Molecular phylogeography of the asp viper *Vipera aspis* (Linnaeus, 1758) in Italy: evidence for introgressive hybridization and mitochondrial DNA capture

F. Barbanera[a,*], M.A.L. Zuffi[b], M. Guerrini[a], A. Gentilli[c], S. Tofanelli[d], M. Fasola[c], F. Dini[a]

[a]*Dipartimento di Biologia, Unità di Protistologia-Zoologia, Università di Pisa, Via A. Volta 6, I-56126 Pisa, Italia;* [b]*Museo di Storia Naturale e del Territorio, Università di Pisa, Via Roma 79, I-56011 Calci (Pisa), Italia;* [c]*Dipartimento di Biologia Animale, Università di Pavia, P.za Botta 9, I-27100 Pavia, Italia;* [d]*Dipartimento di Biologia, Unità di Antropologia, Università di Pisa, Via Derna 1, I-56126 Pisa, Italia*

F. Barbanera: fbarbanera@biologia.unipi.it

M.A.L. Zuffi: marcoz@museo.unipi.it

M. Guerrini: mguerrini@biologia.unipi.it

A. Gentilli: augusto.gentilli@unipv.it

S. Tofanelli: stofanelli@biologia.unipi.it

M. Fasola: fasola@unipv.it

F. Dini: fdini@biologia.unipi.it

*Corresponding Author: F. Barbanera, Dipartimento di Biologia, Unità di Protistologia-Zoologia, Università di Pisa, Via A. Volta 6, I- 56126 Pisa, Italia; phone: +39 050 2211386; fax: +39 050 2211393; e-mail: fbarbanera@biologia.unipi.it

Abstract

Owing to its temperature dependence and low vagility, the asp viper (*Vipera aspis*) is an interesting model species to study the effects of Pleistocene climatic fluctuations on vertebrate genomes. We genotyped 102 specimens from the whole Italian distribution range at three mitochondrial DNA regions (2278 characters, total) and six microsatellite DNA loci (Short Tandem Repeats, STR). The molecular phylogeny was constructed according to Bayesian, Neighbour-Joining, Maximum Parsimony and Maximum Likelihood procedures. All methods grouped individuals of the three morphological subspecies (*V. a. aspis, V. a. francisciredi, V. a. hugyi*) into five different haploclades. Specimens assigned to *hugyi* clustered in two highly differentiated clades, one being sister group to the complex comprising the second clade of *hugyi* (i.e., a paraphyletic status), plus two clades of *francisciredi*. The Bayesian clustering of the STR variability disclosed only two groups, the first including *aspis* and *francisciredi*, the second all *hugyi*. Introgressive hybridization and capture of *francisciredi*-like lineages in the *hugyi* mitochondrial genome were suggested to explain the discordance between mitochondrial and nuclear data. The phylogeographic pattern was compatible with population contractions in three glacial refuges. Plausibility of derived hypothesis was checked using coalescence simulations as *post hoc* tests. Long-term drift and serial founder-effects, rather than selection, appeared the main factors affecting the genetic make-up of the Italian asp viper.

Keywords**:** *V. a. aspis*; *V. a. francisciredi*; *V. a. hugyi*; mitochondrial-nuclear DNA discordance; Pleistocene glacial refuges;

1. Introduction

The asp viper, *Vipera aspis* (Linnaeus 1758), exhibits likely the most variable phenotype among western Palaearctic snakes (Brodmann, 1987; Zuffi and Bonnet, 1999). Its variability in colour patterns led to the description of five subspecies: *V. a. zinnikeri* in Spain and south western France, *V. a. aspis* in France, *V. a. atra* in western Italian Alps and Switzerland, *V. a. francisciredi* in central and northern Italy and in extreme southern Switzerland, and *V. a. hugyi* in southern Italy. However, the taxonomical meaning of these subspecies is still debated (e.g., Kramer, 1958; Zuffi, 2002). Biochemical (Pozio, 1980), biometrical and meristic data (Zuffi and Bonnet, 1999) endorsed the subspecific status of these taxa, whereas karyotype proved its unsuitability (Aprea et al., 2006). The anatomy of male reproductive organs was postulated as a valuable feature in identifying evolutionary lines within Squamata (Gasc 1968; Böhme, 1988; Ziegler and Böhme, 1996; Joger et al., 1997; Glaw et al., 1999; Keogh, 1999) and to discriminate asp viper populations of different subspecies (Zuffi, 2002; Zuffi et al., 2003). Recently, morphological and genetic traits have been deemed synergistic factors weighting for a synonymy of *V. a. aspis* and *V. a. atra*: cf. Golay et al. (2008). Uncertainty thus remains regarding taxonomy of the asp viper.

Quaternary climatic fluctuations have significantly shaped the genetic diversity of natural populations of the temperate northern hemisphere (e.g., Taberlet et al., 1998; Petit et al., 2003; Brito, 2005; Schmitt et al., 2006). Given the association among phenotypic variability, eterothermy, low vagility and latitude dispersion, the Italian asp vipers provide a particularly suitable model group to gain insights in how the phylogeography of European vertebrates was shaped by the glacial transitions. Ursenbacher et al. (2006) postulated Pleistocene refuges for the asp viper in southern France and in southern peninsular Italy by genotyping the studied specimens at the Control Region of the mitochondrial DNA (mtDNA). Nevertheless, the same Authors defined the refuge in southern peninsular Italy as “questionable” since it was inferred on the basis of only four samples.

To address the natural history of the Italian forms of *V. aspis* and their evolutionary processes at different time-scales, a large sample of specimens collected throughout the entire Italian distribution range was investigated using both mitochondrial and microsatellite DNA markers. The molecular phylogeny of the Italian asp viper populations was constructed and compared with the taxonomic interpretations based on morphology. The concordance between the geographic patterns described by two genetic systems and different evolutionary models was tested and a plausible historical scenario is given.

2. Materials and methods

2.1 Sample collection and DNA extraction

The study comprised 102 representatives of *Vipera aspis*, plus one specimen of *V. ammodytes* (Fig. 1; Table 1). The asp vipers were assigned to the known subspecies: *V. a. aspis* (or *V. a. atra*: cf. Golay et al., 2008), *V. a. francisciredi* and *V. a. hugyi*. Specimens with landmarks of hybridism in outwardly visible characteristics were not scored.

In order to extract DNA, either a tiny fragment of skin from ventral scales of wild animals or ethanol preserved internal tissues of dead specimens were chosen. DNA was isolated using Puregene Tissue Core Kit-A (Qiagen, Germany) following manufacturer's instructions.

2.2 Mitochondrial DNA

2.2.1 PCR and sequencing

We amplified both partial Control Region (CR: 550 bp) and Cytochrome-*b* gene (Cyt-*b*: 701 bp) as well as the entire gene codifying for the second subunit of the NADH dehydrogenase (ND2: 1023

bp) of the mtDNA (Table 2). PCRs were run in a MyCycler™ thermal cycler (Biorad, USA) with the following thermal profile: 3 min 94 °C, 30 cycles of 1 min 94 °C, 2 min at the annealing temperature (Cyt-*b*: 42 °C; CR, ND2: 54 °C) and 1 min 72 °C, followed by 7 min 72 °C. For the ND2, annealing lasted for 1 min. Reactions (50 µl) were prepared with 1 µl of Ampli*Taq* Gold DNA Polymerase (1 U/µl, Applied Biosystems, USA), 4 µl of 25 mM $MgCl_2$ (Applied Biosystems), 5 µl of 10X PCR Gold buffer (Applied Biosystems), 5 µl of 2.5 mM dNTP (Sigma Aldrich, Italy), 3 µl of each primer (1 µM) and 20 ng of DNA template. PCR products were purified (Genelute PCR Clean-up Kit, Sigma Aldrich) and directly sequenced on both DNA strands (BigDye® Terminator v. 3.1 Cycle Sequencing Kit, ABI Prism® 310 sequencer, Applied Biosystems).

2.2.2 Phylogenetic analysis

The alignment of 103 Cyt-*b*, ND2 and CR joint sequences was performed with CLUSTALW (v. 1.81: Thompson et al., 1994). We used MRMODELTEST (v. 2.3: Nylander, 2004), a simplified version of MODELTEST (v. 3.06: Posada and Crandall, 1998), and PAUP* (v. 4.0b10: Swofford, 2002) to estimate the best-fitting substitution models for both partitioned and single mtDNA dataset. According to the Akaike Information Criterion (AIC: Akaike, 1974; cf. Posada and Buckley, 2004), we selected the General Time Reversible (GTR) model for the Cyt-*b* (+ G) and ND2 (+ I), the HKY85 (Hasegawa et al., 1985) + I + G model for the CR, and the GTR + I + G model for the Cyt-*b*, ND2 and CR joint dataset. Bayesian analysis was conducted with Metropolis-coupled Markov chain Monte Carlo algorithms as implemented in MRBAYES (v. 3.1.2: Huelsenbeck and Ronquist, 2001). Two independent runs of analysis were conducted for 8,000,000 generations with a sample frequency of 100 (four chains, heating = 0.2, random starting tree) for both partitioned (all parameters unlinked across partitions) and single dataset. We applied the selected best evolutionary models with parameters estimated during the analysis. Convergence between the two runs was

monitored in MRBAYES through the standard deviation of split frequencies, and runs were continued until this value dropped to less than 0.01 (partitioned dataset: 0.009408; single dataset: 0.008081). Then, the convergence of each run towards stationarity was monitored with TRACER (v. 1.4: Rambaud and Drummond, 2007) using likelihood values as well as all other parameters estimated. Stationarity was reached after 2,000,000 of generations. Hence, 20,000 trees were discarded as burn-in, and the remaining 120,002 trees were used to produce 50% majority-rule consensus trees with PAUP*. According to Kass and Raftery (1995), the absolute values of the total harmonic means of the marginal likelihood (partitioned dataset: - 6581; single dataset: - 6943) were subtracted and doubled to get the difference of the log-likelihoods [$2\log_e(B_{10})$ or harmonic means of the marginal log-likelihoods]. Such a difference (= 724) was much greater than 10, hence, we found a very strong support for the partitioned dataset. The output was confirmed with TRACER by directly calculating the Bayes factor (partitioned dataset: - 6563; single dataset: - 6923).

Phylogenetic relationships were also inferred with Neighbour Joining (NJ: Saitou and Nei, 1987) and Maximum Parsimony (MP: Swofford et al., 1996) methods using PAUP*. The NJ tree was constructed using the Transitional model (TIM + I + G algorithm, with: A = 0.31; C = 0.30; G = 0.10; T = 0.28; I = 0.61; α = 1.44) selected using MODELTEST (with AIC), while the MP procedure was set-up as in Barbanera et al. (2007). Finally, a phylogenetic reconstruction based on a Maximum Likelihood analysis (ML) was performed under the General Time Reversible (GTR) model (a = 1.0; b = 21.2; c = 1.4; d = 1.4; e = 1.8; f = 1.0; for other parameters, see above) using a quartet puzzling procedure (10,000 steps) with TREE-PUZZLE (v. 5.2: Strimmer and von Haeseler, 1996). All trees were rooted using *V. ammodytes* as outgroup. While posterior probability values were calculated trough the Bayesian analysis, in the NJ, MP and ML reconstructions the statistical support for each node was evaluated by bootstrapping (BP, 10,000 replicates: Felsenstein, 1985). Haplotype network for combined Cyt-*b* and ND2 mtDNA data (CR was excluded because of its fast

evolving mutational rate) was carried out using the Median Joining method (Bandelt et al., 1999) with NETWORK (v. 4.5.1.0, © 2004-2009 Fluxus Technology).

MEGA (v. 3.1: Kumar et al., 2004) was used to compute the corrected Cyt-*b* distance among haploclades (± s.e., 1000 replicates) and to compare the CR sequences of Ursenbacher et al. (2006) with those of this study. The haplotype sequences were deposited at the Gene Bank (accession numbers: AM944366; AM944739 - AM944799).

Calibration of the molecular clock based on our mtDNA markers was not possible in *V. aspis* due to a lack of fossils. However, calibration for Cyt-*b* based on geological evidence (the emergence of the Isthmus of Panama, 3.5 Mya) is available for the Viperidae, with a suggested divergence rate of 1.4% My^{-1} (95% confidence interval, CI: 1.09% - 1.77%: Wüster et al., 2002; cf. Ursenbacher et al., 2006). The Likelihood Ratio Test (LRT) was run with DAMBE (v. 4.2.13: Xia and Xie, 2001) using our mtDNA markers either separately or combined (in pairs or all together). A heterogeneous evolutionary rate across lineages was always found. The scores (- 2 Log Δ), calculated with and without clock, ranged between 425.7 (Cyt-*b*) and 2401.4 (Cyt-*b* + CR + ND2) (all $p < 10^{-3}$), respectively. However, the Cyt-*b* sequences were tentatively used to estimate time divergence among haploclades by applying the TN93 + G model (Tamura and Nei, 1993; MODELTEST with AIC, $\alpha = 0.266$) and the suggested rate of Wüster et al. (2002).

2.2.3 Genetic diversity and demographic analysis

The partition of the genetic diversity was investigated by AMOVA with ARLEQUIN (v. 3.01: Excoffier et al., 2005) using F_{ST} pairwise distances (1000 permutations). The gene flow was estimated with DNASP by calculating the effective number of migrants per generation (N_em). The N_{ST} estimator of Lynch and Crease (1990) was used, where $N_e m = (1- N_{ST})/2\ N_{ST}$. N_{ST} assumes equilibrium between migration and drift during the time following the coalescence of two random alleles (Griswold and

Baker, 2002). ARLEQUIN was used to calculate the nucleotide diversity (π), the mean number of pairwise differences (k), the haplotype diversity (h), and to check for neutral evolution of the mtDNA sequences (Tajima test: Tajima, 1989). The McDonald-Kreitman test (McDonald and Kreitman, 1991) as implemented in DNASP (v. 4.10: Rozas et al., 2003) was conducted on mtDNA protein-coding genes to investigate the deviation from an equal ratio of non-synonymous to synonymous fixed substitutions at each haploclade using *V. ammodytes* as outgroup.

Inferences of population demographic expansion were obtained with DNASP using different methods (cf. Pilkington et al., 2008). We calculated the *Fs* statistic of Fu (1997), which is based on the probability of having a number of haplotypes greater or equal to the observed number of samples drawn from a constant-sized population. Then, we calculated the R_2 statistic (Ramos-Onsins and Rozas, 2002), which is based on the difference between the number of mutations and the average number of nucleotide substitutions. Ramos-Onsins and Rozas (2002) demonstrated that *Fs* and R_2 statistics have the greatest power to detect population expansion especially when sample sizes are large (~50) or small (~10), respectively. They also found that the power of the R_2 statistic is relatively high when the number of segregating sites is low (< 20). Recently, Ramirez-Soriano et al. (2008) investigated the statistical power in detecting a sudden population expansion, a sudden contraction, or a bottleneck analyzing DNA polymorphism data by means of a wide range of statistics. They found that the most powerful tests were those based on haplotype frequencies, including the Fu's *Fs* and R_2 statistics. In this study, the significance of the *Fs* and R_2 statistics was investigated by examining the null distribution of 5000 coalescence simulations using DNASP. Only significant negative *Fs* and positive R_2 values were retained as evidence of population expansion (Pilkington et al., 2008). Finally, the mismatch distributions (MD) of mtDNA pairwise distances were examined using ARLEQUIN in order to get insight into both demographic and spatial population expansion. In the first case, the more ragged the shape of the distribution, the closest the population

to a stationary model of constant size over a long period (Harpending's raggedness index, r: Harpending et al., 1993). The MD tests use the observed parameters of the expansion to perform coalescent simulations and to create new estimates of the same parameters. Departure from a model of sudden expansion was tested for each mtDNA clade by summing the squared differences (SSD) between observed and estimated MD (Schneider and Excoffier, 1999; Excoffier, 2004). The use of "SSD statistic" referred to the MD test for demographic expansion, whereas that of "SSD* statistic" referred to the MD test for spatial expansion.

2.2.4 Test of glacial refuges

The hypothesis derived in this study, namely the existence of three Pleistocene glacial refuges for the Italian asp viper populations, was tested using the tree simulation approach of MESQUITE (v. 2.6: Maddison and Maddison, 2008; cf. Crottini et al., 2007). The amount of discordance between the reconstructed gene tree and the population tree models was determined by the distribution of Slatkin and Maddison "*S*" values (1989), which measure the minimum number of sorting events required to produce the genealogy within a given model of divergence. For all coalescent simulations, the absolute time (years) was converted to coalescent time (generations) assuming a generation time of 6 years. This value (T) was calculated using the equation $T = \alpha + [s/(1-s)]$ of Lande et al. (2003), where "α" is the age at maturity (3 years: M.A.L. Zuffi, pers. com. 2009) and "s" is the annual adult survival rate (0.75: Flat et al., 1997) of the asp viper, respectively. The analysis was performed using different N_e values (100; 1000; 10,000; 100,000; 1,000,000) with 10,000 coalescence trees simulations. Divergence times were set according to three Pleistocene ice ages in Europe (Gibbard and van Kolfschoten, 2004). The first split (between *hugyi* clade E and clade D) was set to 1.25 Mya (Donau glaciation: 1.5-1.0 Mya); the second split (between *hugyi* and

francisciredi clade D) was set to 0.57 Mya (Gunz glaciation: 0.65-0.5 Mya); the third split (between *francisciredi* clade B e clade C) was set to 0.35 Mya (Mindel glaciation: 0.4-0.3 Mya).

2.3 Microsatellite DNA

All *V. aspis* representatives ($N = 102$) were investigated at six loci of the microsatellite DNA (Short Tandem Repeats, STR) originally isolated from the adder genome (*V. berus*: Carlsson et al., 2003). Touchdown PCR reactions (12.5 µl) were performed as follows: 10 min 94 °C, then 5 cycles 45 s 94 °C, 45 s T_{a1}, and 1 min 72 °C; 25 cycles of 45 s 94 °C, 45 s T_{a2}, and 1 min 72 °C, after that 72 °C 10 min (for T_{a1} and T_{a2} annealing temperature, see Table 3).

MICRO-CHECKER (v. 2.2.3: Van Oosterhout et al., 2004) was used to assess the quality of our microsatellite scoring. The mean observed (H_O) and the expected (H_E) heterozygosity under the Hardy-Weinberg Equilibrium (HWE) were computed using GENEPOP (v. 3.4: Raymond and Rousset, 1995). The partition of the genetic diversity was investigated by AMOVA with ARLEQUIN using F_{ST} pairwise distances (1000 permutations). Gene flow was calculated via the private allele method (Slatkin, 1985) using GENEPOP.

The Bayesian clustering procedure (admixture model with independent allele frequencies) as implemented in STRUCTURE (v. 2.2: Pritchard et al., 2000) was used to identify the K (unknown) populations of origin of the sampled individuals and to simultaneously assign them to these populations. STRUCTURE was run either using or not phenotype prior information. In the first case, the optimal K value, namely that minimizing possible departures from HWE and allelic Linkage Equilibrium (LE), was selected according to Evanno et al. (2005) using the following information: (i) the formula $\Delta K = m[|\ln" P(K)|]/s[\ln P(K)]$, where m stands for "mean" and s for "standard deviation", (ii) $\ln P(K)$ itself and (iii) the α value. Analyses were repeated by dividing the total sample into different populations according to the prior phenotype information of the specimens.

An identification threshold to each cluster was selected ($q_i = 0.90$: Vaha and Primmer, 2006). Individuals were probabilistically assigned to one ($q_i \geq 0.90$) or more ($0.10 < q_i < 0.90$) cluster if their genotypes indicated they were admixed. All simulations were run with 10^6 iterations, following a burn-in period of 10^5 iterations, and were replicated ten times per each *K*-value (1-10).

3. Results

3.1 mtDNA data

The alignment defined a set of 2278 characters, indels included: 449 variables sites were present (without outgroup: 265) and 206 were parsimony informative (without outgroup: 193); 61 haplotypes (H) were found (outgroup = H62; Table 1). Bayesian, NJ, MP (length, 634; consistency index, 0.779; retention index, 0.924) and ML procedures concurrently clustered the haplotypes into five groups (Fig. 2). Henceforth, statistical support for each node was reported in this order: (i) Bayesian, (ii) NJ, (iii) MP and (iv) ML. The clade A (1.00,100, 100, 100) was basal and included all *V. a. aspis* from north western Italy. The clades B, C and D were grouped (1.00, 100, 100, 100) whereas E was isolated. The clades B (0.62, 70, 52, 55) and C (1.00, 100, 100, 94: with vipers from Elba Island) were all *V. a. francisciredi*. The clade D (1.00, 98, 98, 100) included *V. a. hugyi* from southern peninsular Italy. The remaining *V. a. hugyi* specimens clustered into clade E (1.00, 100, 100, 100: with vipers from Montecristo Island). Haplotype network (Fig. 1 in the ESM) confirmed the mtDNA groups inferred by Bayesian, NJ, MP, and ML methods.

The Cyt-*b* based estimates of both distance (± s.e.) and time divergence among haploclades were as follows: *V. a. aspis* (clade A), 2.70 ± 0.24 (2.1-1.7 Mya); *V. a. hugyi* (Sicily and southern Calabria: clade E), 1.92 ± 0.20 (1.5-1.2 Mya); *V. a. hugyi* (southern peninsular Italy: clade D), 1.03 ± 0.14 (0.8-0.6 Mya); *V. a. francisciredi* clade B vs. C, 0.68 ± 0.11 (0.6-0.4 Mya).

The 62.4% of the total mtDNA variability was partitioned among phenotypes (*V. a. aspis*, *V. a. francisciredi*, *V. a. hugyi*) and 37.6% within them (AMOVA: $F_{ST} = 0.62$, $p < 10^{-5}$). The groups B and C showed lower average values of haplotype diversity (h), pairwise differences (k) and nucleotide diversity (π) than groups A, D and E (Table 4, Anova test: h: $F = 4.93$, $p = 0.001$; k: $F = 22.19$, $p < 10^{-3}$; π: $F = 17.25$, $p < 10^{-3}$). The gene flow was: *V. a. aspis* vs. *V. a. francisciredi*, $N_e m = 0.08$; *V. a. francisciredi* vs. *V. a. hugyi* (group D), $N_e m = 0.40$; within *V. a. hugyi* (D vs. E), $N_e m = 0.13$

In the whole sample the evolution of the mtDNA sequences did fit to the neutral model (Tajima's $D = -1.422$; $p = 0.060$). Within haploclades, the Tajima test was significant only for the clade C (Table 4); the McDonald-Kreitman test was always not significant (all $p > 0.280$), the group C being an exception ($p = 0.037$).

All mtDNA clades showed statistically significant negative Fu's *Fs* values, clade A being the only exception; positive R_2 values were statistically significant for the clades B, D and E (Table 4). Demographic expansion could be excluded only for the clade-B population (Fig. 3), while the spatial one could not be rejected out for all clades (not calculable for the clade-A; Table 4).

The distribution of *S*-values for the coalescent simulations ran with MESQUITE under the three glacial refuges hypothesis was recorded for each value of N_e. The *S*-value calculated for our tree genealogy (Bayesian, NJ, MP and ML reconstructions) was 3. It was found that the hypothesis could be never rejected when N_e was set between 100 and 100,000 ($p > 0.05$, each case).

3.2 STR data

Less than 3% of the microsatellite locus-population combinations (16 out of 588) gave evidence of null alleles. No allelic dropout was present. Hence the scoring quality was appropriate. The STR loci were polymorphic in the investigated sample (Table 3). Amplification was successful in 98 out of 102 specimens. The average values of H_O were smaller than H_E for each locus (Table 3, Fisher

exact multilocus test: $p < 10^{-5}$, all loci), also when computed within each phenotypic group (*V. a. aspis*: $H_O = 0.218$, $H_E = 0.591$; *V. a. francisciredi*: $H_O = 0.281$, $H_E = 0.489$; *V. a. hugyi*: $H_O = 0.229$, $H_E = 0.528$; one-sided test, all $p < 10^{-5}$). Departure from HWE within each phenotype was due to heterozygotes deficit. All pairwise locus combinations did deviate from LE (exact probability test, all $p < 10^{-5}$). The AMOVA performed with the sample divided into phenotypic groups (*V. a. aspis*, *V. a. francisciredi*, *V. a. hugyi*: $N = 91$, Montecristo excluded), showed that 31.8% of the total variability was partitioned among groups and 68.2% within them ($F_{ST} = 0.31$, $p < 10^{-5}$).

STRUCTURE indicated that vipers can be split into two groups as the probability of the data was maximum for $K = 2$ [$\Delta K = 898.2$; ln P(K) = - 1428.8; $\alpha = 0.098$]. When phenotype priors were incorporated, the membership proportions were: q_i (I) = 0.97 and q_i (II) = 0.03 for the *V. a. aspis* phenotype (100% assigned); q_i (I) = 0.88 and q_i (II) = 0.12 for the *V. a. francisciredi* phenotype (75.4% assigned); q_i (I) = 0.05 and q_i (II) = 0.95 for the *hugyi*-like vipers (89.5% assigned). In Fig. 4, the membership of the investigated specimens to the disclosed STR clusters is depicted as average per sampling locality. Admixed samples (12 *V. a. francisciredi*, 3 *V. a. hugyi*) from 14 localities were jointly assigned to the two clusters with individual proportion of membership (q_i) comprised between 0.10 and 0.90. Gene flow was as follows: *V. a. aspis* vs. *V. a. francisciredi*, $N_e m = 0.24$; *V. a. francisciredi* vs. *V. a. hugyi*, $N_e m = 0.47$; *V. a. aspis* vs. *V. a. hugyi*, $N_e m = 0.17$.

4. Discussion

4.1 mtDNA and STR pattern

Five main asp viper mtDNA clades were concurrently inferred by four different kind of phylogenetic reconstructions, either allowing (Bayesian) or not (NJ, MP, ML) partitioned analysis (Fig. 2; Fig. 1 in the ESM). The clade A comprised morphological *V. a. aspis* from north western

Italy. On the basis of hemipenes morphology Zuffi (2002) suggested that vipers from north western Italy could be a distinct species (*V. atra*), but Ursenbacher et al. (2006) disclosed a high sequence similarity between French *V. a. aspis* and Italian *V. a. atra*, later confirmed by Golay et al. (2008). The identity (> 99%) between the *V. a. aspis* CR sequences of Ursenbacher et al. (2006) and those showed by the *V. a. aspis* (*V. a. atra*) from north western Italy (this study), confirms that the two subspecies are synonymous. Haploclades B and C included all phenotypic *V. a. francisciredi*, with a certain degree of differentiation between western and eastern regions (Fig. 2). The clades D and E included phenotypic *V. a. hugyi* from southern peninsular Italy and Sicily, respectively (Fig. 2). Hence, the existence of three morphological subspecies for the Italian asp viper was confirmed. However, within *V. a. hugyi*, two diverging clades were found: group D, as sister group of *V. a. francisciredi*, and group E, with an older ancestor, thus suggesting a paraphyletic status (see paragraph 4.3). Furthermore, cryptic mtDNA lineage (*V. a. hugyi*, clade E) inhabiting Sicily and southern Calabria represented a new result with respect to the study of Ursenbacher et al. (2006), that was lacking in samples from the extreme south of Italy.

The analysis of the STR loci showed high discrepancy between mean expected (H_E) and observed (H_O) heterozygosity under HWE (Table 3). This result was due to our samples, which consisted of specimens collected from many different populations throughout the whole Italian Peninsula rather than from single populations. Considering the geographic isolation and the very low vagility of the asp viper, our sampled specimens could be expected to originate from populations with high genetic divergence (Fig. 2). Thus, when pooled all together, heterozygotes were much more rare than expected for a randomly mating population (Wahlund effect; cf. Allendorf and Luikart, 2007).

STR-based Bayesian assignments identified only two groups vs. the five ones inferred by mtDNA markers (Fig. 4), and no partition within *V. a. hugyi* was present. Failure of STR analysis in

identifying distinct mtDNA groups within *V. a. hugyi* may be due to the small sample within investigated localities. The first group comprised 100% of the *V. a. aspis* and 75% of the *V. a. francisciredi* vipers, the second about the 90% of *V. a. hugyi*. The remaining specimens ($N = 15$), unmistakably looking like *V. a. francisciredi* or *V. a. hugyi* in their outwardly appearance, were actually genetic *V. a. francisciredi* x *V. a. hugyi* hybrids, although both mtDNA- and STR-based estimates showed low gene flow ($N_em = 0.40$ and 0.47, respectively) when compared to that computed for Swiss and French *V. aspis* populations ($N_em = 1.6$, Jäggi et al., 2000). However, any comparison with gene flow reported by Jäggi et al. (2000) may be uncertain as they used a different marker (Random Amplified Polymorphic DNA) and because of the many assumptions involved in this kind of estimate (Whitlock and McCauley, 1999; Allendorf and Luikart, 2007). The hybrid vipers were detected not only near the contact zone between the two taxa, but also in central and southern Italy (Fig. 4), that is, inside the domains of the typical *V. a. francisciredi* and *V. a. hugyi* morphotypes (Fig. 4; backcross individuals: see paragraph 4.3).

4.2 Discordant population patterns from mtDNA and STR data

Cases of inconsistency between population structures inferred from mtDNA or from STR variability are well known. A greater population differentiation can be detected using nuclear rather than mitochondrial markers (e.g., by: Piertney et al., 2000; Johnson et al., 2003) but the opposite may occur as well (e.g., by: Borden and Stepien, 2006). Our study falls in this latter case, where the apportionment of the genetic diversity among phenotypes was larger by mtDNA than by STR loci. The ratio of mtDNA to STR F_{ST} was 0.62/0.31 = 2.0. The mtDNA markers inferred multiple clades whereas the STRs only two groups. In the asp viper, significant variations in sex ratio, sex-biased dispersal (Zuffi et al., 1999) and in female phylopatry (Naulleau et al., 1992) can be ruled out. However, mtDNA and STR markers provided different genetic pictures. The easiest explanation

was that mtDNA genetics is determined by an effective population size (N_e) that is 1/4 as large as that of nuclear genes (Birky et al., 1983). Bi-parentally inherited STRs have a higher mutation rate than mtDNA, but they would need thousands of generations to accumulate the same amount of genetic difference in isolated populations. Hence, patterns of intra-population decreased and inter-population increased diversity could be rapidly acquired in fragmented/bottlenecked, low vagile populations such as in the asp viper (Jäggi et al., 2000).

4.3 Introgressive hybridization and mitochondrial DNA capture

The paraphyletic status of *V. a. hugyi* deserved further attention. One possible explanation is that the branching order of the "species tree" might differ from that of the "genes tree" because of incomplete lineage sorting from an ancestral polymorphic gene pool (cf. Hudson and Turelli, 2003). The probability that the trees will be incongruent is high when an internode is short. But this is not the case because the node between the *V. a. hugyi* haplogroups is very well supported and the number of mutational changes between populations is consistent. Further, the retention of ancestral polymorphisms seems ruled out also by the lack of shared haplotypes between *franciscired*i-like and *hugyi*-like mtDNA lineages (Wilson and Bernatchez, 1998).

According to Avise et al. (1990), a paraphyletic status within an intraspecific phylogeny might be due to introgressive hybridization. There are many examples of populations with introgressed mtDNA genome but with diagnostic alleles at nuclear loci (Bernatchez et al., 1995). Regrettably, a mtDNA genealogy that may result from introgressive hybridization can be similar to a genealogy expected by retention of ancestral polymorphisms (Ballard and Whitlock, 2004). Hence, the use of the only mtDNA may not suffice to infer the phylogeography of a species, because genes are inherited as single linkage group and provide only one independent estimate (Moore, 1995). In this study, our mtDNA data assigned part of *V. a. hugyi* to a sister group of *V. a. franciscired*i, yet they

showed distinct nuclear genotypes (Fig. 4) that allowed detection of *V. a. francisciredi* x *V. a. hugyi* hybrids. It was hypothesized that introgression and asymmetric capture of the mtDNA might have occurred, a common phenomenon in hybridizing species (Avise, 2004; Podnar et al., 2005; Good et al., 2008). Generally, the mtDNA is more prone to introgression than nuclear DNA mostly because of selective pressure (local adaptation) and the chance (drift) (Ballard and Whitlock, 2004). Thermal adaptation in ectotherms is a strong candidate for a type of selection that may cause mtDNA introgression, and the relative fitness of different mtDNA lineages might change as a result (Somero, 2002). Both Tajima and McDonald-Kreitman test (Table 4) concurred that only the mtDNA clade C significantly deviated from the neutral model, a result that can explain the *p* value (= 0.06) obtained performing the Tajima test for the whole sample. We felt confident in considering that such a deviation from neutrality was due rather to demographic than to selection processes (cf. Ballard and Whitlock, 2004). The drift can account for the introgression of the *francisciredi*-like maternal lineage into the mtDNA genome of *V. a. hugyi* from southern peninsular Italy, traces of selection, if any, having been deleted by more recent demographic dynamics (Fig. 3: clade C). Similarly, Ferris et al. (1983) found that mtDNA could flow at the hybrid zone between two species of house mice in Scandinavia due to founder event. The asp viper might have suffered from drift associated to low N_e values, a common feature in current populations (Jäggi et al., 2000). The *francisciredi*-like mtDNA introgression into *V. a. hugyi* mtDNA genome would have also benefited from the higher fitness of *V. a. francisciredi* (Luiselli and Zuffi, 2002), which helped spreading of the *francisciredi*-like lineage during backcrossing to *V. a. hugyi* (cf. Whitmore, 1983) through a series of founder effects and/or under long-term drift in small populations.

4.4 A plausible phylogeographic scenario

Glacial refuges would characteristically harbour organisms with genetic variability that would be

higher than that of the derived populations, formed by a subset of the original gene pool. Another related prediction is that intraspecific diversity should decline away from refuges as a consequence of successive founder events during post-glacial colonization (Hewitt, 2000).

Dating evolutionary events is a challenging task, to be undertaken with extreme caution. Our rough estimates dated the separation of the Italian *V. a. aspis* between 2.1 and 1.7 Mya. Within *V. a. hugyi*, the split of populations from Sicily and southern Calabria would be the oldest pattern (1.5-1.2 Mya), whereas the split of populations from southern peninsular Italy from *V. a. franciscisredi* would be more recent (0.8-0.6 Mya). Ursenbacher et al. (2006) suggested that southern France harboured a glacial refuge for the asp viper. In our study, *V. a. aspis* clade A (north western Italy) was the basal (Fig. 2) and hold very high genetic variability. However, no clear demographic dynamics were inferred likely because of the low sample size (Table 4). Lambeck and Bard (2000) predicted that the sea level during last glaciations was ca. 100 m lower than today at the border between France and Italy (Gulf of Genoa: Fig. 1). This might have allowed vipers from southern France to colonize the Italian coastal areas, presently submerged. Yet our data cannot reliably support such a hypothesis.

It is known that Quaternary land bridges connecting Calabria with Sicily (Fig. 1) allowed continental and island faunas to merge during glacial expansions (Nilsson, 1983). As occurred, for instance, to the rock partridge (Lacertidae, *Podarcis sicula* Rafinesque-Schmaltz 1810: Podnar et al., 2005; Galliformes: *Alectoris graeca* Meisner 1804: Randi et al., 2003) and to the Italian wall lizard (Podnar et al., 2005). When our estimates of the asp viper mtDNA diversity were considered, *V. a. hugyi* clade E (Sicily and southern Calabria) showed the highest value of genetic diversity. The *Fs*, R_2 and MD tests did concurrently fit to a model of both demographic and spatial population expansion (Fig. 3, Table 4). Hence, Sicily represented the oldest Italian asp viper glacial refuge.

Clade D showed very high values of genetic diversity. Therefore, southern peninsular Italy was

another glacial refuge, as suggested also by Garrigues et al. (2005) and Ursenbacher et al. (2006). It benefited from merging routes from both northern and southern regions (Fig. 5), to instead of with Sicily was a true *cul-de-sac*, with a single colonization route from the land bridge with Calabria. Hence, the stronger statistical support obtained for both demographic and spatial population expansion in southern peninsular Italy than in Sicily did not come as a surprise (Table 4).

In central Italy, *V. a. francisciredi* clade B showed relatively high genetic diversity (Table 4). Overall, both demographic and spatial expansion was statistically supported (*Fs*, R_2, and MD test with SSD* statistics), the MD test ($p = 0.023$, SSD statistic) being an exception. However, although all tests we employed may be sensitive to unknown structure within populations, Ptak and Przeworski (2002) stressed that the MD test (SSD statistic) was actually the less powerful. Further, the clade B had the lowest, yet reliable, statistical support in the phylogenetic trees, and this might have influenced the SSD statistic of the MD test. Hence, we confidently consider clade B as an additional glacial refuge too (the most recent one: 0.6-0.4 Mya). In northern Italy, *V. a. francisciredi* clade C showed the lowest value of genetic diversity. It was assumed that asp vipers from central Italy colonized the northern regions only recently. However, although spatial population expansion could not be rejected, only contrasting results were obtained when demographic expansion was tested (Table 4). Overall, the pattern of decreasing genetic diversity from either north or south to central Italy marked out the main colonization routes of the asp viper.

In conclusion, the estimated divergence time among haploclades corresponded to three Pleistocene ice ages in Europe (Donau, Gunz and Mindel glaciations: Gibbard and van Kolfschoten, 2004). When the existence of the above-mentioned refuges was tested using the coalescence tree simulation approach of MESQUITE, the hypothesis was not rejected (see Results; Fig. 5). The two largest glacial refuges were geographically contiguous (R2, for *V. a. hugyi*; R3, for *V. a. francisciredi*: Fig. 5). These regions experienced the strongest demographic and spatial asp viper

population expansions. A result that fits with the present discovery of a scattered distribution of *V. a. francisciredi* x *V. a. hugyi* hybrids (Fig. 4).

4.5 A history apart: the asp viper of Montecristo Island

The islands of Elba and Montecristo have experienced different geological histories, their geographic proximity notwithstanding (Fig. 1). Elba was likely colonized by asp vipers moving along land bridges with peninsular Italy during marine regressions (Fig. 2: clade C). Our results support the hypothesis that exclusively humans mediated the colonization of Montecristo, as this latter was never connected with the mainland or with other islands (Krijgsman et al., 1999). The *V. a. hugyi* clade E (Fig. 2) included all Montecristo vipers, with closest relationships with the Sicilian populations (the ancient *Magna Graecia*, inhabited by people of Greek origin). The geographer Strabon (58 BC - 25 AC, in: Lassère, 1967) reported that vipers were thrown as weapons during attack to vessels coming from Africa and Sardinia to pirate the rich Etruscan towns. They were protected as commercial partners by Greeks, who, to the purpose, established a military base on Montecristo Island. The colonization of this island by asp vipers likely originated from *V. a. hugyi* specimens, carried out by the ancient militia from *Magna Graecia* during centuries VIII to III BC. This is the first genetic evidence that *V. a. montecristi* Mertens 1956 should be placed in synonymy with *V. a. hugyi*, as suggested by Pozio (1980), Corti et al. (1991) and Zuffi and Bonnet (1999).

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References

Akaike, H., 1974. Information theory as an extension of the maximum likelihood principle. In: Petrov, B.N., Csaki, F. (Eds.), Second International Symposium on Information Theory. Akademiai Kiado, Budapest, pp. 276-281

Allendorf, F.,Luikart, G., 2007. Conservation and the genetics of populations. Blackwell, Malden, USA

Aprea, G., Gentilli, A., Zuffi, M.A.L., Odierna, G., 2006. The karyology of *Vipera aspis*, *V. atra*, *V. hugyi*, and *Cerastes vipera*. Amphibia-Reptilia 27, 113-119

Ashton, K.G., de Queiroz, A., 2001. Molecular systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snake. Mol. Phylogenet. Evol. 21, 176-189

Avise, J.C., 2004. Molecular Markers, Natural History, and Evolution, 2nd edn. Sinauer Associates, Sunderland, Massachusetts

Avise, J.C., Ankney, C.D., Nelson, W.S., 1990. Mitochondrial gene trees and the evolutionary relationship of mallard and black ducks. Evolution 44, 1109-1119

Ballard, J.W.O., Whitlock, M.C., 2004. The incomplete natural history of mitochondria. Mol. Ecol. 13, 729-744

Bandelt, H.-J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37-48

Barbanera, F., Guerrini, M., Hadjigerou, P., Panayides, P., Sokos, C., Wilkinson, P., Khan, A.A., Khan, B.Y., Cappelli, F., Dini, F., 2007. Genetic insight into Mediterranean chukar (*Alectoris chukar*, Galliformes) populations inferred from mitochondrial DNA and RAPD markers. Genetica 131, 287-298

Bernatchez, L., Glemet, H., Wilson, C.C., Danzmann, R.G., 1995. Introgression and Fixation of Artic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 52, 179-185

Birky, C., Maruyama, T., Fuerst, P., 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics 103, 513-527

Böhme, W., 1988. Zur Genitalmorphologie der Sauria: Funktionelle und Stammesgeschichtliche Aspekte. Bonn. Zool. Monogr. 27, 3-176

Borden, W.C., Stepien, C.A., 2006. Discordant Population Genetic Structuring of Smallmouth Bass, *Micropterus dolomieu* Lacepède, in Lake Erie Based on Mitochondrial DNA Sequences and Nuclear DNA Microsatellites. J. Gt. Lakes Res. 32, 242-257

Brito, P. 2005. The influence of Pleistocene glacial refugia on tawny owl genetic diversity and phylogeography in western Europe. Mol. Ecol. 14, 3077-3094

Brodmann, P., 1987. Die Giftschlangen Europas und die Gattung Vipera in Afrika und Asien. Kümmerly-Fre, Bern

Carlsson, N., Isaksson, M., Hoggren, M., Tegelström, H., 2003. Characterization of polymorphic microsatellite markers in the adder, *Vipera berus*. Mol. Ecol. Notes 3, 73-75

Corti, C., Nistri, A., Poggesi, M., Vanni, S., 1991. Biogeographical analysis of the Tuscan herpetofauna (Central Italy). Rev. Esp. Herp. 5 (1990), 51-75

Crottini, A., Andreone, F., Kosuch, J., Borkin, L.J., Litvinchuk, S., Eggert, C., Veith, M., 2007. Fossorial but widespread: the phylogeography of the common spadefoot toad (*Pelobates fuscus*), and the role of the Po Valley as a major source of genetic variability. Mol. Ecol. 16, 2734-2754

Evanno, G., Reganut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611-2620

Excoffier, L., 2004. Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons fro the infinite-island model. Mol. Ecol. 13, 853-864

Excoffier, L., Laval, G., Schneider, S., 2005. ARLEQUIN ver. 3.0: An integrated software package for population genetics data analysis. EBO 1, 47-50

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783-791

Ferris, S.D., Sage, R.D., Huang, C.M., Nielsen, J.T., Ritte, U., Wilson, A.C., 1983. Flow of mitochondrial DNA across a species boundary. Proc. Natl. Acad. Sci. U.S.A. 80, 2290-2294

Flatt, T., Dummermuth, S., Anholt, B.,R., 1997. Mark-Recapture Estimates of Survival in Populations of the Asp Viper, *Vipera aspis aspis*. J. Herpetol. 31, 558-564

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915-925

Garrigues, T., Dauga, C., Ferquel, E., Choumet, V., Failloux, A.-B., 2005. Molecular phylogeny of *Vipera* Laurenti, 1768 and the related genera *Macrovipera* (Reuss, 1927) and Daboia (Gray,

1842), with comments about neurotoxic *Vipera aspis aspis* populations. Mol. Phylogenet. Evol. 35, 35-47

Gasc, J.-P., 1968. Morphologie des hémipénis chez *Vipera ursinii ursinii* (Bonaparte) et discussion biogéographique sur la répartition des espèces du genre *Vipera* en Europe occidentale. Bull. Mus. Nat. Hist. Nat. 40, 95-101

Gibbard, P.L., van Kolfschoten, T., 2004. The Pleistocene and Holocene epochs. In: Gradstein, F.M., Ogg, J.G., Smith, A.G. (Eds.), A Geologic Time Scale. Cambridge University Press, Cambridge, pp. 441-452

Glaw, F., Vences, M., Ziegler, T., Böhme, W., Köhler, J., 1999. Specific distinctness and biogeography of the dwarf chameleons *Brookesia minima*, *B. peyrierasi* and *B. tuberculata* (Reptilia: Chamaeleonidae): evidence from hemipenial and external morphology. J. Zool. 247, 225-238

Golay P., Monney J.-C., Conelli A., Durand T., Thiery G., Zuffi M.A.L., Ursenbacher, S., 2008. Systematics of the Swiss asp viper: some implications for the European *Vipera aspis* (Linnaeus 1758) complex (Serpentes: Viperidae). A tribute to Eugen Kramer. Amphibia-Reptilia 29, 71-83

Good, J.M., Hird, S., Reid, N., Demboski, J.R., Steppan, S.J., Martin-Nims, T.R., Sullivan, J., 2008. Ancient hybridization and mitochondrial capture between two species of chipmunks. Mol. Ecol. 17, 1313-1327

Griswold, C.K., Baker, A.J., 2002. Time to most recent common ancestor and divergence times of populations of common chaffinches (*Fringilla coelebs*) in Europe and North Africa: insights into Pleistocene refugia and current levels of migration. Evolution 56, 143-153

Harpending, H.C., Sherry, S.T., Rogers, A.R., Stoneking, M., 1993. Genetic structure of ancient human populations. Curr. Anthropology 34, 483-496

Hasegawa, M., Kishino, H., Yano, T-A., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160-174

Hewitt, G.M., 2000. The genetic legacy of Quaternary ice ages. Nature 405, 907-913

Hudson, R.R., Turelli, M., 2003. Stochasticity overrules the 'three-times rule': genetic drift, genetic draft, and coalescent times for nuclear loci versus mitochondrial DNA. Evolution 57, 182-190

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754-755

Jäggi, C., Wirth, T., Baur, B., 2000. Genetic variability in subpopulations of the asp viper (*Vipera aspis*) in the Swiss Jura mountains: implications for a conservation strategy. Biol. Conserv. 94, 69-77

Joger, U., Lenk, P., Baran, I., Böhme, W., Ziegler, T., Heidrich, P., Wink, M., 1997. The phylogenetic position of *Vipera barani* and *V. nikolskii* within the *Vipera berus* complex. In:

Böhme, W., Bischoff, W., Ziegler, T. (Eds.), Herpetologia Bonnensis. Societas Europaea Herpetologica, Bonn, pp. 185-194

Johnson, J.A., Toepfer, J.E., Dunn, P.O., 2003. Contrasting patterns of mitochondrial and microsatellite population structure in fragmented populations of greater prairie-chickens. Mol. Ecol. 12, 3335-3347

Kass, R.E., Raftery, A.E., 1995. Bayes Factors. J. Am. Stat. Assoc. 90, 773-795

Keogh, J.S., 1999. Evolutionary implications of hemipenal morphology in the terrestrial Australian elapid snakes. Zool. J. Linn. Soc. 125, 239-278

Kramer, E., 1958. Eine neue Rasse der Aspisviper asu dem südwestlichen Frankreich *Vipera aspis zinnikeri* n. subsp. Vierteljahrsschrift Naturforschende Gesellschaft Zürich 103, 321-326

Krijgsman, W., Hilgen, F.J., Raffi, I., Sierro, F.J., Wilson, D.S., 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature 400, 652-655

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brief. Bioinform. 5, 150-163

Kumazawa, Y., Ota, H., Nishida, M., Ozawa, T., 1996. Gene rearrangement in snake mitochondrial genomes: highly concerted evolution of control region-like sequences duplicated and inserted into a tRNA gene cluster. Mol. Biol. Evol. 13, 313-329

Lambeck, K., Bard, E., 2000. Sea-level change along the French Mediterranean coast for the past 30000 years. Earth Planet. Sci. Lett. 175, 203-222

Lande, R., Engen, S., Saether, B.E., 2003. Stochastic population dynamics in ecology and conservation. Oxford University Press, Oxford, UK

Lassère, F., 1967. Strabon. Géographie. Tome III (Livres V et VI), Paris

Lenk, P., Kalyabina, S., Wink, M., Joger, U., 2001. Evolutionary Relationships among the True Vipers (Reptilia: Viperidae) Inferred from Mitochondrial DNA Sequences. Mol. Phylogenet. Evol. 19, 94-104

Luiselli, L., Zuffi, M.A.L., 2002. Female life-history traits of the aspic viper (*Vipera aspis*) and sand viper (*V. ammodytes*) from the Mediterranean region. In: Schuett, G.W., Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of Vipers. CPG/Biological Sciences Press, Carmel, Indiana, pp. 279-284

Lynch, M., Crease, J., 1990. The analysis of population survey data on DNA sequence variation. Mol. Biol. Evol. 7, 377-394

Maddison, W.P., Maddison, D.R., 2008. MESQUITE: a modular system for evolutionary analysis. Version 2.6. Available at http://mesquiteproject.org.

McDonald, J.H., Kreitman, M., 1991. Adaptive protein evolution at the Adh locus in *Drosophila*. Nature 351, 652-654

Moore, W., 1995. Inferring phylogeny from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evolution 49, 718-726

Naulleau, G., Bonnet, X., Duret, S., 1992. Dèplacements et domaines vitaux des femelles reproductrices de Vipères aspic *Vipera aspis* (Reptilia, Viperidae) dans le Centre Ouest de la France. Bull. Soc. Herp. Fr. 78, 5-18

Nilsson, T., 1983. The Pleistocene: Geology and Life in the Quaternary Ice Age. Ferdinand Enke Verlag, Stuttgart, West Germany

Nylander, J.A.A., 2004. MRMODELTEST, version 2. Program distributed by the author

Petit, R.J., Aguinagalde, I., de Beaulieu, J.-L., Bittkau, C., Brewer, S., Cheddadi, R., Ennos, R., Fineschi, S., Grivet, D., Lascoux, M., Mohanty, A., Müller-Starck, G., Demesure-Musch, B., Palmé A., Martín, J. P., Rendell, S., Vendramin, G.G., 2003. Glacial Refugia: Hotspots But Not Melting Pots of Genetic Diversity. Science 300, 1563-1565

Piertney, S.B., MacColl, A.D.C., Bacon, P.J., Racey, P.A., Lambin, X., Dallas, J.F., 2000. Matrilineal genetic structure and female mediated gene flow in red grouse (*Lagopus lagopus scoticus*): an analysis using mitochondrial DNA. Evolution 54, 279-289

Pilkington, M.M., Wilder, J.A., Mendez, F.L., Cox, M.P., Woerner, A., Angui, T., Mobasher, Z., Batini, C., Destro-Bisol, G., Soodyall, H., Strassmann, B.I. and Hammer, M.F., 2008. Contrasting Signatures of Population Growth for Mitochondrial DNA and Y Chromosomes among Human Populations in Africa. Mol. Biol. Evol. 25, 517-525

Podnar, M., Mayer, W., Tvrtkovic, N., 2005. Phylogeography of the Italian wall lizard, *Podarcis sicula*, as revealed by mitochondrial DNA sequences. Mol. Ecol. 14, 575-588

Posada, D., Crandall, K.A.,1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818

Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Systematic Biol. 53, 793-808

Pozio, E., 1980. Contributo alla sistematica di *Vipera aspis* (L.) mediante analisi elettroforetica delle proteine contenute nel veleno. Natura Milano 71, 28-34

Pritchard, J.K., Stephens M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945-959

Ptak, S.E., Przeworski, M., 2002. Evidence for population growth in humans is confounded by fine-scale population structure. Trends Genet. 18, 559-563

Rambaud, A., Drummond, A.J., 2007. TRACER v.1.4, available at http://beast.bio.ed.ac.uk/Tracer

Ramirez-Soriano, A., Ramos-Onsins, S.E., Rozas, J., Calafell, F., Navarro, A., 2008. Statistical Power Analysis of Neutrality Tests Under Demographic Expansions, Contractions and Bottlenecks With Recombination. Genetics 179, 555-567

Ramos-Onsins, S.E., Rozas, J., 2002. Statistical properties of new neutrality tests against population growth. Mol. Biol. Evol. 19, 2092-2100

Randi, E., Tabarroni, C., Rimondi, S., Lucchini, V., Sfougaris, A., 2003. Phylogeography of the rock partridge (*Alectoris graeca*). Mol. Ecol. 12, 2201-2214

Raymond, M., Rousset, F., 1995. GENEPOP (version 3.1) is an update version of GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86, 248-249

Rozas, J., Sanchez-Del Barrio, J.C., Messeguer, X., Rozas, R., 2003. DNASP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496-2497

Saitou, N., Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425

Schmitt, T., Habel, J.C., Zimmermann, M., Müller, P., 2006. Genetic differentiation of the marbled white butterfly, *Melanargia galathea*, accounts for glacial distribution patterns and postglacial range expansion in southeastern Europe. Mol. Ecol. 15, 1889-1901

Schneider, S., Excoffier, L., 1999. Estimation of past demographic parameters from the distribution of pairwise differences when the mutation rates vary among sites: application to human mitochondrial DNA. Science 303, 223-22

Slatkin, M., 1985. Rare alleles as indicators of gene flow. Evolution 39, 53-65

Slatkin, M., Maddison., W. P., 1989. A cladistic measure of gene flow inferred from the phylogenies of alleles. Genetics 123, 603-613

Somero, G.N., 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and cost for living. Integr. Comp. Biol. 42, 780-789

Strimmer, K., von Haeseler, A., 1996. Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. Mol. Biol. Evol. 13, 964-969

Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony. Version 4.0b10. Sinauer Associates: Sunderland, Massachusetts

Swofford, D.L., Olsen, G.J., Waddel, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis. D.H., Moritz, C., Bable, B.K. (Eds.), Molecular Systematics, 2nd edn. Sinauer Associates, Sunderland, Massachusetts, pp. 407-514

Taberlet, P., Fumagalli, L., Wust-Saucy, A.-G., Cossons, J.-F., 1998. Comparative phylogeography and postglacial colonization routes in Europe. Mol. Ecol. 7, 453-464

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585-595

Tamura, K., Nei, M.,1993. Estimation of the number of the nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512-526

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680

Ursenbacher, S., Conelli, A., Golay, P., Monney, J.-C., Zuffi, M.A.L., Thiery, G., Durand, T., Fumagalli, L., 2006. Phylogeography of the asp viper (*Vipera aspis*) inferred from mitochondrial DNA sequence data: Evidence for multiple Mediterranean refugial areas. Mol. Phylogenet. Evol. 38, 546-552

Vaha, J.-P., Primmer, C.R., 2006. Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. Mol. Ecol. 15, 63-72

Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol. Ecol. Notes 4, 535-538

Whitlock, M., McCauley, D., 1999. Indirect measures of gene flow and migration: F_{ST} does not

equal $1/4N_m + 1$. Heredity 82, 117-125

Whitmore, D.H., 1983. Introgressive Hybridization of Smallmouth Bass (*Micropterus dolomieui*) and Guadalupe Bass (*M. treculi*). Copeia 3, 672-679

Wilson, C.C., Bernatchez, L., 1998. The ghost of hybrids past: fixation of arctic charr (*Salvelinus alpinus*) mitochondrial DNA in an introgressed population of the lake trout (*S. namaycush*). Mol. Ecol. 7, 127-132

Wüster, W., Salomão, M.D.G., Quijada-Mascareñas, J.A., Thorpe, R.S., BBBSP., 2002. Origins and evolution of the South American pitviper fauna: evidence from mitochondrial DNA analysis. In: Schuett, G.W., Höggren, M., Douglas, M.E., Greene, H.W. (Eds.), Biology of Vipers. CPG/Biological Sciences Press, Carmel, Indiana, pp. 111-128

Xia, X., Xie, Z., 2001. DAMBE: Data analysis in molecular biology and evolution. J. Hered. 92:371-373

Ziegler, T., Böhme, W., 1996. New results on sex recognition in squamate reptiles. Kleintierpraxis 41, 585-590

Zuffi, M.A.L., 2002. A critique of the systematic position of the asp viper subspecies *Vipera aspis aspis* (Linnaeus, 1758), *Vipera aspis atra* Meissner, 1820, *Vipera aspis franciscired*i Laurenti, 1768, *Vipera aspis hugy*i Schinz, 1833 and *Vipera aspis zinnikeri* Kramer, 1958. Amphibia-Reptilia 23, 191-213

Zuffi, M.A.L., Bonnet, X., 1999. Italian subspecies of the asp viper, *Vipera aspis*: patterns of variability and distribution. Ital. J. Zool. 66, 87-95

Zuffi, M.A.L., Gentilli, A., Razzetti, E., Scali, S., 2003. Transition hybridization areas in parapatric *Vipera aspis* subspecies from northern Italy. Biota 3 (2002), 191-196

Zuffi, M.A.L., Macchia, M., Ioalè, P., Giudici, F.,1999. Winter activity in a coastal population of *Vipera aspis.* Revue D Ecologie-la Terre Et La Vie 54, 365-374

Figures legend

Fig. 1. Sampling sites ($N = 76$) of the 102 specimens of *Vipera aspis* and one *V. ammodytes*. Dotted lines mark the boundaries between *V. a. aspis* and *V. a. francisciredi* in north western Italy, and between *V. a. francisciredi* and *V. a. hugyi* in southern Italy (from Zuffi, 2002). Names are given for some regions, cities, and islands cited in the text. Details for each site are given in Table 1

Fig. 2. NJ tree computed by PAUP* for the aligned haplotypes using *V. ammodytes* as outgroup. Numbers at internodes: bootstrap percentage values computed in the NJ (above internodes, left side), 50% majority-rule consensus MP (above internodes, right side) and ML (below internodes, left side) trees; posterior probability values computed in the Bayesian analysis (below internodes, right side). Rectangular boxes mark out the clades (A to E). The phenotype of the specimens is indicated, and the map of Italy shows the origin of the specimens of each clade

Fig. 3. Distribution of the pairwise differences (observed: dotted; expected: line) calculated for each

mtDNA clade (A to E, Fig. 2). Clade E does not include Montecristo Island. Harpending's raggedness index r is given for each clade, with the *p* value of the SSD statistic from the mismatch distribution under a model of sudden expansion (Table 4)

Fig. 4. Bayesian admixture analysis of asp viper genotypes (six STR loci) computed by STRUCTURE with $K = 2$. Each locality is represented by a pie chart (white, cluster I; black, cluster II), whose width is proportional to the estimated membership (q_i) to the *K* clusters. The sampling localities with all admixed specimens ($N = 15$) are reported together with their average q_i values

Fig. 5. *V. aspis* post-glacial main colonization routes (thick lines) originating from the disclosed glacial refuges (R1, R2 and R3), and merging into the present range. The thin lines show secondary re-expansion routes. A schematic representation of the hypothesis (three glacial refuges) tested with MESQUITE is given with the relative time scale corresponding to three Pleistocene ice ages in Europe (see Results)

Figures legend (Electronic Supplementary Material, ESM)

Fig. 1 ESM. Haplotype network (Cyt-*b* and ND2 combined mtDNA sequences) computed by NETWORK. For the sake of clarity, the number of mutational changes was reported only for the main connections. The three different morphological asp viper subspecies as well as a scale to infer the number of haplotypes for each pie were indicated

Table 1

Phenotype	Ref.	Specimens (*n*)	Field / Collection	Collection site (Province)	mtDNA haplotype	Accession code
V. a. aspis	1	1	Field	Sassello (SV)	H1	AM944366
	2	1	Field	Torino (TO)	H2	AM944739
	3	1	Field	Argentera (CN)	H3	AM944740
	4	1	Field	Carrosio (AL)	H4	AM944741
	5	1	Field	Varzi (PV)	H5	AM944742
	6	1	Field	Val Larone (SV)	H6	AM944743
	7	1	Field	Val Ferret (AO)	H7	AM944744
V. a. francisciredi	29	1	Field	Copogna (MC)	H8	AM944745
	21	1	Field	Pianoro (BO)	H8	AM944745
	16	1	Field	Belluno (BL)	H8	AM944745
	19	4	Field	Bosco Baredi (UD)	H8 (2), H11 (2)	AM944745, -748
	47	1	Field	Lesina (FG)	H9	AM944746
	49	1	Field	Mattinata (FG)	H10	AM944747
	27	1	Field	Fabriano (AN)	H11	AM944748
	20	2	Field	Ravenna (RA)	H11	AM944748
	30	1	Field	Monte Igno (MC)	H12	AM944749
	50	1	Field	Apricena (FG)	H13	AM944750
	31	1	Field	Garulla (MC)	H14	AM944751
	18	1	Museum of Natural History, Trieste	Latisana (UD)	H15	AM944752
	33	1	Field	Talamone (GR)	H16	AM944753
	32	1	Roman Herpetological Association	Monte Malbe (PG)	H17	AM944754
	38	1	Field	Gallese (VT)	H18	AM944755
	40	2	Field	Fonte Cerreto (AQ)	H19	AM944756
	43	2	Field	Marcigliana (Roma)	H19	AM944756
	39	1	Field	Borgorose (RI)	H19	AM944756
	44	1	Field	Castel Guido (Roma)	H19	AM944756
	42	1	Field	Mentana (Roma)	H19	AM944756
	41	1	Field	Vicovaro (Roma)	H19	AM944756
	28	1	Field	Montelago (MC)	H20	AM944757
	48	1	Field	Sannicandro (FG)	H21	AM944758
	46	1	Field	Pietraroja (BN)	H22	AM944759
	45	1	Field	S. Maria Pugliano (FR)	H23	AM944760

	13	4	Field	Bosco Fontana (MN)	H24	AM944761
	11	2	Field	Val Taleggio (BG)	H24, H28	AM944761, -765
	8	4	Field	Baragge (NO)	H24	AM944761
	10	1	Field	Castagnolo (PV)	H24	AM944761
	22	1	Field	Castelnuovo (LU)	H24	AM944761
	12	1	Field	Garda Bresciana (BS)	H24	AM944761
	14	1	Field	Cavalese (TN)	H25	AM944762
	23	1	Field	Gallicano (LU)	H26	AM944763
	24	1	Field	Rifiglio (AR)	H27	AM944764
	15	1	Museum of Natural History, Trento	Varena (TN)	H28	AM944765
	35	1	Field	Acquabona (Elba, LI)	H29	AM944766
	9	1	Field	Fagiana (MI)	H29	AM944766
	36	1	Field	S. Lucia (Elba, LI)	H29	AM944766
	37	1	Field	Bagnolo (Elba, LI)	H30	AM944767
	25	1	Field	Arnino (PI)	H31	AM944768
	26	2	Field	Tombolo (PI)	H31	AM944768
V. aspis hugyi	64	1	Field	Belvedere (CS)	H32	AM944769
	52	1	Field	Matera (MT)	H33	AM944770
	57	2	Field	Frassanito (LE)	H34	AM944771
	58	2	Field	San Cataldo (LE)	H35, H36	AM944772, -773
	54	1	Field	Laterza (BA)	H37	AM944774
	55	1	Field	Putignano (BA)	H38	AM944775
	53	1	Field	Santeramo in Colle (BA)	H39	AM944776
	59	1	Field	Ugentu (LE)	H40	AM944777
	61	1	Field	Colle Giunca (CS)	H41	AM944778
	60	1	Zoological Museum, Cosenza	Pollino (CS)	H41	AM944778
	63	1	Zoological Museum, Cosenza	Cosenza (CS)	H42	AM944779
	65	1	Zoological Museum, Cosenza	Serre Catanzaresi (CZ)	H43	AM944780
	62	1	Zoological Museum, Cosenza	Sila Grande (CS)	H44	AM944781
	56	1	Field	Policoro (MT)	H45	AM944782
	51	1	Field	Monte Croccia (MT)	H46	AM944783
	66	1	Zoological Museum, Firenze	Badolato (CZ)	H47	AM944784
	67	1	Zoological Museum, Firenze	Cardinale (CZ)	H48	AM944785
	34	9	Field	Montecristo (LI)	H49, H50 (7), H51	AM944786, -787, -788
	73	1	Zoological Museum, Firenze	Madonie (PA)	H52	AM944789

	68	1	Field	Aspromonte (RC)	H53	AM944790
	70	1	Zoological Museum, Firenze	Bosco Ficuzza (PA)	H54	AM944791
	71	1	Field	Mazara del Vallo (TP)	H55	AM944792
	69	3	Field	Etna (ME)	H56, H57, H60	AM944793, -794, -797
	74	2	Field	Anapo (SR)	H58	AM944795
	75	1	Field	Pantalica (SR)	H58	AM944795
	76	1	Field	Buccheri (SR)	H59	AM944796
	72	1	Zoological Museum, Firenze	Madonie (PA)	H61	AM944798
V. ammodytes	17	1	Field	Monte Prat (UD)	H62	AM944799

Asp viper specimens investigated in this study: phenotype expressed, reference number assigned (Fig. 1), number of specimens analysed, specimen origin, collecting location, phenotype and mtDNA haplotype (Fig. 2), Gene Bank accession code for each haplotype. Sampling was carried out between 2004 and 2007

Table 2

Primer	5'- 3' sequence	Marker	Reference
L14845	AAACATTTCAACCTGRTGAAATTTC	Cyt-*b*	Lenk et al. (2001)
H15557	AATAGGAAGTATCATTCGGGTTTGATG	Cyt-*b*	Lenk et al. (2001)
L16571VA	CTCTTTCCAAGGCCTCTGGCT	CR	Kumazawa et al. (1996)
H690	GTTGAGGCTTGCATGTATA	CR	Kumazawa et al. (1996)
L4437b	CAGCTAAAA AAGCTATCGGGCCCATACC	ND2	Kumazawa et al. (1996); Ashton and de Quieroz (2001)
HtRNA-Trp	GGCTTTGAAGGCTMCTAGTTT	ND2	Kumazawa et al. (1996); Ashton and de Quieroz (2001)

The primers used for each mtDNA marker are reported together with their reference

Table 3

Locus	Primer sequence (5' – 3')	Repeat motif	T_a (° C)	Size range (bp)	N_A	H_O	H_E
3	Fw: CAAGAAATGGAGATGAGC	$(AC)_{12}$	TD 52/50	149 - 173	13	0.417	0.715
	Re: GAAACCTATGAGCCAGTA						
11	Fw: GCAGCAGTCAGGACCGTTA	$(TC)_7$	TD 60/55	104 - 124	11	0.484	0.848
	Re: CCCCTTTCCTCTCCTTCTT						
21	Fw: CCAGTGGCACATAAGTAG	$(AC)_7$	TD 52/50	140 - 156	9	0.193	0.639
	Re: GTTCCATCATCAAAACAT						
37	Fw: CTAAAGATGTCTTAGGGTCACT	$(TC)_{10}$	TD 52/50	273 - 283	6	0.095	0.603
	Re: ATCCAGCCAGAACTGAT						
64	Fw: AGGCTCTGCTAAATGACC	A_8 … $(TG)_5TT(TG)_2$	TD 55/52	234 - 262	11	0.170	0.727
	Re: GATCCCCTGAATTGATTA						
71	Fw: TTGGCAAGAATCGAGGAGCTG	$(AC)_9(TC)_3(AC)_5$	TD 62/58	116 - 122	4	0.186	0.453
	Re: TGTGCCGACTTTTTGTGCTGA						

The characteristics of the investigated STR loci are shown. Fw, forward; Re, reverse; T_a (° C), annealing temperature; TD, touchdown PCR; N_A, number of alleles per locus; H_O, mean observed heterozygosity; H_E, mean expected heterozygosity

Table 4

Clade	Vipers (*N*)	Sites (*N*)	Haplotype (*N*)	Haplotype diversity (*h* ± s.d.)	Pairwise differences (*k* ± s.d.)	Nucleotide diversity (π ± s.d., %)	Tajima's *D* (*D*; *p*)	Fu's *Fs* (*Fs*; *p*)	R_2 (R_2; *p*)	*p* SSD	*p* SSD*
A	7	32	7	1.00 ± 0.08	13.3 ± 6.8	0.58 ± 0.34	**0.098**; 0.54	**- 1.114**; 0.16	**0.158**; 0.26	0.113	-
B	31	37	16	0.90 ± 0.36	7.6 ± 3.6	0.33 ± 0.18	**- 0.749**; 0.25	**- 9.768**; < 10^{-5}	**0.082**; 0.013	0.023	0.27
C	24	15	8	0.73 ± 0.09	2.0 ± 1.2	0.09 ± 0.06	**- 1.739**; 0.017	**- 4.441**; 0.002	**0.145**; 0.13	0.670	0.58
D	17	52	15	0.98 ± 0.03	12.3 ± 5.9	0.54 ± 0.29	**- 0.897**; 0.18	**- 6.429**; 0.007	**0.010**; < 10^{-5}	0.784	0.14
E*	14	50	12	0.97 ± 0.04	14.0 ± 6.7	0.61 ± 0.33	**- 0.479**; 0.34	**- 3.884**; 0.028	**0.100**; 0.040	0.320	0.50

Estimates of genetic diversity (mean ± s.d.), Tajima's *D*, Fu's *Fs* and R_2 values (in bold, with *p*) computed for each mtDNA clade (A-E, Fig. 2). The *p* values of the SSD statistic from the MD test for both demographic (SSD) and spatial (SSD*) expansion are reported. SSD* statistic could be not calculated for the clade A. Sites: number of segregating sites; E*: Montecristo vipers excluded.