0.26 for mitochondrial and nuclear DNA, respectively). Results indicate that 3 populations possibly went through a bottleneck and 1 population showed low genetic diversity compared with the others. Although a significant isolation by distance was detected for both markers, strong differentiation was also observed between geographically close populations, probably due to the ragged landscape that constitutes a serious barrier to gene flow owing to the limited dispersal capability of the viper. Despite some discrepancies between the 2 markers, 8 Management Units have been identified and should be considered for future management projects.

Key words: conservation, cytochrome b, genetic structure, microsatellites, snake, Vipera ursinii ursinii

Changes in genetic diversity and population structure have potential implications for the persistence and viability of population or species both on the short and long term (Frankham et al. 2009). Because loss of diversity is often associated with inbreeding and reduction of reproductive fitness (Reed and Frankham 2001; Reed et al. 2003), increasing efforts are undertaken for monitoring genetic differences and preserving high levels of diversity in wild populations (Beebee and Rowe 2008). Recent molecular techniques allow conducting of noninvasive sampling (Beebee and Rowe 2008) in order to estimate population differentiation based on different types of genetic markers and define Management Units (MUs). According to Moritz (1994), if populations are reciprocally monophyletic for

mitochondrial DNA (mtDNA) haplotypes and show significant divergence of allele frequencies at nuclear loci, then these populations should be defined as separate MUs and managed independently. Genetic analyses therefore appear of primary importance for conservation planning of threatened species and are becoming increasingly common (see review in Vernesi et al. 2008).

Historical event(s) of natural and/or human origin can lead to habitat fragmentation, isolation of populations, and potentially to the recognition of distinct MUs. The genetic consequences of fragmented landscapes are well known (see review in Keyghobadi 2007). Fragmentation often leads to more isolation for populations (Bender et al. 1998) in decreasing habitat patch size and therefore enhances the threat of extinction for populations and species (Giordano et al. 2007; Noël et al. 2007). In small and fragmented populations, theoretical studies predict a high risk of genetic drift, which—associated with reduced gene flow—results in erosion of genetic diversity within populations and simultaneously increases differentiation among patches within the fragmented landscape (Young and Clarke 2000).

The Orsini's viper (*Vipera ursinii*) is probably the most threatened snake in Europe (Ujvari et al. 2002; Edgar and Bird 2006). The species is classified in the "Endangered species" category by International Union for Conservation of Nature (Baillie et al. 2004) and in the Annex I of the Washington convention. In Europe, the species is protected by the Berne Convention, the Habitat-Fauna-Flora directive, and several Life Nature programs. The very scattered distribution of the complex *V. ursinii* in Europe (Figure 1a, Penloup et al. 1998) represents the main threat of extinction for this taxa. In Western Europe (Italy and France) and in the Balkan Peninsula, *V. ursinii* inhabits alpine/subalpine dry meadow steppe, whereas the species occupies lowland meadows in Central and Eastern Europe (Nilson and Andrén 2001). In addition to rarity and endemism, the Orsini's viper accumulates numerous factors enhancing the risk of local extinction: a weak dispersal capability (Baron 1997), a slow growth rate, and a late sexual maturity (Baron et al. 1996). Moreover, rural land abandonment and the global warming both contribute to the expansion of unfavorable bushy environment (Luterbacher et al. 2004), which constitutes a supplementary threat. Indubitably, the evolutionary history of the Orsini's viper is tightly linked to the history of open environment and therefore to variations of the upper forest limits fluctuating with climatic conditions (Quézel and Médail 2003). Although most lowland populations decreased drastically (Edgar and Bird 2006), mountain populations are less endangered but strongly fragmented (Corbett 1989). In both habitats, local extinction of populations already occurred in several countries (Austria, Hungary, Romania, and France; Nilson and Andrén 2001), but the reasons are not fully understood. The high threat lying on the Orsini's viper has led to a recent increase in scientific studies focusing especially on ecological and conservation aspects (Baron et al. 1996; Ujvari et al. 2000; Krecsák and Zamfirescu 2001; Crnobrnja-Isailović 2002; Krecsák et al. 2003; Tomovic et al. 2004; Toth et al. 2005; Ujvari et al. 2005; Lyet et al. 2009). Genetic studies conducted in Hungary and Ukraine, on 2 related taxa (subspecies *V. ursinii rakosiensis* and species *Vipera renardi*, respectively), revealed a very limited genetic diversity (measured with mtDNA markers; Ujvari et al. 2002; Ujvari et al. 2005). Moreover, genetic reduction seems to affect dramatically individual and population survival in *V. ursinii* (Ujvari et al. 2002) as well as in the closely related taxon *V. berus* (Madsen et al. 1999; Madsen et al. 2004).

French populations (subspecies *V. ursinii ursinii*) constitute the westernmost limit of the distribution area of the species (Figure 1a) and occur in the southern Prealps/Alps at an altitude between 900 and 2150 meters (Penloup et al. 1998). The viper is not present in lowlands, and populations are thus confined to a limited number of mountain massifs of the French alpine region, surrounded by unfavorable forested valleys (Figure 1b). In this context, we defined a population as a site containing vipers from a homogeneous and unfragmented habitat. According to this definition, *V. u. ursinii* has been recorded in 17 populations (Figure 1b). However, the subspecies has not been found in 2 of them for 30 years and in one of them for 15 years, thus leading to the number of 14 confirmed populations. Several projects (the French "Plan National de restauration" [National Restoration Program], the LIFE project, etc.) were carried out since 1992 in order to improve knowledge about the distribution and the biology of the species in France (prospecting of potential new populations, capture-mark-recapture studies started in 2 populations).

Our study focused on the genetic impact of fragmentation in the French populations of *V. u. ursinii* and its implications for conservation strategies. Here, the genetic pattern of French populations is evaluated in combining both mitochondrial (cytochrome *b* sequences) and nuclear (microsatellites) markers. First, we compared current levels of genetic variability within 11 populations, and we determined past changes in population size using models based on the distribution of the current variability. Second, we evaluated the differentiation between sites in order to assess the degree of isolation among populations. Third, we delineated homogeneous genetic groups (MUs) that would be essential for short-term management and conservation of the French Orsini's viper populations. Finally, suggestions for conservation planning are provided in order to reduce the risk of extinction for these populations.

Materials and Methods

Population Sampling

The 14 populations presently recognized in southeastern France range from 25 to about 2500 ha. The minimum distance between individuals belonging to different populations was 2000 m, whereas samples from 1 population were separated by a maximum distance of 1000 m. We sampled between 7 and 20 *V. u. ursinii* originating from 11 of the 14 known populations (total number: 157 individuals, Table 1) as well as 1 sample from Italy (Grand Sasso). All
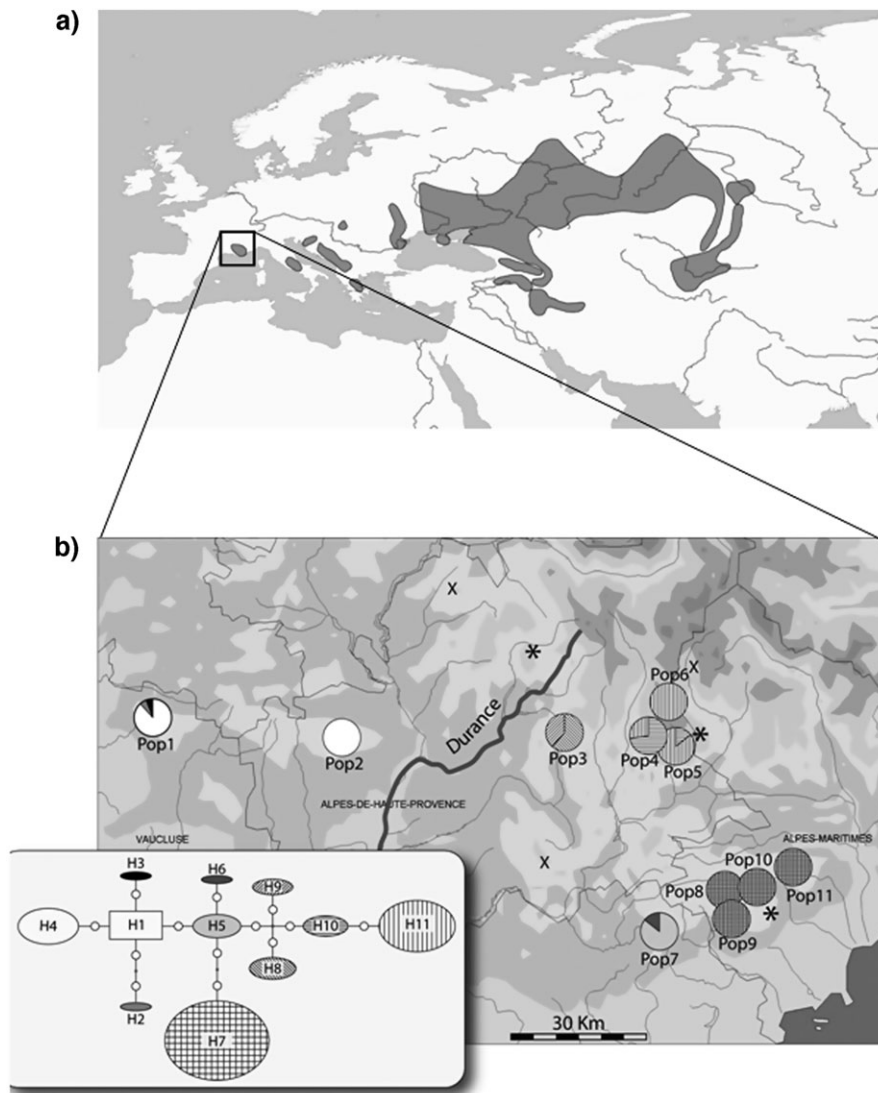
Figure 1. (**a**) Distribution of the taxonomic complex of *Vipera ursinii* around the world (Nilson and Andrén 2001). (**b**) Populations of the 17 southeastern French populations (*corresponds to unsampled populations and X to extinct populations) and distribution of the observed cytochrome *b* haplotypes (see Table 1). The different levels of gray refer to the relief: Light gray corresponds to lowlands and dark gray to alpine habitat. In the bottom left side: parsimony network between the 11 haplotypes and white dots represent substitutions. For each haplotype, the size is proportional to their observed frequencies.

captured individuals were sexed, measured, weighed, and marked through scale clipping. The exact location was also noted using a Global Position System (GPS) or an accurate map. Tissue samples (part of 2 ventral scales) were taken, and genomic DNA was subsequently extracted using QIAamp DNA Mini Kit (Qiagen) following the manufacturer's procedure.

Mitochondrial Analyses

The cytochrome *b* (cyt *b*; 1116 bp) was amplified and sequenced using the primers L14724Vb and H15914Vb (Ursenbacher et al. 2006). Sequences were obtained for all

French individuals, for the Italian sample and for 1 individual of the closely related taxon *V. eriwanensis* (Arpaçay, Turkey, Collection Laboratoire de Biogéographie et Écologie des Vertébrés BEV.8856), that has been used as outgroup. Similar results were obtained when 2 additional individuals of *V. berus* were included as outgroups (data not shown).

Polymerase chain reactions (PCR) were conducted in 40 μl reaction volume with the conditions described in Ursenbacher et al. (2006). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 or ABI Prism 3100 (Applied Biosystems). Electropherograms were read and

Table 1 Genetic diversity for the 11 sampled populations of *Vipera ursinii ursinii* in France (*N* = sample size, *f* = number of females, *m* = number of males, and *i* = number of individuals for which the sex is undetermined).

Localities	N (f/m/i)	Mitochondrial data					Microsatellite data					
		Haplotypes	π	F_S (P)	D (P)	Goodness-of-fit, P	H_O	H_E	A_R	F_{IS}	A_T	A_P
Loc1	17 (10/4/3)	H1, H2, H3	0.00032	−0.96 (0.10)	−1.71 (0.02)	0.20	0.422	0.495	3.02	0.149	23	3
Loc2	20 (9/5/6)	H4	0	—	—	—	0.253	0.327	2.07	0.228	15	0
Loc3	18 (16/2/0)	H8, H9	0.00090	2.85 (0.88)	1.78 (0.97)	<0.01	0.593	0.618	3.89	0.041	31	6
Loc4	11 (6/2/3)	H10, H11	0.00039	0.78 (0.44)	0.67 (0.91)	—	0.455	0.545	3.44	0.166	23	3
Loc5	14 (8/6/0)	H10, H11	0.00024	0.19 (0.28)	−0.34 (0.22)	0.17	0.369	0.387	2.76	0.045	19	0
Loc6	15 (7/5/3)	H11	0	—	—	—	0.467	0.447	2.31	−0.043	15	0
Loc7	14 (7/7/0)	H5, H6	0.00024	0.19 (0.32)	−0.34 (0.19)	0.06	0.595	0.526	3.32	−0.131	24	0
Loc8	10 (6/2/2)	H7	0	—	—	—	0.600	0.576	3.61	−0.042	23	1
Loc9	11 (4/0/7)	H7	0	—	—	—	0.455	0.470	3.79	0.032	26	0
Loc10	20 (16/3/1)	H7	0	—	—	—	0.617	0.582	3.94	−0.059	28	1
Loc11	7 (3/2/2)	H7	0	—	—	—	0.500	0.542	4.17	0.077	25	1
Global	157	H1–H11	0.00259	0.83 (0.68)	0.63 (0.78)	<0.01	0.484	0.501	5.07	—	252	15

Mitochondrial data: haplotypes = name of haplotypes (see Figure 1); π = nucleotide diversity; F_S = Fu's F_S; D = Tajima's D; P = probability of the test. Microsatellite data based on 6 microsatellite loci: H_O = observed heterozygosity; H_E = expected heterozygosity, A_R = allelic richness (based on 7 diploid individuals); F_{IS} = heterozygote deficit within populations, A_T = number of total alleles per population, and A_P = number of private alleles.

translated into amino acids to check for possible amplification of pseudogenes (Numt) using Sequence Navigator software (Applied Biosystems). No stop codons or unusual amino acid substitutions were detected, supporting the mitochondrial origin for the sequences obtained (GenBank accession numbers FM954982–FM955139 for *V. u. ursinii* and FN870958 for *V. eriwanensis*).

mtDNA sequences were aligned using the ED editor of the software Must (Philippe 1993). We used ModelTest (Posada and Crandall 1998) to determine the appropriate model of sequence evolution (model HKY with transition/transversion ratio = 3.34 and nucleotide frequencies A = 0.303, C = 0.317, G = 0.104, and T = 0.276). Four phylogenetic methods using different reconstruction principles were performed: maximum parsimony (MP), maximum likelihood (ML), neighbor-joining (NJ), and Bayesian inferences (BI). NJ and MP analyses were conducted using Paup* v4.10b (Swofford 2002), and ML analyses were carried out using Phyml v2.4.4 (Guindon and Gascuel 2003). In MP, ML, and NJ methods, robustness of nodes was assessed with 1000 bootstrap replications. Bayesian posterior probabilities were generated with MrBayes v3.0 (Huelsenbeck and Ronquist 2001) using 4 chains run for 10^6 iterations. Each chain was sampled every 50th generation with the first 200 000 iterations (2000 trees) discarded as burn-in. Two different runs were conducted in order to avoid possible local optima.

Relationships among unique haplotypes were described by a statistical parsimony network constructed with the program TCS1.21 (Clement et al. 2000). The hypothesis of population growth from low-diversified founder individuals was evaluated using pairwise mismatch distributions of substitution differences for each population and for the whole data set. Observed and expected distributions were tested for goodness-of-fit using parametric bootstrapping (10 000 replicates) with Arlequin v3.0 (Schneider et al. 2000). For the whole data set (except the Italian individual)

and each separate population, excess of rare alleles, indicating a recent expansion, was also tested with the Fu's F_S test (Fu 1997) and Tajima's D test (Tajima 1989). Nucleotide diversities as well as population differentiation (F_{ST}) were estimated using Arlequin. The unrooted NJ tree between the different localities was computed using Populations v1.2.28 (Langella 1999) based on the average number of pairwise differences between localities calculated by Arlequin. In addition, we used the Mantel's test (Mantel 1967) to assess the hypothesis of isolation by distance (IBD) by comparing geographic distances and F_{ST} values using Fstat 2.9.3 (Goudet 1995).

Microsatellite Analyses

So far, no specific microsatellite locus has been isolated for *V. ursinii*. We used already published microsatellite primers identified for a closely related species, *V. berus* (5.9% divergence as measured with the cytochrome *b*; Garrigues et al. 2005). Of the 19 microsatellites identified for *V. berus* (Carlsson et al. 2003; Ursenbacher et al. 2009), we selected 6 loci (Vb3, Vb64, Vb71, Vb-A11, Vb-A8, and Vb-D17) that did not show signs of null allele occurrence (see below). PCR amplifications were conducted in 25 µl volumes using the respective protocol for each locus. Microsatellite PCR products were analyzed on a CEQ 8000 Genetic Analysis System (Beckman Coulter).

Microsatellite data were checked for null alleles and mis-scoring with Micro-Cheker (Van Oosterhout et al. 2004) for each population. We estimated allele frequency, allelic richness (A_R), and observed and expected heterozygosity (H_O and H_E) using Fstat. Heterozygote deficit within population (F_{IS}) and exact tests for Hardy–Weinberg proportions for multiple alleles were conducted using Fstat with 1000 permutations. Linkage disequilibrium between loci was also examined for all couples of loci with Fstat. Genetic differentiation between populations was

investigated using Fstat to estimate *F*-statistics (Wright 1951). IBD was tested with Fstat by comparing pairwise geographic distances (log transformed) with pairwise (F_{ST}/ $[1 - F_{ST}]$) using a Mantel's test. This transformation is known to be more accurate in detecting IBD than F_{ST} versus geographic distance (Rousset 1997). An unrooted NJ tree between the different localities was computed using Populations v1.2.28 (Langella 1999) based on the distance calculated through the Cavalli-Sforza & Edwards Dc method (Cavalli-Sforza and Edwards 1967). Branch supports were based on 1000 random permutations.

To test for signs of bottleneck, we used the program Bottleneck (Cornuet and Luikart, 1996; Piry et al. 1999). The 2-phase model (TPM; di Rienzo et al. 1994) with parameters set to 95% of single-step mutations (SSM; Ohta and Kimura 1973) and a variance among multiple steps equal to 12 was chosen as suggested in Piry et al. (1999). To assess the robustness of these tests, additional simulations with different parameter values (80–99% of SSM, 2–24 of variance among multiple steps) were performed. Based on the number of loci used, the Wilcoxon test (Bauer 1972) was chosen to examine heterozygosity excess. In addition, the Bayesian approach implemented in Msvar (version 0.4.1b; Beaumont, 1999) was conducted to detect population declines and/or expansions using multilocus microsatellite genotypes. This program performs coalescent simulations to estimate posterior distributions of demographic parameters, such as the rate of population change $r = N_0/N_1$ (N_0 = current effective number of chromosomes and N_1 = number of chromosomes at the time of population decline or expansion). A value smaller than 1 for $\log_{10}(r)$ indicates a declining population. To check for stability, we ran 6 independent computations for each model of population using different configuration parameters and starting values. Each run lasted 2×10^9 iterations, with 20 000 sampling completed every 10 000 steps. For each run, results were analysed using Tracer v1.4 (Rambaut and Drummond 2007) demonstrated that the first 2–5% of the simulation were unstable, and thus, the first 10% of the samples were discarded as burn-in. Initial priors were defined as following: starting value of $r = 0.01$; starting value of $t_f = 4.0$; lower and upper bounds on respective theta, r and t_f: $-8/2$, $-6/1$, and $-4/2$. After preliminary simulations, some priors were modified according to the results.

Bayesian individual assignment approach as implemented in Structure v2.2.3 (Pritchard et al. 2000) was tested using a model of admixture with a number of clusters (*K*) ranging from 1 to 11. Likelihood values for 20 repetitions for each *K* value were estimated after 1.5×10^6 iterations (0.5×10^6 considered as burn-in). We also implemented the Evanno et al. (2005) method, which uses a second order rate of change (the ΔK value) to determine the most likely value of *K*. In addition, substructure was investigated with the same procedure (ML or ΔK values, respectively) using the groups of populations defined in the first structure analysis.

Statistical tests (analyses of variance [ANOVAs]) were conducted using SPSS 13.0 (SPSS Inc., Chicago, IL).

Results

Mitochondrial Analyses

The 157 French *V. u. ursinii* cyt *b* sequences provided 11 haplotypes without insertion and deletion. It is noticeable that 6 populations were characterized by a single haplotype (Pop2, H4; Pop6, H11; and Pop8-11, H7; Figure 1b), one of them (H7) being found in 4 populations (Pop8-11). The divergence between the 11 haplotypes was low (a maximum of 6 substitutions; mean divergence: 0.31%), and the differentiation intra- as well as interlocalities was also low (mean *P* distance: 0.21 ± 0.03% and 0.28 ± 0.15%, respectively). A different haplotype (H12) characterized the Italian sample but only limited divergence was observed between this sequence and French haplotypes (minimal divergence: 0.27%; mean divergence: 0.43%).

Phylogenetic relationships between haplotypes (Figure 2a) revealed a limited resolution, with the exception of a clade joining haplotypes H1–H4, characterizing Pop1 and Pop2. The sample of *V. u. ursinii* from Italy appears in a sister position to the in-group clade of the French samples with low support. The parsimony network between French haplotypes (Figure 1b) showed a similar genetic structure with few substitutions between haplotypes. The structure observed was highly correlated with the geography, related haplotypes belonging to neighboring populations (see e.g., H10 and H11 found in Pop4-6).

Despite a limited number of substitutions between haplotypes, F_{ST} values indicated a marked genetic differentiation of the localities, most comparisons between pairs being highly significant ($P < 0.005$; Table 2). Nonsignificant genetic differentiation was only observed between Pop4 and Pop6 ($P = 0.084$) and between Pop8–Pop11, which shared the same haplotype (Figure 1b). The high genetic differentiation was strengthened by a significant IBD (Mantel test: $r^2 = 0.245$; $P < 0.001$; Figure 3a). The unrooted NJ tree based on pairwise genetic distances between populations (Figure 2b) evidenced 3 main groups: Pop1 and Pop2, Pop4–Pop6, and Pop8–Pop11. Mismatch distributions performed for the whole data set and for each population did not reveal significant evidence of rapid growth from low-diversified founder population except in Pop1, for which Tajima's *D* tests was significant ($D = -1.71$; $P = 0.021$) although Fu's F_S was not significant ($F_S = -0.96$; $P = 0.10$; Table 1).

Microsatellite Analyses

Cross amplification of microsatellite markers developed for other species can often result in amplification difficulties, such as the lack of PCR products or high frequency of null alleles. Analyses of the 6 selected microsatellite loci did not present signs of null alleles in the French *V. u. ursinii* populations, except for Vb-D17 in Pop11 and for Vb-A11 in Pop2, where an excess of homozygotes was observed. However, tests for HW equilibrium did not show deviation from the expected frequencies for all populations. In
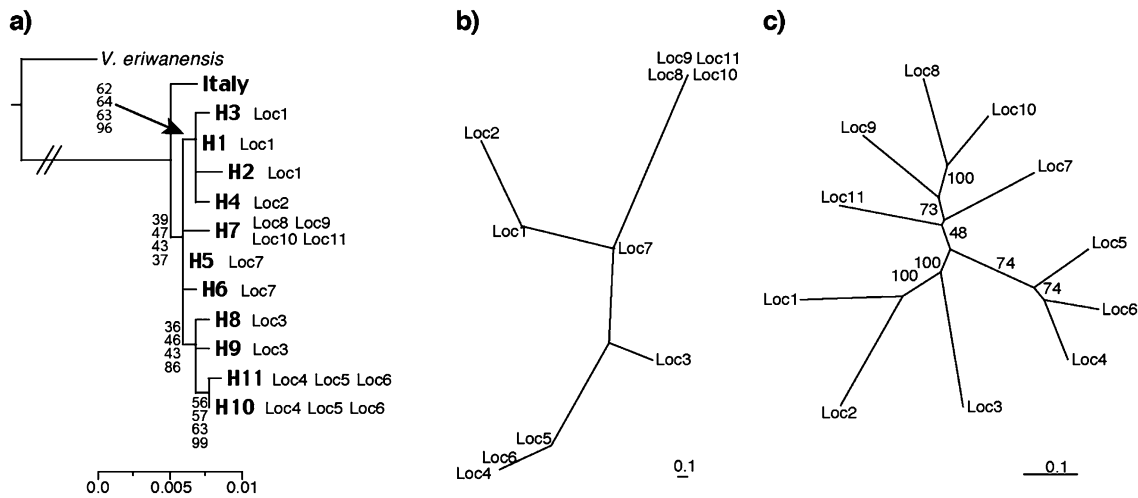
Figure 2. (a) ML phylogram derived from the cytochrome *b* (1116 bp) for the 11 French and 1 Italian haplotypes of *Vipera ursinii ursinii*. Values at nodes represent (from top to bottom) bootstrap support for NJ, MP, likelihood analyses, and Bayesian posterior probability (in %); (**b–c**) NJ trees from genetic distances between the 11 French populations of *V. u. ursinii* based on mitochondrial (**b**) and nuclear (**c**) markers. Mitochondrial distances were calculated as the average number of pairwise differences between populations. Microsatellite distances were evaluated using 6 loci with the Cavalli-Sforza & Edwards Dc distance, and values at nodes were obtained from 1000 random permutations calculated with POPULATIONS v1.2.28 (Langella 1999; see Materials and Methods for details).

addition, no linkage disequilibrium between loci was detected. Low genetic diversity (mean A_R = 3.30) was noted, especially in Pop2 (mean A_R = 2.07), where the observed heterozygosity was significantly lower than in the other localities (ANOVA: F = 4.74; P = 0.033). However, the expected heterozygosity (H_E) and the A_R were not significantly lower in this population compared with the other ones (H_E: F = 2.91, P = 0.093; A_R: F = 3.65, P = 0.061; Table 3). To a lesser extent, Pop4 and Pop6 also had lower genetic diversity (Table 1). Using Bottleneck, significant signs of bottleneck (heterozygosity excess) were identified in Pop4 (P = 0.047), Pop8 (P = 0.031), and Pop10 (P = 0.047), and by contrast, significant deficiency in heterozygosity was found for Pop11 (P = 0.039). Similar

results were obtained with different parameters (variance from 2 to 24 and 80–99% of SSM mutation model in TPM model). With Msvar, $\log_{10}(r)$ was lower than 1 (i.e., population decline) in most populations, and the most significant reductions of population size were detected in Pop1, Pop 2, and Pop11 for which the present size is estimated to be 1.5×10^{-7}, 3.4×10^{-7}, and 1.4×10^{-6} of the historical size, respectively.

F-statistics demonstrated that only 1 couple of sites (Pop4 and Pop5) did not show significant genetic divergence (Table 2). The highest mean F_{ST} value was observed for Pop2, which was significantly higher than other localities (ANOVA: F = 41.0; P < 0.001). Significant IBD was detected within the region studied (r^2 = 0.380;

Table 2 Pairwise F_{ST} estimates for 6 microsatellite loci (above the diagonal) and the cytochrome *b* (under the diagonal) between 11 French populations of *Vipera ursinii ursinii*

	Loc1	Loc2	Loc3	Loc4	Loc5	Loc6	Loc7	Loc8	Loc9	Loc10	Loc11
Loc1		0.312	0.252	0.412	0.347	0.427	0.384	0.376	0.437	0.358	0.307
Loc2	0.861		0.411	0.577	0.520	0.582	0.470	0.455	0.574	0.415	0.485
Loc3	0.783	0.880		0.281	0.206	0.334	0.230	0.280	0.255	0.218	0.228
Loc4	0.955	1.000	0.819		0.024 (NS)	0.133	0.317	0.349	0.301	0.331	0.289
Loc5	0.888	0.964	0.660	0.739		0.163	0.235	0.294	0.238	0.282	0.191**
Loc6	0.922	0.978	0.764	0.084 (NS)			0.374**	0.377	0.325	0.372	0.320**
Loc7	0.764	0.949	0.687	0.960	0.481*			0.153	0.154	0.136	0.102**
Loc8	0.930	1.000	0.841	1.000	0.859	0.912	0.928		0.186	0.041	0.207**
Loc9	0.932	1.000	0.846	1.000	0.946	0.968	0.931	0 (NS)		0.180	0.149**
Loc10	0.949	1.000	0.880	1.000	0.949	0.969	0.949	0 (NS)	0 (NS)		0.198
Loc11	0.921	1.000	0.823	1.000	0.964	0.978	0.917	0 (NS)	0 (NS)	0 (NS)	
Mean microsatellites	0.361	0.480	0.269	0.301	0.250	0.340	0.255	0.272	0.280	0.253	0.248
Mean mtDNA	0.891	0.963	0.798	0.856	0.839	0.802	0.886	0.661	0.663	0.672	0.656

Mean pairwise F_{ST} is calculated for both markers. Significance was determined by 55 000 permutations. NS, not significant; P > 0.05; *P < 0.05; **P < 0.01; for all other pairwise estimates, P < 0.005.
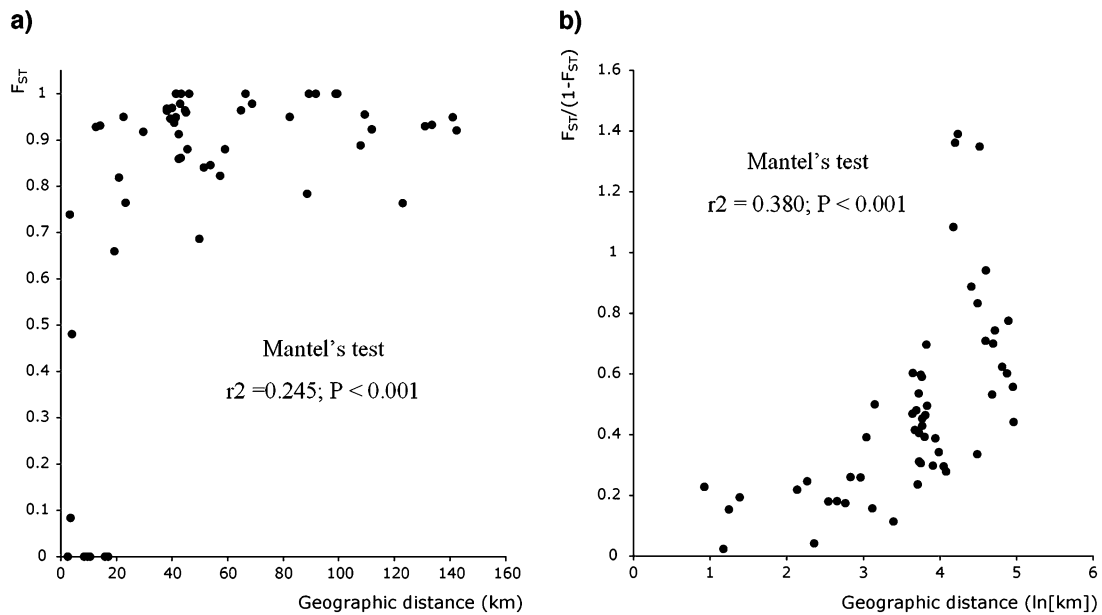
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Figure 3. IBD observed between French *Vipera ursinii ursinii* populations using (**a**) the divergence between the mitochondrial cytochrome *b* (1116 bp; Mantel test: $r^2 = 0.245$; $P < 0.001$) and (**b**) 6 microsatellite markers (Mantel test: $r^2 = 0.380$; $P < 0.001$). We used the uncorrected distance for the mitochondrial analysis due to some $F_{ST} = 1$ (with the correction $F_{ST}/[1 − F_{ST}] = \infty$).

$P < 0.001$; Figure 3b). However, pairs of nearby locations (<4 km, i.e., ln(4) = 1.39 in Figure 3b) presented high genetic divergence (Pop4-Pop6, Pop5-Pop6, and Pop8-Pop9), whereas Pop8 and Pop10 were genetically close although they are more than 10 km (i.e., ln(10) = 2.30 in Figure 3b) far from each other. Graphical representation of genetic differentiation between localities (Figure 2c) showed strong grouping between (Pop1 + Pop2) + Pop3 and Pop8 + Pop10. Weaker groupings were obtained between (Pop8 + Pop10) + Pop9 and (Pop4 + Pop6) + Pop5.

The number of genetically homogeneous groups was also assessed using the Structure software. The ML value was observed for $K = 7$ (Pop1/Pop2/Pop3/Pop4 + Pop5 + Pop6/Pop7+ most individuals of Pop11/Pop8 + Pop10/ Pop9 + some individuals of Pop11; Figure 4a,b). Calculation of ΔK (Evanno et al. 2005) from the structure output produced a modal value of the statistic for $K = 3$ (Pop1 + Pop2/Pop4 + Pop5 + Pop6/Pop3 + Pop7 + Pop8 + Pop9 + Pop10 + Pop11; Figure 4a). Although the highest value of ΔK was obtained for $K = 3$, a second mode was observed for $K = 7$ (Figure 4a). The height of the modal values of ΔK indicates the strength of the population subdivision signal (Evanno et al. 2005), here suggesting a deep subdivision for $K = 3$ and a less pronounced differentiation for $K = 7$. In fact, when similar simulations were run on the 3 groups observed for $K = 3$, subdivision of populations was still detected in the first (Pop1/Pop2) and the third group (Pop3/Pop8 + Pop10/Pop7 and some individuals of Pop11/Pop9 and Pop11). Thus, when the substructure was analyzed, the Evanno et al. (2005) method gave the same assignments as the ML method.

Discussion

Limited Genetic Diversity or Not?

The evaluation of genetic diversity is of major concern for threatened species. In addition, comparisons with other sister populations/species are required in order to compare current levels of genetic diversity among populations and species. For the Orsini's viper, mitochondrial as well as nuclear markers revealed a quite limited genetic diversity in French populations (mean divergence between cyt *b* haplotypes: 0.31%). Analysis of the mitochondrial control region sequences of 2 related taxa showed a lack of genetic variation within 3 specimens from a single Hungarian population of *V. u. rakosiensis* and low variation for 8 specimens from 2 Ukrainian *V. renardi* populations (Ujvari et al. 2005). Moreover, based on nuclear markers under selection (major histocompatibility loci [MHC]), the same authors found very low or no polymorphism in Hungarian populations, whereas the 2 Ukrainian populations showed high MHC diversity (Ujvari et al. 2002). This discrepancy between nuclear and mitochondrial variability in the Ukrainian vipers suggested a rather slow Control Region evolution rate. This observation has been documented in numerous snakes or birds, probably resulting from the occurrence of at least 2 CR copies (Questiau et al. 1998; Ashton and de Queiroz 2001; Randi et al. 2001; Ursenbacher et al. 2006). Limited genetic variability was also observed within populations of *V. berus* for 2 mitochondrial markers (cyt *b* and CR; Ursenbacher et al. 2006) as well as for 13 microsatellites (Ursenbacher et al. 2009). These results suggest that the low diversity

Table 3 Characteristics of microsatellites in *Vipera ursinii ursinii* with the total number of private alleles at the population level

Locus	Size range (di- or trinucleotide)	Number of alleles	H_O	H_s	H_t	Number of private alleles
Vb-A11	142–176 (di)	11	0.537	0.553	0.791	4
Vb-A8	164–188 (di)	11	0.605	0.645	0.831	2
Vb-D17	216–269 (tri)	17	0.658	0.718	0.873	4
Vb3	157–183 (di)	12	0.601	0.614	0.846	1
Vb64	247–255 (di)	3	0.242	0.227	0.447	1
Vb71	121–161 (di)	6	0.261	0.25	0.461	3

H_O = observed heterozygosity for all individuals, H_s = mean heterozygosity on all populations, and H_t = total heterozygosity for all individuals.

measured with mitochondrial and nuclear markers found in French populations of *V. u. ursinii* might be frequent in vipers.

Our results regarding population reductions were inconclusive. Bottleneck indicated little support for any population bottlenecks, whereas according to Msvar results, 3 populations (Pop1, Pop2, and Pop11) have undergone bottlenecks. The discrepancy between the 2 methods might be attributed to BOTTLENECK, which is known to present some limitations as mentioned by Piry et al. (1999). For instance, bottlenecks could be detected only when occurring between 2Ne and 4Ne generations ago with this method. Additionally, the present study was conducted on limited size populations located at the westernmost limit of its distribution area, and the pattern could be different in large unfragmented populations in the core of the distribution area. To conclude about historical changes in French populations would necessitate analyzing additional populations from other countries.

Restricted genetic divergence was also observed between Italian and French mitochondrial haplotypes. Genetic distances within French haplotypes (mean: 0.28 ± 0.15%; maximum value: 0.54%) were similar to differences measured between the Italian sample and some French haplotypes (mean divergence: 0.43%; minimal divergence: 0.27%). Moreover, the low support for the sister taxa relationship between Italian and French haplotypes (Figure 2a) would suggest a recent shared history for both populations. Palaeoecological information (Van Andel and Tzedakis 1996) indicates the occurrence of steppic vegetation favorable to *V. u. ursinii* between the Mediterranean coast and the Alps during the last ice age. Thus, we could infer that the Apennines and southern French Alps populations were connected during cold periods, allowing past gene flow. Even if a single individual from Italy cannot provide information about the level of genetic variation in Italian populations, the low divergence between French and Italian haplotypes is a preliminary datum supporting such expectations.

Intrapopulation Variability

French populations are characterized by limited intrapopulation variability, with 6 localities presenting no cyt *b* haplotypic polymorphism (Pop2, Pop6, and Pop8–Pop11; Table 1). The maximum haplotype richness (3 haplotypes) was found in only 1 population (Pop1).

Compared with the others, Pop2 had extremely low genetic variability (−37% mean A_R and −48% mean H_O) as well as a high level of differentiation (+60% mean F_{ST}). This result cannot be explained by a reduced territory (potential suitable area of about 500 ha) or by a location at the boundary of the distribution area of the species in France (Figure 1a). Human culling and subsequent drift during the late 19th and early 20th century is more likely the reason of this observation. Indeed, Musset (2004) noted the destruction of 2500 vipers during a few years in the very close surrounding area of Pop2. Even if this culling has stopped since 1973, it might have caused the lower genetic diversity in this population. Indeed, the same observation has been reported in *V. berus* by Ursenbacher et al. (2009).

For most populations, there is no indication of recent population growth: mismatch distribution curves and neutrality tests (Fu's F_S and Tajima's D) suggested constant population size (Table 1). The sole exception is Pop1 in which only Tajima's D test was significant, but the hypothesis of a recent population growth would require more samples and/or more genetic data.

Differentiation and Isolation Patterns between French Populations

As often reported for populations in highly fragmented habitats (see review in Keyghobadi 2007), we observed a strong differentiation among several of the 11 populations as revealed by F_{ST} values (Table 2) that appeared significant in 87% and 98% of pairwise comparisons for mitochondrial and nuclear markers, respectively. Microsatellite (genetic distances and structure analyses; Figures 2c and 4) and mitochondrial (phylogenetic reconstructions from populations or haplotypes; Figure 2a,2b) analyses suggested a split between westernmost (Pop1 and Pop2) and the remaining eastern populations. The genetic structure within eastern and central localities is more complex. Low genetic divergence (both for mitochondrial and nuclear markers) was observed between Pop4, Pop5, and Pop6 as well as between Pop8 and Pop10, suggesting that gene flow still occurs (or had recently occurred) between these localities. By contrast, Pop3 and Pop7 cannot be associated with other populations on the basis of both genetic markers. Finally, dissimilar signals between mitochondrial data and microsatellites were observed for Pop11 and Pop9. Results of structure (using the ML or the Evanno et al. 2005 methods) suggested that individuals of Pop11 are split between Pop7
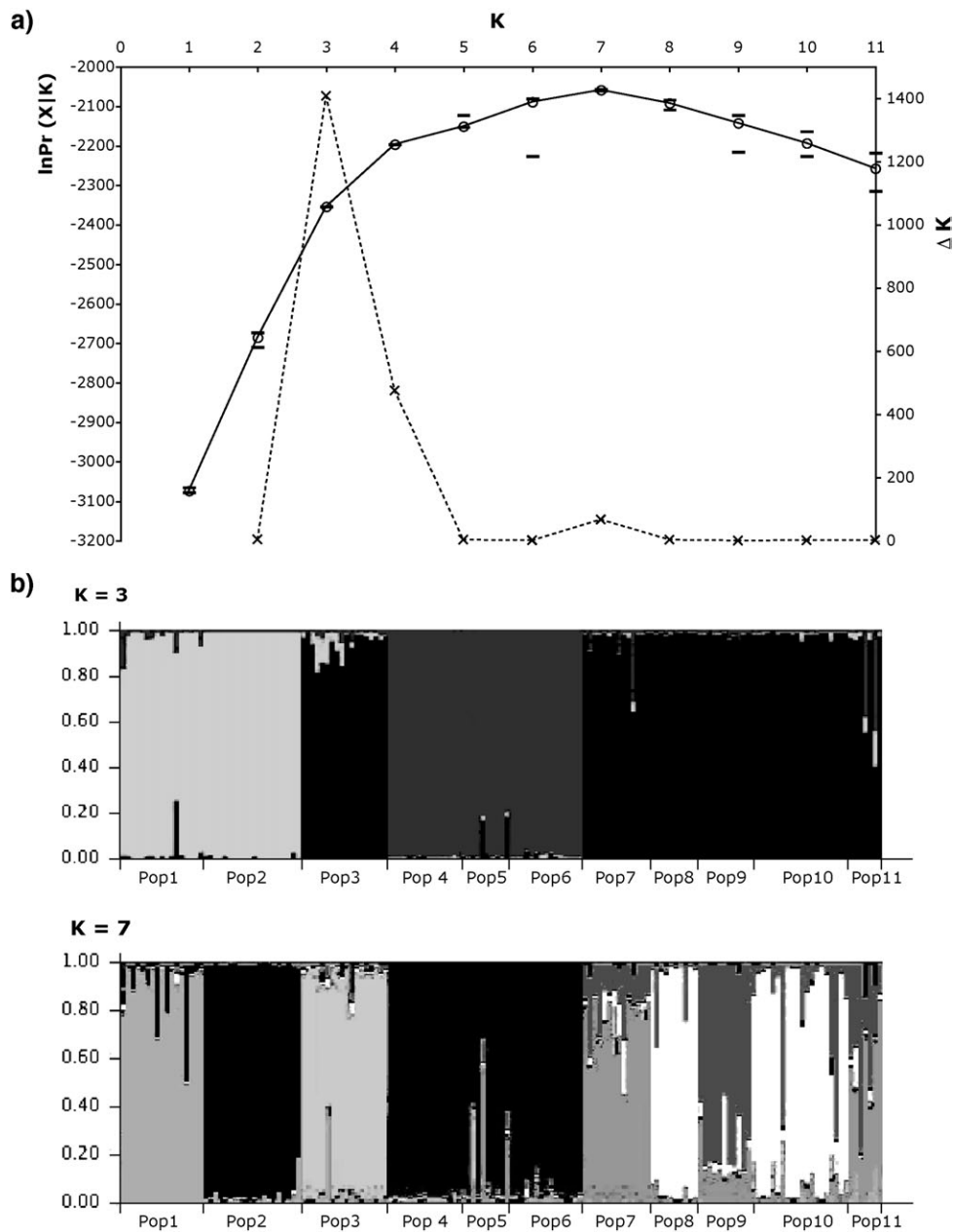
Figure 4. (a) Plot of the likelihood of the mean ln Pr $(X|K)$ (circle and plain line) with minimal and maximal values (dashes) of 10 runs for $K = 1–11$. The maximal value was observed for $K = 7$. Right scale (cross and dotted lines) indicates ΔK calculated as $\Delta K = m|L''(K)|/s[L(K)]$, as suggested by Evanno et al. (2005). The modal value of this distribution indicates the occurrence of 3 clusters. (b) Individual assignment calculated with STRUCTURE based on $K = 3$ and $K = 7$.

and Pop9 (Figure 4b). However, none of the haplotypes observed in Pop7 was detected in Pop11, which shares the same haplotype as Pop8–Pop10. Several hypotheses (lack of cyt *b* resolution, asymmetric dispersion between males and females, etc.) can be put forward to explain the discrepancy between the 2 markers. Another explanation (not mutually exclusive from the previous) would be that microsatellites would depict a more recent history (disruption) not observable with the mtDNA because of differential molecular rates of evolution between the 2 markers.

However, more samples would be necessary to further explore these different hypotheses.

Significant IBD was found for both mitochondrial and nuclear markers (Figure 3), which was predictable for a species with low dispersal capability in highly fragmented habitats. Although IBD is a strong structuring factor, other parameters (e.g., forest, river, or valley) might also explain the high differentiation observed between populations, particularly between geographically close populations. Indeed, all populations genetically differentiated are separated

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by deep valleys that form strong barriers for the Orsini's vipers (e.g., Pop7/Pop8), such as eastern and western populations separated by the Durance River (the deepest valley, see Figure 1b). Even for nearby sites (<4 km such as between Pop8 and Pop9, Pop4 and Pop6, or Pop 5 and Pop6) high genetic differentiation can be observed. Thus, even a small valley could constitute a real barrier between adjacent populations. Moreover, forest does not seem to represent the sole factor causing differentiation of populations because unforested habitat is observed between differentiated populations, such as Pop10 and Pop11. On the contrary, populations currently undifferentiated or connected by gene flow are not only geographically close but also localized on the same mountain massif (Pop4, Pop5, and Pop6). Definition of new microsatellites and increase of sampling will be useful to conduct analysis of landscape genetics in order to understand the role of topography and habitat quality (Giordano et al. 2007) in the genetic differentiation of the French Orsini's viper populations.

Implications for the Conservation of French Populations

Both mitochondrial and microsatellite analyses confirmed the strong isolation between most sampled sites in France. Based on our analyses, different groups of populations can be distinguished and specific MUs can be suggested accordingly. First, Pop1 and Pop2, although related, should be treated separately due to the high nuclear differentiation and the occurrence of unique haplotypes in both populations. In the eastern part of the French distribution, Pop4–Pop6 as well as Pop8 + Pop10 appear geographically and genetically close (from both markers) and should be each treated as a single unit. By contrast, Pop3 and Pop7 are more isolated geographically, and their unique genetic information indicates that they should be considered as independent units. A more complex pattern with contradictory results between mitochondrial and microsatellite analyses emerges for Pop11, leading us to regard this population as a single unit. Therefore, for the 11 French locations studied, 8 distinct MUs should be considered for future management programs (Pop1, Pop2, Pop3, Pop4 + Pop5 + Pop6, Pop7, Pop8 + Pop10, Pop9, and Pop11). It can be noticed that the different MU's represent a large range of areas (from 55 ha for Pop1 to 2852 ha for Pop4 + Pop5 + Pop6). Even the smallest MU (Pop1) is pertinent because of the limited dispersal capability of the species (maximum movement observed is 200 m; Baron 1997). Given the high fragmentation observed between these different groups, we should consider them as completely isolated and adopt specific management plans. Indeed, the disappearance of 1 group would mean the permanent extinction of the local population due to the lack of natural recolonization. Moreover, a special survey (by increasing the sampling size as well as the number of microsatellite loci) should be conducted in Pop2 due to its limited genetic variability, which has been shown to have a high impact on newborn mortality and abnormality occurrence in vipers (Madsen et al. 1999; Ujvari et al. 2002; Madsen et al. 2004).

The reduction of altitudinal meadows due to forest expansion (resulting from rural land desertion and the global warming) drove the snake on the upper parts of mountains and therefore accelerates the present fragmentation observed for *V. u. ursinii*. However, when populations occur on the top of mountains, sometimes occupying very small areas (confined to "sky islands" of suitable alpine habitat), the risk of natural disappearance during this century is particularly high. Consequently, populations located on the highest mountains (and presently not on the top) will probably have the best chance of surviving because they will keep the possibility to move up with the forest limit. We can therefore consider Pop3 and Pop4–Pop6 (located on mountains reaching about 2000 m) as the most favorable sites for the species future. However, these populations are genetically related (see Figure 1b) and thus do not include the total variability of French populations. As a consequence, conservation planning should focus on 2 different aspects: 1) a specific protection of the most mid-term threatened populations/metapopulations, such as the smallest populations with the lowest genetic diversity (Pop1 and Pop2) and 2) a long-term protection of the species in order to limit the impact of forest upward shift. The latter aspect would concern populations that would have kept the possibility to colonize new areas in the upper parts of mountains (Pop3 and Pop4–Pop 6). Such an approach could guarantee a long-term survival of *V. u. ursinii* in the French Alps. Moreover, traditional fire and grazing have progressively decreased after the decline in pastoral practices throughout the 20th century, resulting in a progressive degradation of grasslands (Cernusca et al. 1999; Chauchard et al. 2007). A beneficial approach would be to promote pastoral activity as a modern tool allowing controlled fire and management of grasslands, widening the natural habitat of the Orsini's viper. In order to prevent population splitting due to encroachment and forest expansion, prescribed burning and tree cutting have already been used (as done e.g., for Pop3, Lyet et al. 2009) within the context of the Life-Nature program focusing on biology and distribution of *V. u. ursinii* in France. However, long-term effects of such habitat preservation on conservation of the Orsini's viper populations remain to be investigated.

This study highlights the particular threats encountered by alpine species that are often locally endemic and restrained to suitable mountainous habitat (see other reptile examples such as *Iberolacerta bonnali*, Crochet et al. 2003; *Cyclodomorphus praealtus*, Koumoundouros et al. 2009). As other species confined to sky islands (Browne and Ferree 2007; Holycross and Douglas 2007; Koumoundouros et al. 2009), French populations of the Orsini's viper appear geographically and genetically isolated from each others with low dispersal capability and thus should be considered as separate conservation units (Moritz 1994; Palsboll et al. 2006). Moreover, alpine species are usually physiologically adapted to cold climates (Osborne et al. 2000) and therefore particularly sensitive to temperature increases (Callaghan et al. 2004). Consequently, alpine species can be regarded as early indicators of ecosystem changes and as such need

particular attention and steady management to assess species response to climatic fluctuations.

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