

ORIGINAL ARTICLE

Genetic and ecological data reveal species boundaries between viviparous and oviparous lizard lineages

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Identification of cryptic species is an essential aim for conservation biologists to avoid premature extinctions of ‘unrecognized’ species. Integrating different types of data can undoubtedly aid in resolving the issue of species delimitation. We studied here two lineages of the common lizard *Zootoca vivipara* that display different reproductive mode (the viviparous *Z. v. vivipara* and the oviparous *Z. v. carniolica*) and that overlap their distributional ranges in the European Alps. With the purpose of delimiting species’ boundaries, we analyzed their ecological, genetic and natural history features. More than 300 samples were collected and analyzed at cytochrome b and 11 microsatellites loci for investigating genetic variation, population structure, individual relatedness and evolutionary histories of the two lineages. Additionally, we compared their ecological niches using eight ecological variables. Genetic data showed contrasting patterns of genetic structure between the two lineages, different demographic dynamics and no hybridization events. Also strong ecological differences (such as temperature) emerged between the two lineages, and niche overlap was limited. Taken together, these results indicate that *Z. v. vivipara* and *Z. v. carniolica* should be recognized as two separate species, and particular conservation consideration should be given to the oviparous lineage that tends to live in areas threatened by increasing impact of human activities. However, recent and rapid climate warming might determine an increasing risk for the persistence of the viviparous lineage, being adapted to cold environments. *Heredity* advance online publication, 1 July 2015; doi:10.1038/hdy.2015.54

INTRODUCTION

Accurate species delimitation is a basic objective in evolutionary systematics for understanding extant levels of biodiversity and adopting effective conservation strategies. Identifying cryptic species is recognized as fundamental to avoid premature extinctions of ‘unrecognized’ species, in particular in the light of current ongoing global changes (Koh *et al.*, 2004). It has been widely recognized that using only morphological traits for taxonomic inference tends to underestimate cryptic biodiversity, while genetic and phylogenetic tools contribute greater resolution and accuracy for taxonomy and systematics (Bickford *et al.*, 2007). However, in some cases, using only phylogenetic methods can give inconclusive results. To resolve species delimitation fully, a multidisciplinary approach, defined ‘integrative taxonomy’, has emerged with the aim of combining many different types of data (for example, phylogenetics, morphology, behavior, population genetics, ecology; Dayrat, 2005).

The integrative taxonomy approach has been recently successful for many species groups, by combining morphological data with additional molecular phylogenies (for example, Singhal and Moritz, 2013), behavioral information (for example, Bourguignon *et al.*, 2013) or ecological data (for example, Raxworthy *et al.*, 2007). For instance, closely related species may show adaptations that allow them to exploit distinct environments, therefore showing differences for their

ecological niche, although detecting these differences may be challenging. Only recently robust analytical tools have been developed for an objective assessment of niche differences (Broennimann *et al.*, 2012). In this study, we analyze species delimitation in the lacertid genus *Zootoca* using an integrative approach.

The lizard *Zootoca vivipara* is among the reptiles with the widest distribution range and northernmost distribution limits for a lizard (Surget-Groba *et al.*, 2001). However, this widespread lizard has strong intraspecific structure, with striking different reproductive mode among lineages. Specifically *Z. v. vivipara*, corresponding to mitochondrial clade E according to Surget-Groba *et al.* (2006), and occupying the great majority of the European distributional range of the species, is a viviparous lineage: embryos develop inside eggs within the body of the mother followed by birth to live offspring. Meanwhile two allopatric oviparous lineages, namely *Z. v. louislantzi* (Arribas, 2009) and *Z. v. carniolica* (Mayer *et al.*, 2000), live in the Pyrenees and in the Central Eastern Alps, respectively. No morphological traits clearly distinguish any *Z. v. vivipara* lineages (Guillaume *et al.*, 2006), while mitochondrial and nuclear sequences data clearly resolved phylogenetic relationship, with *Z. v. carniolica* being the sister lineage to all the others. Although distributional ranges of *Z. v. vivipara* and *Z. v. louislantzi* do not overlap in Western Europe, *Z. v. vivipara* and *Z. v. carniolica* are parapatric in the European Alpine chain. This pattern was likely generated by quaternary temperature oscillations

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Received 20 February 2015; revised 18 May 2015; accepted 18 May 2015

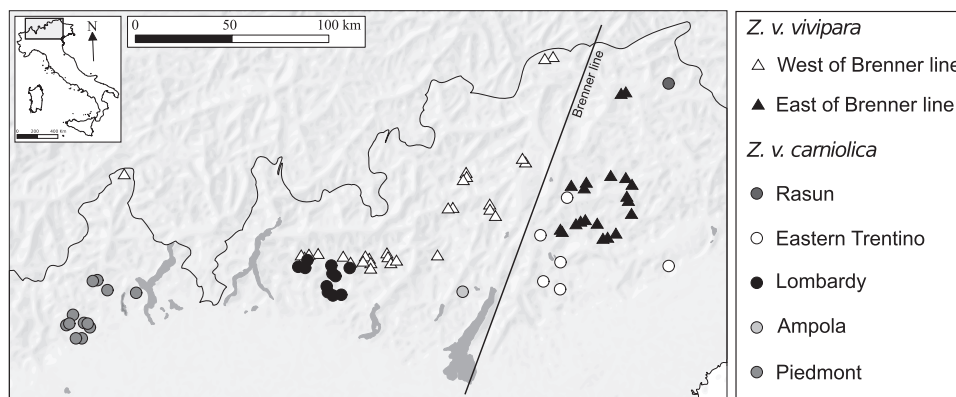


Figure 1 Sampling sites of common lizard across the Italian Alps. Triangles and circles represent locations where *Z. v. vivipara* and *Z. v. carniolica* were collected, respectively (according to mtDNA results). Different gray scales indicate the homogeneous genetic groups identified by STRUCTURE analyses and describe the two and five clusters inferred for *Z. v. vivipara* and *Z. v. carniolica*, respectively. Lakes are shown in dark gray.

that strongly influenced the demographic history of the species, likely promoting the evolution of viviparity (Surget-Groba *et al.*, 2006)

In reptiles, viviparity often evolves as an adaptation to cold climates (Pyron and Burbrink, 2014). Pleistocene climatic changes forced presumably still oviparous *Z. vivipara* populations to disperse to suitable southern areas of Europe. Pleistocene glacial movements have acted as a strong selective pressure in some populations between the Balkan Peninsula and Southern Russia, where viviparity likely arose, and probably facilitated the recolonization of the entire continent during interglacial periods (Surget-Groba *et al.*, 2001). Conversely, surviving populations in isolated Italian refugia (currently classified as *Z. v. carniolica*), remained oviparous and well adapted to warmer temperatures (Surget-Groba *et al.*, 2002). Recolonization pathways brought the two allopatrically differentiated lineages into secondary contact in the Alps. In the southern margin of species distribution the two lineages tend to live in moist mountainous habitat with *Z. v. carniolica* also present in a few relict spots in the Po plain (Surget-Groba *et al.*, 2002). In fact, oviparous and viviparous lineages are presumed to be better adapted to warmer and colder environmental conditions, respectively, and a differential altitudinal distribution has been reported, with the viviparous lineage tending to live at higher altitudes (Cornetti *et al.*, 2014). However, the two lineages show a definite overlap of their altitudinal distributions in the Alps, as confirmed by recent findings of syntopy areas (Lindtke *et al.*, 2010; Cornetti *et al.*, 2015), and quantitative analyses of niche differentiation between them are lacking.

This system provides the opportunity for combining molecular, life history and ecological data to assess species delimitation between *Z. v. vivipara* and *Z. v. carniolica*. Elucidating the taxonomic status of the *carniolica* lineage has major conservation implications. Overall, *Z. vivipara* is a widespread, least concern species (IUCN, 2014). However, local extinctions and population declines have been reported for *Z. v. carniolica*; during the past century, many low-altitude populations have disappeared, probably because of the high anthropization level in lowlands and/or because of climate changes (Sindaco *et al.*, 2006). Clear delimitation between the two lineages would therefore imply that *Z. v. carniolica* is a taxon of conservation concern. Furthermore, if the two species show strong ecological differences, management strategies should be definitively different among them.

In this study we apply an integrative approach combining mitochondrial phylogeny, population genetic and life history analyses using autosomal microsatellites, and ecological niche data for providing an accurate image of species boundaries.

MATERIALS AND METHODS

Sample collection and molecular techniques

Lizard samples were collected across the Alpine chain with the objective to cover most of the overlapping distribution of *Z. v. carniolica* and *Z. v. vivipara* (Figure 1 and Supplementary Table S1). Since their distributions are strictly correlated with moist habitat, our sampling primarily focussed on locations with this kind of habitat. Some of the intervening, unexplored zones might be suitable habitat, and we cannot exclude that the two subspecies can be found in other locations. Lizards were captured by hand, collecting ~0.5 cm of tail tissue and immediately released. Individual GPS coordinates of each sample and altitude were recorded. Tissue samples were stored in 95% ethanol until DNA extraction. The sample set ($N = 304$) used in the present study was obtained by merging tissue collections (191) used in Cornetti *et al.*, (2014) and newly collected samples (113, summer 2012, thus far unpublished). Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN Inc, Hilden, Germany) following manufacturer's instructions. The newly collected samples were amplified and sequenced for a 385 base pair fragment of mtDNA cytochrome b (cytb) according to Cornetti *et al.* (2014) for lineage identification. In addition, and for the first time on the entire sample set, 11 nuclear microsatellites (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115 and Lv-1-139 from Boudjemadi *et al.* (1999); B107, B114, C103, D109 from Remon *et al.* (2008)) were amplified in four multiplexes according to the thermocycling conditions specified in Supplementary Table S2. This study is distinct from two other recent works in this system. Cornetti *et al.* (2014), used sequences of mitochondrial and nuclear genes to examine *Z. v. carniolica* using a phylogenetic approach, supported by dating the divergence between the species; no microsatellites were used. Cornetti *et al.* (2015) examined hybridization dynamics at very fine scale, with different samples. Here, we compare the ecological and genetic patterns (based on microsatellites marker for the first time on the entire data set) at broad scale in the two lineages where they overlap their distributional ranges (European Alps). None of the sample analyzed in Cornetti *et al.* (2015) is included in the present study. Although we here use some of the samples of Cornetti *et al.* (2014), we have additional samples, and genotyped all samples at nuclear microsatellites specifically for this work.

Data analysis: mtDNA and microsatellites

Sequences were successfully obtained for all samples and aligned with deposited haplotypes (AY714882-AY714929) coming from Alpine area. This data set was used to build a median joining network using Network 4.612 (Bandelt *et al.*, 1999) in order to discriminate between the two *Z. vivipara* lineages since no morphological traits clearly distinguish them (Guillaume *et al.*, 2006). Cytb variability was assessed using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010) by analyzing nucleotide diversity, mean number of pairwise differences (intra-lineages) and F_{st} comparison (between lineages).

The 11 microsatellites were first examined for presence of null alleles, allele dropout and scoring errors using MicroChecker (Van Oosterhout *et al.*, 2004).

ARLEQUIN (Excoffier and Lischer, 2010) was used to calculate number of alleles (N_a), expected (H_e) and observed (H_o) heterozygosity, deviation from Hardy–Weinberg equilibrium and linkage disequilibrium between pairs of loci, while allelic richness (A_r) was calculated with Fstat (Goudet, 1995). Before intralineage analysis, we used Factorial correspondence analysis implemented in Genetix (Belkhir *et al.*, 2004) to exclude any possible discrepancy between mitochondrial and nuclear assignment, classifying each individual as *Z. v. vivipara* or *Z. v. carniolica* according to mtDNA determination.

For detecting possible hybridization events, we used the Bayesian algorithms of NewHybrids 1.1 (Anderson and Thompson, 2002) on the entire data set ($N=304$). NewHybrids calculates, for each individual posterior probability of assignment in different categories: pure parental populations, F1 hybrids, F2 hybrids or backcrosses. We used using default settings and tried both uniform and Jeffreys-like priors. We ran 500 000 iterations after a burn-in of 100 000.

The most likely number of genetic clusters (K) in our sample set was evaluated using a hierarchical analysis with STRUCTURE 2.3.2 (Pritchard *et al.*, 2000). We initially ran 10 replicates consisting of 1 000 000 iterations after a burn-in of 250 000 on the entire data set ($N=304$) with K ranging from 1 to 10. After the identification of two clearly distinct genetic clusters (see Results section) corresponding to the two *Z. v. vivipara* lineages, STRUCTURE was also used to investigate population structure within *Z. v. carniolica* ($N=114$) and *Z. v. vivipara* ($N=190$) with the same setting as above. The most likely number K that best explained genetic structure in the entire data set and within lineages, was estimated using STRUCTURE HARVESTER (Dent and Von Holdt, 2012), analyzing both $L(K)$ (Pritchard *et al.*, 2000) and ΔK (Evanno *et al.*, 2005). Additional analyses, for investigating intralineage genetic structure and presence of barriers to gene flow, were performed in BARRIER 2.2 (Manni *et al.*, 2004, see Supplementary Methods).

Evidence of possible reduction in effective population size (N_e) was investigated using M_P_VAL (Garza and Williamson, 2001), as well as the Wilcoxon test implemented in bottleneck (Piry *et al.*, 1999). Both methods contemplate a complex mutational model, that microsatellites markers usually exhibit (Di Rienzo *et al.*, 1994; Peery *et al.*, 2012); we ran M_P_VAL setting the mean size of multistep mutation and the proportion of multistep events to 3.1 and 0.22, respectively, as suggested by Peery *et al.* (2012). In bottleneck, we used a two-phase mutational model (with 78% stepwise mutation model and 22% infinite allele model) and a variance among multiple steps equal to 12 (Piry *et al.*, 1999). M-ratio is expected to decrease in shrinking populations, indicating a bottleneck in the distant past, if its value is <0.68 (Garza and Williamson, 2001). We explored a wide range of possible pre-bottleneck effective population sizes performing the analyses using the following θ values: 1, 2, 5, corresponding to 500, 1000 and 2500 individuals (using the common estimate of mutation rate of 5.0×10^{-4} mutations/generation/locus; Estoup and Angers, 1998). The statistical significance of M-ratio was obtained after 10 000 simulations of a population at equilibrium. The Wilcoxon test, in contrast, determines if a population suffered a recent reduction in population size ($0.2-4xN_e$ generation before present; Luikart and Cornuet, 1998) by evaluating the transient heterozygote excess in bottlenecked populations.

Isolation by distance

The correlation between genetic similarity and geographic distance was examined for the two lineages. Specifically, we constructed spatial autocorrelograms, a way of visualizing decay of relatedness between individuals over distance (isolation by distance). With SPAGeDi (Hardy and Vekemans 2002) we estimated the kinship coefficient derived by Loiselle *et al.*, (1995) for all pairs of individuals within distance classes chosen so that they composed a similar number of comparisons. The purpose of this analysis was to observe whether the two species had similar degrees of relatedness among pairs of individuals on a landscape. We also created autocorrelograms with other different measures of kinship and relatedness and obtained similar results.

Ecological niche differences

We considered eight environmental variables to compare the Grinnelian niche of the two lineages. Three variables describing the thermal environment (average, minimum and maximum annual temperature) and three variables representing water availability (annual, winter and summer precipitation,

potential evapotranspiration) were obtained from WorldClim (Hijmans *et al.*, 2005). We also considered potential evapotranspiration, a climatic parameter representing the amount of atmospheric energy that is strongly related to reptile distribution (Rodríguez *et al.*, 2005), and the normalized difference vegetation index, a proxy of primary productivity, obtained from time-series maps of the VEGETATION program (<http://www.vgt.vito.be>).

We used two approaches to compare the niches of the two lineages. First, we used the principal component analysis (PCA)-env approach to perform multivariate comparisons of niche overlap (Broennimann *et al.*, 2012). PCA-env measures niche overlap on the basis of occurrence and environmental data, and has shown to be the one of the most reliable techniques for niche comparison (Broennimann *et al.*, 2012). This method performs a PCA on the environmental spaces that is available to the two lineages. PCA-env uses a kernel density function to compute the density of occurrences in the multivariate PCA space, in order to take into account potential bias caused by unequal sampling effort. Niche overlap and niche similarity are calculated using the Schoener's D metric (Warren *et al.*, 2008). Schoener's D ranges between 0 (lack of overlap) and 1 (complete overlap), and is particularly suitable to analyze overlap in Grinnelian niches (Warren *et al.*, 2008). The significance of niche differences/similarity between lineages was tested by comparing the observed D -values to null distributions, using equivalency and similarity tests (Warren *et al.*, 2008; Broennimann *et al.*, 2012). The equivalency tests assess whether niches of two species are equivalent, by evaluating if overlap is constant when randomly reallocating occurrences of both lineages among the two ranges. The similarity test evaluates if the niche occupied by one lineage is more similar to the niche of the other species than expected by chance, by testing if the observed overlap between the niches of the two lineages is different from the overlap between the niche of one lineage and the 'niches' of locations selected randomly from the range of the other lineage (Warren *et al.*, 2008; Broennimann *et al.*, 2012). Significance of tests was assessed through 999 random permutations. This analysis was repeated at both fine and broad spatial scale. At fine scale, the study area was the same analyzed for genetic analyses (that is, Italy, north of $45^{\circ}30'N$), and we considered all the *Z. v. vivipara* and *Z. v. carniolica* localities available for Northern Italy (see Figure 1) for which many high-resolution data were available. For this analysis, all variables were obtained at the resolution of 30 arc-seconds, that is, $\sim 650 \times 920$ m within the study area. To confirm that the fine-scale results correspond to the situation occurring over the whole range of the taxa, the analysis was repeated at broad spatial scale, using the whole Palearctic realm as a study area. For this analysis, we included *Z. v. carniolica* and *Z. v. vivipara* records covering the whole range of the two subspecies (Surget-Groba *et al.*, 2002; Surget-Groba *et al.*, 2006; Lindtke *et al.*, 2010; Supplementary Figure S1). The spatial accuracy of most of literature records was limited, as only locality names were available, therefore this analysis was performed at the 0.1° resolution ($\sim 8 \times 11$ km).

To better depict differences for individual environmental variables (that is, for which variables there are differences between the two lineages), after the PCA-env analyses we used density profiles to compare the niche along the eight environmental variables (for example, Rodder, 2013). Comparisons were performed using the *sm.density.compare* function of the *sm* package in R (Bowman and Azzalini, 2010); significance was tested using 10 000 bootstraps.

RESULTS

MtDNA

Cytb analysis suggested that across the Italian Alps 114 samples were ascribable to *Z. v. carniolica* and 190 samples to *Z. v. vivipara* (Figure 2a). In *Z. v. carniolica*, we found 12 different haplotypes, three of which have never been observed before (KM522844–KM522846), while in the *Z. v. vivipara* samples there were six haplotypes, one of which is novel (KM522847). *Z. v. carniolica*, the oviparous lineage, proved to be about one order of magnitude more variable at mtDNA than the viviparous lineage (nucleotide diversity: 0.011 ± 0.006 compared with 0.001 ± 0.001 ; mean number of pairwise differences: 4.26 ± 2.12 compared with 0.59 ± 0.47).

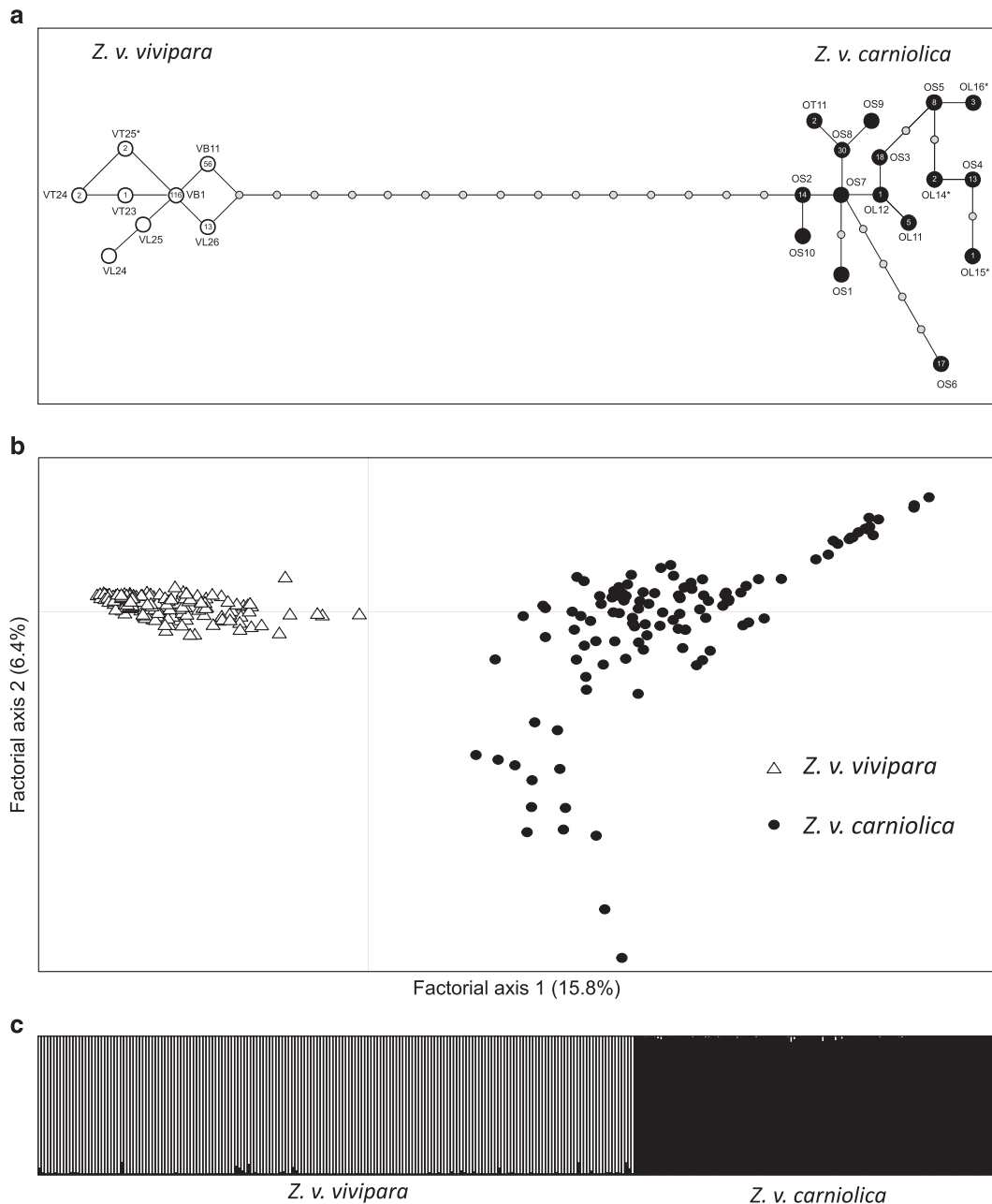


Figure 2 Median joining network of cytb haplotypes found in the Alps; *Z. v. vivipara* and *Z. v. carniolica* haplotypes are colored in white and black, respectively; number inside the circles represent the frequency, while asterisks mark sequences discovered in this study for the first time (a); factorial correspondence analysis based on six microsatellite loci discriminating two distinct groups defined according to mtDNA haplotypes (b); STRUCTURE plot describing the individual ancestry proportion of belonging to one out of the two lineages (c).

Microsatellites

All 11 nuclear microsatellite loci were polymorphic. Because of null alleles, according to MICROCHECKER results, locus Lv-1-139 was excluded from further analysis; no significant presence of null alleles, allele dropout and scoring errors were found for the remaining ten loci. In addition, no deviation from Hardy–Weinberg equilibrium and linkage disequilibrium between pairs of loci were detected. Likely because of the high genetic divergence between the two lineages, some markers could not be analyzed simultaneously in the whole data set. More specifically we could not obtain reliable fragment lengths for the loci C103 and D109 in *carniolica* samples. Moreover, after repeatedly finding private microvariants (incomplete repeats for given alleles) in

carniolica samples for loci Lv-3-19 and Lv-4-7, we sequenced both fragments in some homozygous individuals and found a recurring deletion in the flanking region of these loci. When all 304 samples were analyzed together, Lv-3-19 and Lv-4-72 were, therefore, treated with caution. In fact, for intraspecific comparisons, we conducted analyses on two data sets, with and without Lv-3-19 and Lv-4-72. Since we obtained similar results with both marker sets, we presented the more conservative results obtained with six microsatellites (B107, B114, Lv-4-alpha, Lv-2-145, Lv-4-X and Lv-4-115).

Factorial correspondence analysis clearly separated individuals of the two lineages (based on mtDNA) into two groups, with no overlapping samples (Figure 2b) and any evidence of contrasting

assignment based on mtDNA and microsatellites. The high genotypic divergence was also confirmed also by the mean number of private alleles per locus, 6.3 and 7.5 in *Z. v. carniolica* and *Z. v. vivipara*, respectively and significant F_{st} (0.1198) and R_{st} (0.5782) values for comparison between lineages.

Intraspecific genetic structure and hybridization

STRUCTURE results revealed that the most likely number of genetic groups was two according to the ΔK approach that generally shows the uppermost clustering level (Supplementary Figure S2; Evanno *et al.*, 2005), corresponding exactly to the two *Z. vivipara* lineages studied here. We then specifically tested for hybridization in our sample set running a STRUCTURE analysis fixing $K=2$: the results clearly rejected the occurrence hybrid individuals (Figure 2c) In addition, NewHybrids assigned all samples to their pure parental lineage with a probability above 99%.

Intra-lineage genetic structure and life history

The pattern of genetic variation in microsatellites (indicated by scattering of individuals, see Figure 2b) appeared wider in *Z. v. carniolica* than in *Z. v. vivipara*, suggesting a more pronounced genetic structure within the oviparous lineage than in the viviparous one. However, more detailed analyses were performed for understanding genetic variation within lineages using eight (B107, B114, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115, Lv-3-19 and Lv-4-72) and nine loci (B114, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115, Lv-3-19, Lv-4-72, C103 and D109) for *Z. v. carniolica* and *Z. v. vivipara*, respectively (marker B107 was monomorphic in *Z. v. vivipara*).

The intralinear genetic structure investigated using STRUCURE revealed that for *Z. v. carniolica*, the most likely number of distinct genetic clusters was five, as both $L(K)$ and ΔK showed the highest mean posterior probability values for $K=5$ (Supplementary Figure S5). The clustering analysis clearly divided the distributional range of

oviparous lineage in three parts: Western Alps (Piedmont); Central Alps (Lombardy); and Eastern Alps. In addition, two single populations (Ampola and Rasun) constituted distinct groups (Figure 3). All clusters displayed significant pairwise F_{st} values, varying from 0.15 to 0.35. In contrast, *Z. v. vivipara* lineage, showed weaker substructure, with two statistically supported clusters, according to ΔK (though the highest posterior probability for $L(K)$ was obtained for five groups ($K=5$), mean q -values rarely exceeded 0.5, meaning that inconsistent and ambiguous groups were found, Figure 3, Supplementary Figure S6, with a significant pairwise F_{st} (0.032, $P=0.001$). The results obtained with STRUCTURE were consistent with BARRIER output (Supplementary Figures S7-9). Further genetic analyses were conducted within the seven groups (five in *carniolica* and two in *vivipara*) identified by STRUCTURE. The five *Z. v. carniolica* clusters included populations from 14 to 32 individuals. Number of alleles ranged from 4.4 (Ampola) to 9.5 (Piedmont); similarly, allelic richness assessed Ampola cluster as the less genetically variable. Observed heterozygosity varied from 0.47 to 0.59, while expected heterozygosity from 0.57 to 0.77. On the other hand, the two *vivipara* groups, perhaps because of higher sample size (96 and 94 samples), displayed more genetic variation (Table 1).

Four out of five clusters of *Z. v. carniolica* showed significant evidence of reduction in population size according to M_{P_VAL} results, while no evidence of bottleneck events were highlighted in *Z. v. vivipara*. No significant signal of heterozygosity excess was found using the Wilcoxon test implemented in bottleneck, meaning that neither *vivipara* nor *carniolica* clusters suffered from recent reduction in effective population size (Table 1).

Isolation by distance

The two species showed different patterns in spatial genetic structure as summarized in the autocorrelograms (Figure 4). *Z. v. carniolica* displayed much higher relatedness over the first 50 km of distance (>0.10),

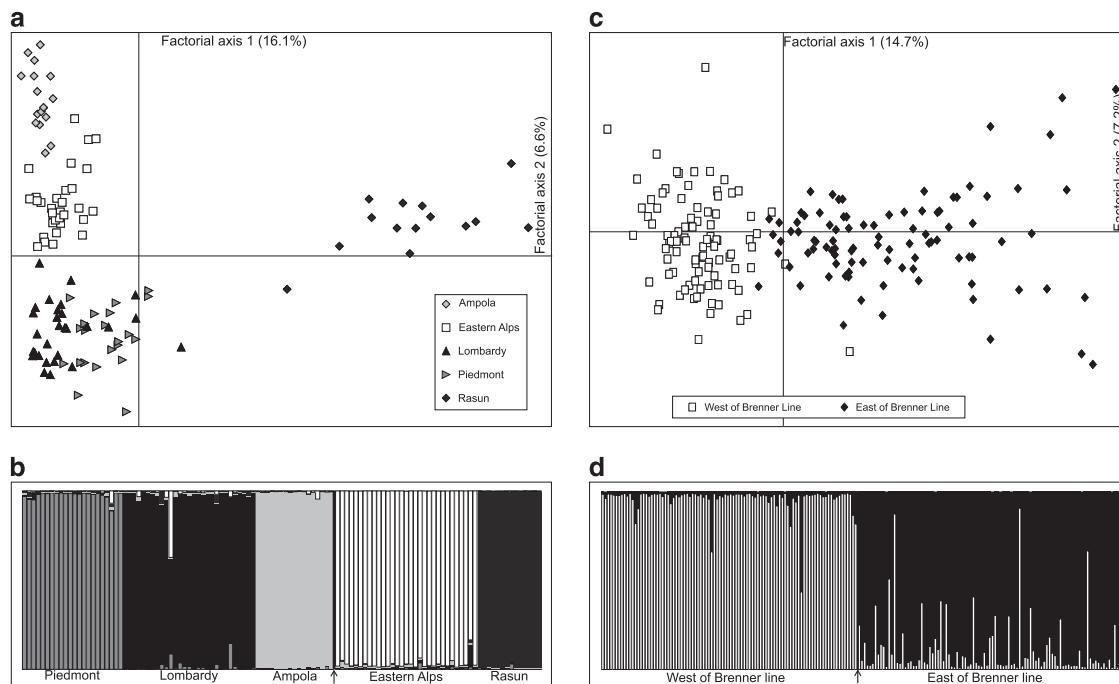


Figure 3 Factorial correspondence analysis of genotypic variation (a and c) and clustering analyses (b and d) of *Z. v. carniolica* (a and b) and *Z. v. vivipara* (c and d) lineages across the Italian Alps. In the STRUCTURE plots, the individuals are ordered by longitude (from West to East) and the 'Brenner line' is indicated by a vertical black bar and an arrow.

Table 1 Microsatellites genetic variability and demographic inferences about *Z. vivipara* populations using M-ratio test and the Wilcoxon test of heterozygosity excess (*P*-value)

Population	<i>N</i>	<i>N_a</i>	<i>A_r</i>	<i>H_o</i>	<i>H_e</i>	<i>M-ratio</i>	<i>P-value (M_P_VAL)</i>			<i>Wilcoxon test (bottleneck)</i>
							$\theta = 1$	$\theta = 2$	$\theta = 5$	
<i>Z. v. carniolica</i>										
Ampola	17	4.4	3.9	0.51	0.57	0.59	0.003	0.011	0.028	0.464
Eastern Alps	32	9.6	6.5	0.59	0.65	0.68	0.032	0.062	0.107	0.990
Piedmont	29	9.5	7.1	0.59	0.77	0.76	—	—	—	0.527
Lombardy	22	6.8	5.5	0.58	0.69	0.54	0.00004	0.001	0.002	0.726
Rasun	14	5.5	5.2	0.47	0.69	0.43	0.00001	0.00001	0.00001	0.097
<i>Z. v. vivipara</i>										
East of Brenner line	96	14.8	14.5	0.71	0.79	0.72	—	—	—	0.632
West of Brenner line	94	11.2	11.0	0.63	0.73	0.70	—	—	—	0.367

Abbreviations: *N_a*, number of alleles; *A_r*, allelic richness; *H_o* and *H_e*, observed and expected heterozygosity. Value in bold are significant using different values of θ .

while *Z. v. vivipara* had quick decay of relatedness such that it is near zero by 40 km. Moreover, F1, the relatedness in the first distance class (that is, the pairs of individuals closest to each other) was near 0.15 for *Z. v. carniolica*, while it was 0.08 for *Z. v. vivipara*. This fine-scale structure shows high relatedness in *Z. v. carniolica* for near and moderate distances. This is consistent with the stronger clustering that appears in STRUCTURE.

Niche differences

In the fine-scale analysis (high resolution, limited to Northern Italy) niche overlap between the two lineages was limited (Schoener's $D=0.333$, Supplementary Figure S3d). The equivalency between the two niches was significantly lower than expected by chance ($P=0.002$), while similarity tests showed that the two niches are not more similar than expected by chance (all $P>0.27$; Supplementary Figures S3e and f).

Density profiles showed significant differences for all the considered environmental variables (bootstrap test: $P<0.001$ for all variables considered). Specifically, the *vivipara* lineage was associated with environments significantly colder, drier and with lower primary productivity (Figure 5). Results remained consistent if the analysis was performed over the whole range of the two subspecies: the equivalency between the two niches was much lower than expected by chance (Schoener's $D=0.409$, $P=0.002$). Density profiles confirmed significant differences for all the considered environmental variables, except maximum summer temperature (Supplementary Figure S4).

DISCUSSION

Identification of species' boundaries is essential for describing and understanding species' natural history, for implementing conservation strategies, and for assessing and monitoring biodiversity. For instance, the most comprehensive and worldwide recognized list of threatened species (the Red List drafted by the International Union for Conservation of Nature) focuses mainly on species, although it also assesses subspecies or populations (IUCN, 2014). Effective conservation policies to counteract biodiversity loss will depend on accurate knowledge of extant biodiversity, including precise delimitation of species' boundaries and description of the ecological, genetic and natural history features of each species. In this context, the multi-disciplinary approach defined 'integrative taxonomy' can be of particular assistance for recognizing conservation units. Combining

mtDNA analyses, population genetics and ecological niche analyses allowed us to identify species boundaries in a lacertid lizard inhabiting European Alps. Specifically we found: (a) that the two lineages display contrasting patterns of genetic structure; (b) no evidence of hybridization between the two lineages; (c) that the two lineages occupy different ecological niches; and (d) evidence of genetic fragmentation, decline and isolation for some *Z. v. carniolica* populations.

Before the discovery of the first oviparous population, ascribable to the genus *Zootoca* in Slovenia and Carinthia (Austria, Mayer *et al.*, 2000), it was assumed that only one lineage of *Z. vivipara* inhabited the Alps—the widespread, viviparous and currently classified *Z. v. vivipara*. This cryptic differentiation in natural history was probably ignored because the two lineages (*Z. v. vivipara* and *Z. v. carniolica*) cannot be unequivocally distinguished by morphology (Guillaume *et al.*, 2006). However, the two lineages differ at a major phenotypic trait (reproductive mode), and experienced long independent evolutionary histories, accumulating genetic divergence in allopatry, before their secondary contact in the Alpine chain (Surget-Groba *et al.*, 2006; Cornetti *et al.*, 2014). We confirmed here with an ample data set that the two lineages, currently classified as 'subspecies', live in sympatry and without hybridization in the Alps, and exploit distinct niches.

The initial mitochondrial survey, necessary for lineage identification, not only confirmed the profound genetic divergence at mitochondrial level (Figure 2a) but also interestingly revealed that the oviparous *Z. v. carniolica* is about 10 times more variable at *cytb* than *Z. v. vivipara*. This finding explains different demographic patterns for the two lineages; while the oviparous lineage remained isolated in refugia in the southern margin of Alpine chain and Po Plain during glacial period (Surget-Groba *et al.*, 2002), the viviparous one colonized the Alps only recently from East (from Balkans or southern Russia, Surget-Groba *et al.*, 2001). We also found an west–east decrease in nucleotide diversity in *Z. v. carniolica* (Table 2), with many locations in Northern–Eastern Italy showing no variation at mitochondrial DNA. This pattern is consistent with a scenario where oviparous populations remained isolated in a northwestern Italian refugium and then colonized northern Italy, Austria and Slovenia. Populations persisting in glacial refugia are generally characterized by high levels of genetic diversity as a result of a longer demographic history, while populations that recently colonized new areas typically show low genetic variation (Hewitt, 2000).

Lack of gene flow between populations due to habitat loss and modification can reduce effective population sizes and increase genetic drift in isolated populations. Although *Z. v. carniolica* displayed higher genetic variation than *Z. v. vivipara* at mtDNA, we found signals of genetic fragmentation in two oviparous populations showing reciprocally private haplotypes (that is, Ampola and Rasun, with OS6 and OS2 haplotypes, respectively), likely as a result of habitat discontinuity. Our finding, also confirmed by microsatellites data (see below) are concordant with the hypothesis that a combination of climate warming (Fenner *et al.*, 2007) and anthropization (for example, drainage of peatbogs, Semenzato *et al.*, 1996) have been contributing to reduction in ground surface of wetlands and thus reducing and fragmenting suitable habitat for *Z. vivipara* in Northern (especially Northeastern) Italy.

Microsatellite data confirm the long, independent evolutionary histories of oviparous and viviparous lineages, and support the hypothesis that they likely started to diverge long ago (at least 2.6

Mya, according to Cornetti *et al.*, 2014). Difficulties in correctly amplifying the same set of microsatellites, occurrence of private deletions in *carniolica* samples in the flanking region of two markers and high percentage of private alleles in each lineage supported the hypothesis of an ancient separation time between the two lineages (Surget-Groba *et al.*, 2001; Cornetti *et al.*, 2014). In addition, we could confidently exclude the presence of hybrid individuals in our sample set (Figure 2b), meaning that pre- or post-zygotic barriers likely arose in allopatry and promoted reproductive isolation after *vivipara/carniolica* secondary contact. However, detailed investigations in the rare syntopic locations are needed to shed light on this aspect. Previous studies showed somehow contrasting results. For instance, Lindtke *et al.* (2010) hypothesized the occurrence of natural hybridization between *Z. v. vivipara* and *Z. v. carniolica*, on the basis of phenotypic traits, such as egg-retention time and eggshell thickness. Still, genetic data would be required to confirm the actual hybridization in Carinthia (Austria, Lindtke *et al.*, 2010). Indeed, fine-scale analyses on a very-narrow contact zone found genetic reproductive isolation between the two lineages (Cornetti *et al.*, 2015). It should also be remarked that gene flow exists between other mtDNA lineages of *Z. vivipara*. For instance, Mila *et al.*, 2013 found asymmetric introgression among divergent mtDNA lineages of *Z. v. louslantzi* in the Pyrenees. However, the divergence time between such lineages was about 1 million years ago, much more recently than *Z. v. vivipara* and *Z. v. carniolica*.

The two lineages also showed contrasting patterns of genetic structure. In particular, while *carniolica* presented a well-defined genetic subdivision, *vivipara* did not, reflecting opposite demographic histories (Figure 3). The absence of clear genetic structure in *Z. v. vivipara* could reveal either extant gene flow or a recent and continuous expansion of the lineage across the Alps. The gene flow explanation is plausible since *vivipara* tends to live at higher altitudes than *carniolica* (Cornetti *et al.*, 2014) and therefore it is likely less affected by the consequences of recent anthropogenic modifications and habitat discontinuity. Similarly, demographic processes such as recent colonization events and range expansion after last glacial maximum could explain the lack of genetic differentiation, since subpopulation divergence takes a long time to accumulate

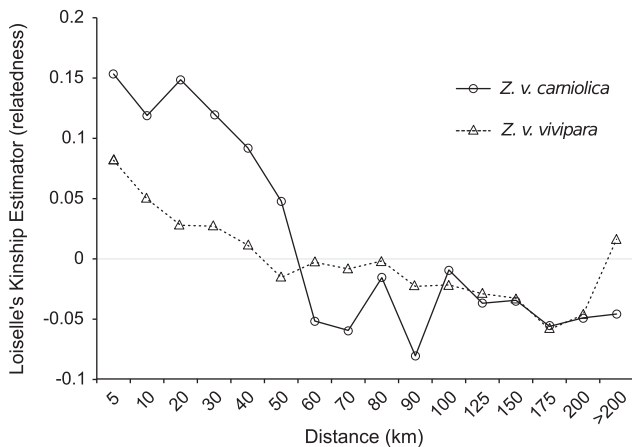


Figure 4 Spatial autocorrelograms for all pairs of individuals within distance classes, comparing *Z. v. vivipara* (dashed lines) and *Z. v. carniolica* (continuous lines).

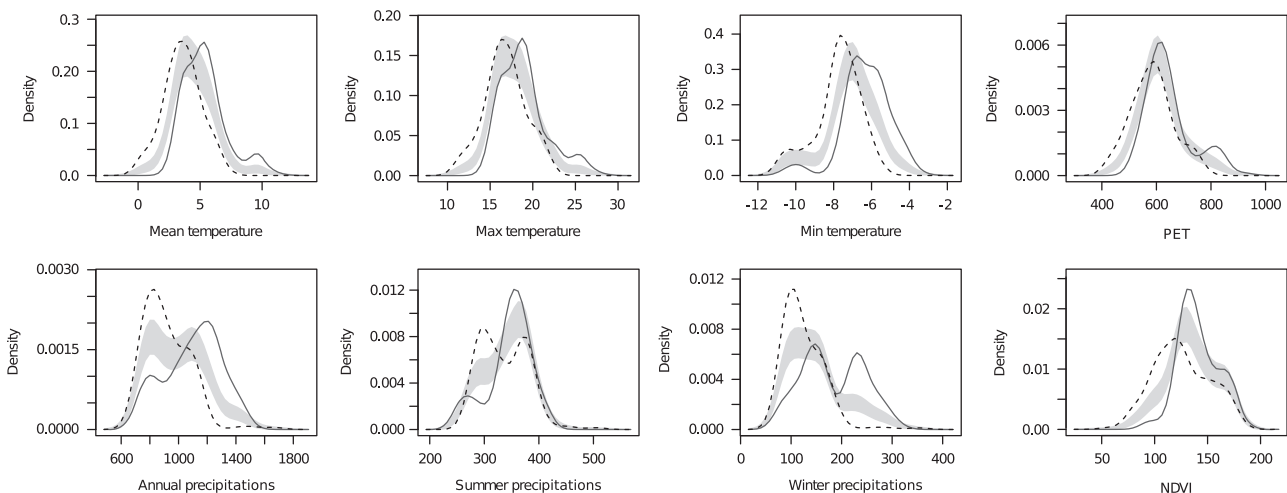


Figure 5 Density profiles comparing the frequency of *Z. v. vivipara* (dashed lines) and *Z. v. carniolica* (continuous lines) along the eight environmental variables: results of the fine-scale analysis. The gray areas represent 95% confidence interval obtained through bootstraps. If the density profile of one taxon is above the 95% confidence interval, it is significantly associated to these environmental conditions; if it is below the 95% confidence interval, it is significantly less frequent than expected by chance. See Supplementary Figure S4 for the broad scale analysis.

Table 2 MtDNA variation in *Z. v. carniolica* and *Z. v. vivipara* lineages

Population	<i>N</i>	<i>H</i>	<i>h</i>	π
<i>Z. v. carniolica</i>				
Ampola	17	1	0	0
Eastern Alps	32	2	0.1210	0.0005
Piedmont	29	5	0.7266	0.0052
Lombardy	22	3	0.4372	0.0017
Rasun	14	1	0	0
<i>Z. v. vivipara</i>				
East of Brenner line	96	4	0.4789	0.0012
West of Brenner line	94	4	0.3747	0.0009

Abbreviations: *H*, number of haplotypes; *h*, haplotype diversity; π , nucleotide diversity.

(Hewitt, 2000). It is also noteworthy that, while the mtDNA shows a much higher diversity for *Z. v. carniolica*, the microsatellite DNA variability shows an opposite pattern. This makes sense as the mtDNA shows the historical structure and the viviparous lineage probably colonized only recently the Alps, while microsatellites reflect current population structure and the recent fragmentation and bottlenecks in the oviparous lowland populations.

Only a longitudinal subdivision in two groups emerged for the *Z. v. vivipara* lineage, dividing populations from western and Eastern Alps, along the so-called 'Brenner line', (including the Adige Valley up to the Brenner Pass, Figure 1; Kerner, 1870). This was confirmed for *Z. v. carniolica*, although showing a stronger genetic structure (five well-defined groups, Figure 3a). When put in the broader context of the European Alps biogeography, the observed pattern of east–west differentiation across the Adige Valley confirms what was detected in some alpine animal and plant species. For instance, molecular data have ascertained genetic differentiation between western and eastern Adige Valley in the high-altitude butterfly *Erebia euryale* (Haubrick and Schmitt, 2007). Some authors (Penck and Bruckner, 1909; Van Husen, 1987) have suggested that two refugia of alpine plant species were present during the glacial oscillations in this region: one in southern Adamello west of the Adige Valley and the other in the Southwestern Dolomites east of the Adige. Indeed, there are plant species such as *Phyteuma globulariifolium*, an Alpine form of rampion, and the alpine speedwell, *Veronica alpina*, that can be genetically subdivided into two major groups, east and west of the Adige Valley (Schönswetter *et al.*, 2002; Albach *et al.*, 2006). More recently, the area between Lake Garda and Innsbruck, including the Adige Valley, has been invoked as a zone of genetic discontinuity delimiting Eastern and Western Alpine populations of many other species (Thiel-Egenter *et al.*, 2009). Interestingly, the 'Brenner line', was first recognized as delimiting eastern and western distributions of plant species in the nineteenth century (Kerner, 1870). The outcome of our survey corroborates the hypothesis that the 'Brenner line' might act as an important barrier for historical recolonization and contemporary gene flow for many alpine species (for example, Crestanello *et al.*, 2009; Thiel-Egenter *et al.*, 2009) and suggests that it may be particularly important for species showing limited dispersal, such as small vertebrates.

Differences in isolation by distance patterns were also observed, with *Z. v. carniolica* displaying higher relatedness at short distances than *Z. v. vivipara*. These results are congruent with the evidence that anthropization and urban sprawl in lowlands/low-elevation valleys has a potential negative effect on biodiversity in the Alps (Nobis *et al.*, 2009) as increasing occurrence of unsuitable habitats might prevent

gene flow (for example, Trizio *et al.*, 2005), and increase inbreeding. For instance, we identified two populations constituting two distinct groups: Ampola and Rasun. These populations are emblematic examples of isolated populations of conservation concern; they showed the lowest estimated number of alleles, allelic richness and heterozygosity (Table 1), and no genetic variation at mtDNA. These results likely reflect the consequences of genetic drift on small and isolated populations.

Conservation managers, in order to prioritize species/populations to be preserved, are usually concerned by changes in N_e , especially recent demographic collapse, which may be revealed in genetic data. One method for detecting a recent reduction in population size (bottleneck) showed no significant reduction in the two lineages of *Z. vivipara*, while different demographic dynamics were revealed by a method for estimating bottleneck occurrence in a more distant past (M_P_VAL). Specifically, while no evidence of population reduction was highlighted for the viviparous lineage, four out of five clusters of *Z. v. carniolica* likely suffered from bottlenecks according to M-ratio results. This indication was confirmed by preliminary Approximate Bayesian Computation analyses (unpublished results), although different demographic dynamics could be biased by unbalanced population sizes.

Ecological differences between the two lineages are strong, and the differentiation was evident both in fine-scale and in range-wide analyses. Although *Z. v. vivipara* and *Z. v. carniolica* are sympatric in the Alpine chain, cases of syntopy are extremely rare, and the two lineages show a niche overlap of only 0.33, a value much lower than expected under equivalency expectations (Supplementary Figure S3). In practice, the two lineages show significant differences for nearly all the analyzed variables. The oviparous lineage tends to occupy more productive habitats, showing precipitation about 50% higher than areas occupied by the viviparous populations (Figure 5). Furthermore, strong differences are detected for variables representing the thermal environment (in particular mean and minimum annual temperature). For example, while *vivipara* is associated to areas with mean annual temperature of about 3 °C, *carniolica* is more frequent in habitats where mean temperature is above 5 °C (Figure 5, Supplementary Figure S4). Such strong differences for ecological niches are likely related to the different reproductive mode showed by the two lineages, and to the biological/ecological processes that determined the transition from oviparity to viviparity. In fact in reptiles the transition toward viviparity is consistently associated with colonization of harsh and cold climates (Pyron and Burbrink, 2014). For instance, *Z. v. vivipara* individuals exhibit a higher cold tolerance than oviparous ones (Voituron *et al.*, 2004) and offspring that develop inside the body of the females for longer are more likely to survive freezing temperatures (Blackburn, 2005), allowing the colonization of high latitudes and altitudes. Differences in thermal tolerance likely determine the distinct altitudinal distribution of the two lineages in the Alps, with the viviparous living at higher altitude than oviparous one, although an overlap of their altitudinal distribution has been documented in the Alpine chain (Cornetti *et al.*, 2014).

The two lineages of *Z. vivipara* have different evolutionary histories, contrasting local and regional genetic structure and well-separated ecological niches. These results and the lack of evidence of hybridization suggest that *Z. v. vivipara* and *Z. v. carniolica* should be considered two separate species and two distinct conservation units, with different management strategies tailored according to their genetic structure and habitat. Particular attention should be given to the oviparous lineage that inhabits low-altitude habitats, which is threatened by increasing impact of human activities in Po Plain

and Southern Alps, especially since population decline and local extinctions have been already reported in this area (Sindaco *et al.*, 2006). If the distinctiveness between the two lineages is overlooked, there is the risk of an unnoticed decline and extinction of the oviparous lineage. Meanwhile *vivipara* has a very broad geographic range (Supplementary Figure S1), mostly stable populations, and is considered a 'least concern' species (IUCN, 2014). Nevertheless, it has also been demonstrated that if evolution of viviparity favored successful colonization of cold climate for reptiles, viviparous lineages are, at the same time, associated with an increased risk of extinction due to rapid climate warming since they are likely to remain adaptively constrained to cold environments (Pincheira-Dinoso *et al.*, 2013) posing, hence, a threat for *Z. v. vivipara* population persistence, as well. In sum, the application of integrative taxonomy allowed the correct identification of evolutionary significant units that should be managed differently with *Z. v. carniolica* managed to avoid extinction because of habitat loss, and *Z. v. vivipara* managed for the impact of climate change.

DATA ARCHIVING

Newly discovered cytb haplotypes are achieved in GenBank (KM522844 - KM522847). Microsatellite genotypes available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.dr4s8>.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Barbara Crestanello, Samuele Ghielmi, Giovanni Giovine and Alexis Marchesini for their help during sampling. Andrea Gandolfi is warmly thanked for his help in figure editing. This research was partially funded by Autonomous Province of Trento (Italy) as part of the ACE-SAP project (University and Scientific Research Service regulation number 23, 12 June 2008) and by FIRS>T (FEM International Research School of Trentino). SH was funded as a Postdoctoral Fellow at the National Institute for Mathematical and Biological Synthesis (NIMBioS), an Institute sponsored by the National Science Foundation through NSF Award #DBI-1300426, with additional support from the University of Tennessee, Knoxville.

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Supplementary Information accompanies this paper on Heredity website (<http://www.nature.com/hdy>)

Supplementary Information

Genetic and ecological data reveal species boundaries between viviparous and oviparous lizard lineages

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Supplementary Methods

Geographic discontinuities in gene flow at intra-lineage level (i.e. *Z. v. vivipara* and *Z. v. carniolica* separately) were detected using spatial autocorrelation methods applied in BARRIER 2.2 (Manni *et al.*, 2004). Barriers were calculated using F_{st} pairwise distance matrix among populations along with geographic coordinates for each of the analysed location. The software creates a network of geo-referenced positions using Delauney triangulation and applies Monmonier's algorithm to identify areas of high genetic distance within the network.

Results obtained with BARRIER were consistent with the genetically homogeneous clusters identified by STRUCTURE 2.3.2 (Pritchard *et al.*, 2000) within each lineage. Specifically, for *Z. v. carniolica* (Supplementary Figure S7) the largest barrier divides Ampola, Rasun and Eastern Alps populations in the East (A, where Ampola is really close to Garda Lake and Brenner Line) and Lombardy and Piedmont populations in the West. The second largest barrier isolates Rasun (B), the third separates Lombardy and Piedmont (C), the fourth identifies a single population (D, Segusino) not identified by STRUCTURE and the fifth makes Ampola population isolated (E).

For *Z. v. vivipara* (Supplementary Figure S8), the largest barrier was found exactly on the Brenner Line (A), the second one (B) separates Alpe Luson population, the third (C), fourth (D) and fifth (E) largest barriers are identified in the western side of Brenner Line, confirming that the eastern side represents a single population as outlined by STRUCTURE analysis (see below).

We ran a hierarchical STRUCTURE analysis for *Z. v. vivipara*. Specifically, after identifying

two main genetic clusters, we investigated the genetic structure of each of the two groups (i.e. east and west of the Brenner Line) independently. We found that the East group likely consists of one single cluster (Supplementary Figure S9a-b), while the most likely number of genetic groups in the West cluster is three, according to both LK and ΔK (Supplementary Figure S9c-d). The STRUCTURE plot relative to the latter group highlights that longitude seems to explain the genetic subdivision (Supplementary Figure S9e). From these results we conclude that there probably is a weak substructure in the Western group (weaker than the other lineage (i.e. *Z. v. carniolica*), see Figure 3) that determined the secondary peak in Supplementary Figure S6b.

Supplementary figures

Figure S1. Distribution of sampling localities with associated genetic data, covering the whole range of *Z. v. carniolica* (pink dots) and *Z. v. vivipara* (green triangles).

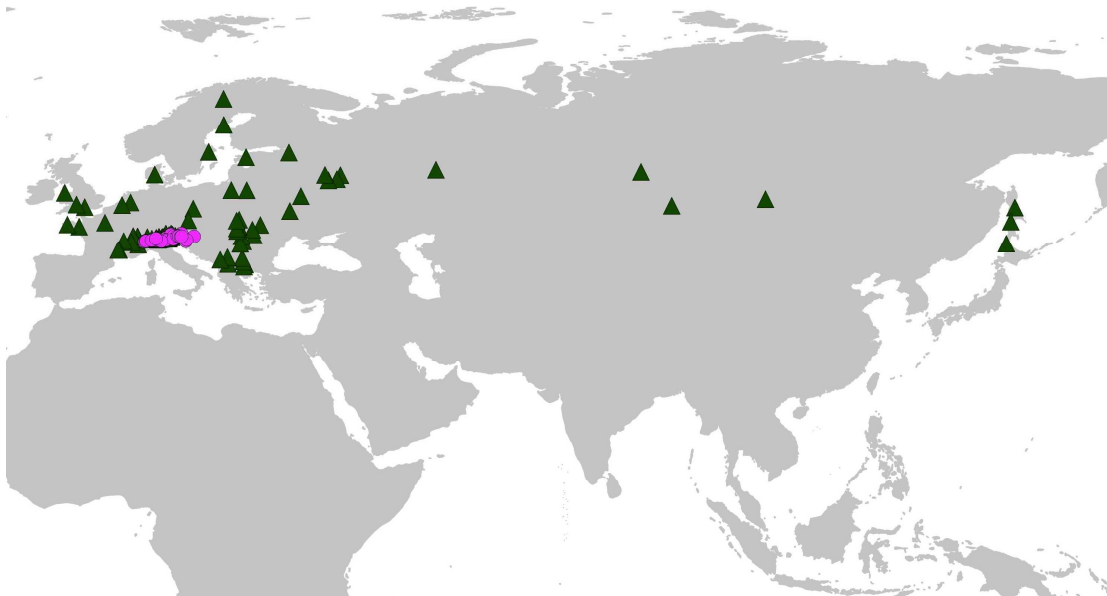


Figure S2. Estimates of best (K) number of genetically homogeneous groups according to the methods by (a) Evanno et al. (2005) and by (b) Pritchard et al. (2000) in the entire dataset.

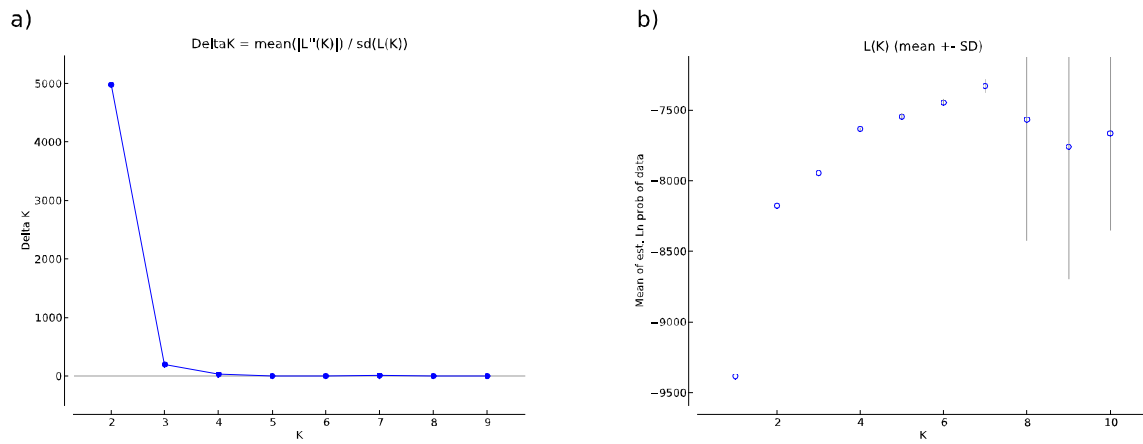


Figure S3. Results of the PCA-env test, comparing the realized ecological niche of *Z. v. vivipara* (dashed lines) and *Z. v. carniolica* (continuous lines). a) Niche of the *vivipara* lineage and b) of the *carniolica* lineage along the two first axes of the PCA. Grey shading represents the density of occurrences of the two lineages. The solid and dashed lines represent, respectively, 100% and 50% of the available (background) environment. (c) Contribution of the eight environmental variables on the two axes of the PCA and the percentage of inertia explained. d-f) Observed niche overlap D between the two ranges (bars with a red diamond) and simulated niche overlaps (grey bars) on which tests of niche equivalency d) and similarity (e-f) tests are calculated. The significance of tests is also shown. See Broennimann *et al.* (2012) for additional details.

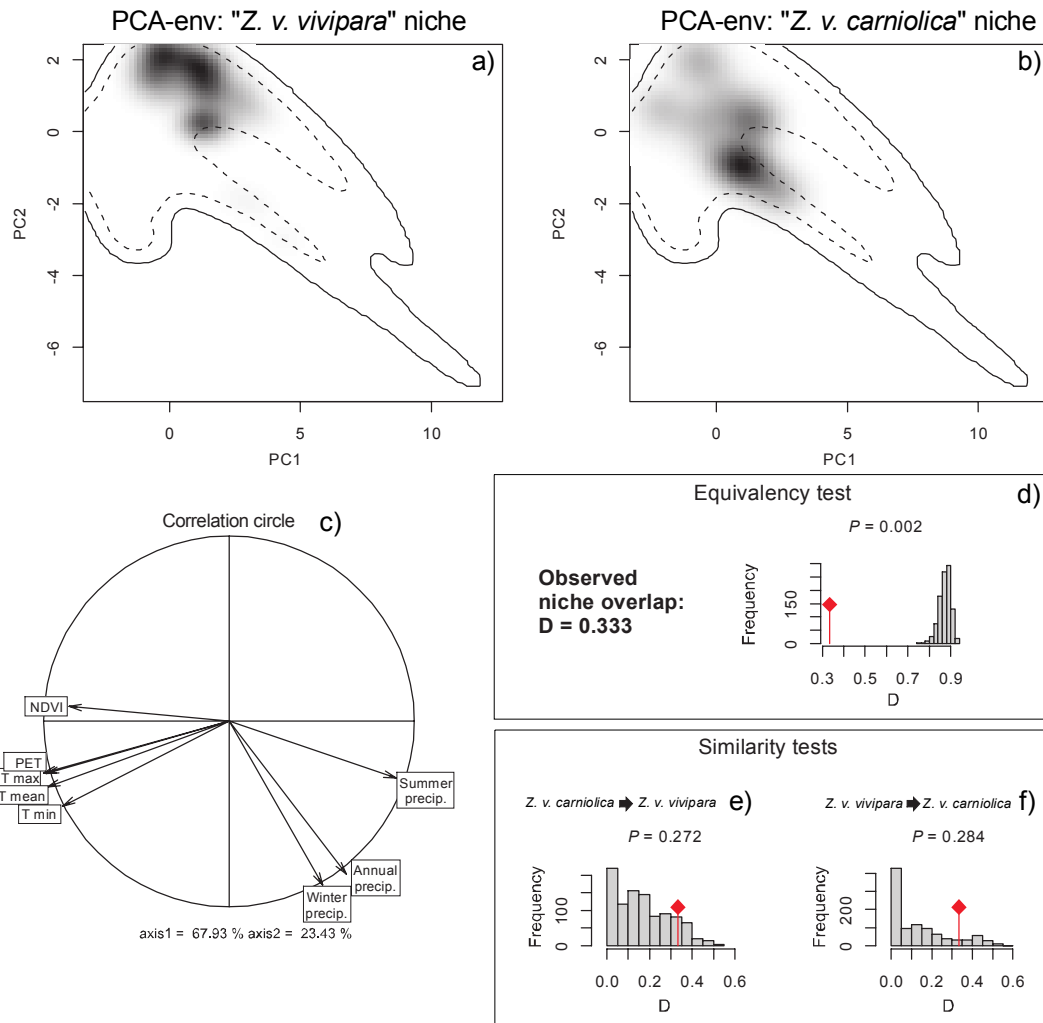


Figure S4. Density profiles comparing the frequency of *Z. v. vivipara* (dashed lines) and *Z. v. carniolica* (continuous lines) along the eight environmental variables: results of the broad scale analysis. The blue areas represent 95% CI obtained through bootstraps. If the density profile of one taxon is above the 95% CI, is significantly associated to these environmental conditions; if it is below the 95% CI, it is significantly less frequent than expected by chance.

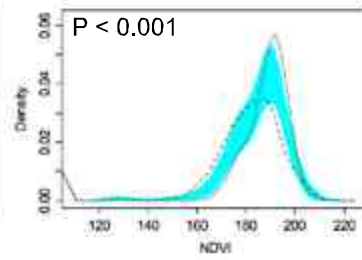
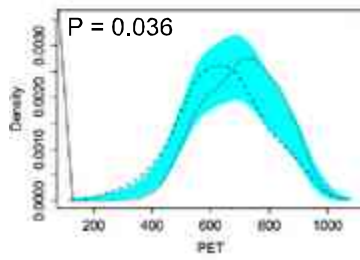
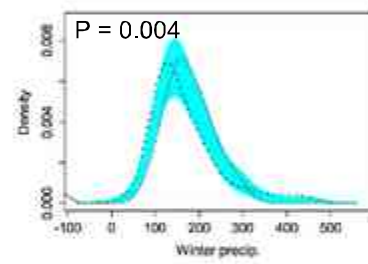
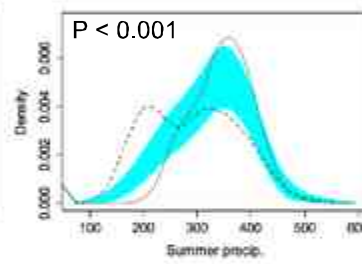
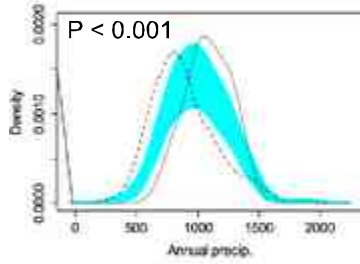
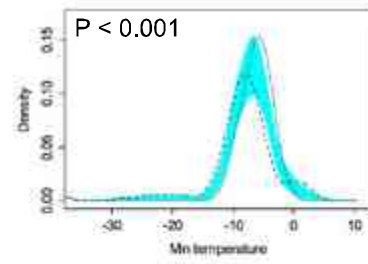
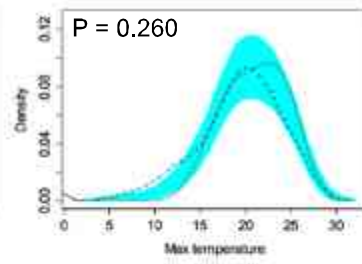
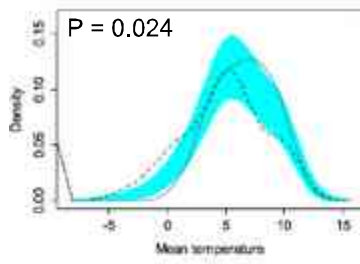


Figure S5. Estimates of best (K) number of genetically homogeneous groups according to the methods by (a) Pritchard *et al.* (2000) and by (b) Evanno *et al.* (2005) in *Z. v. carniolica*.

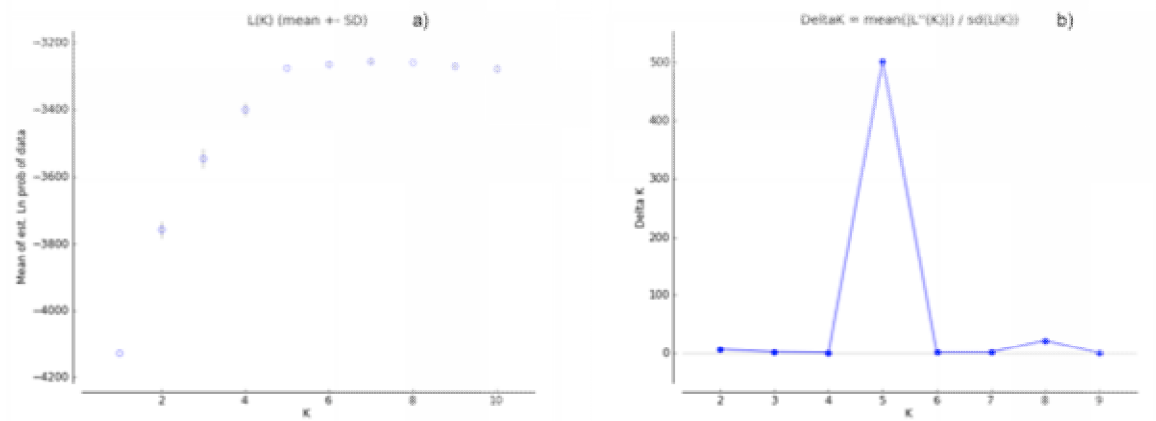


Figure S6. Estimates of best (K) number of genetically homogeneous groups according to the methods by (a) Pritchard *et al.* (2000) and by (b) Evanno *et al.* (2005) in *Z. v. vivipara*.

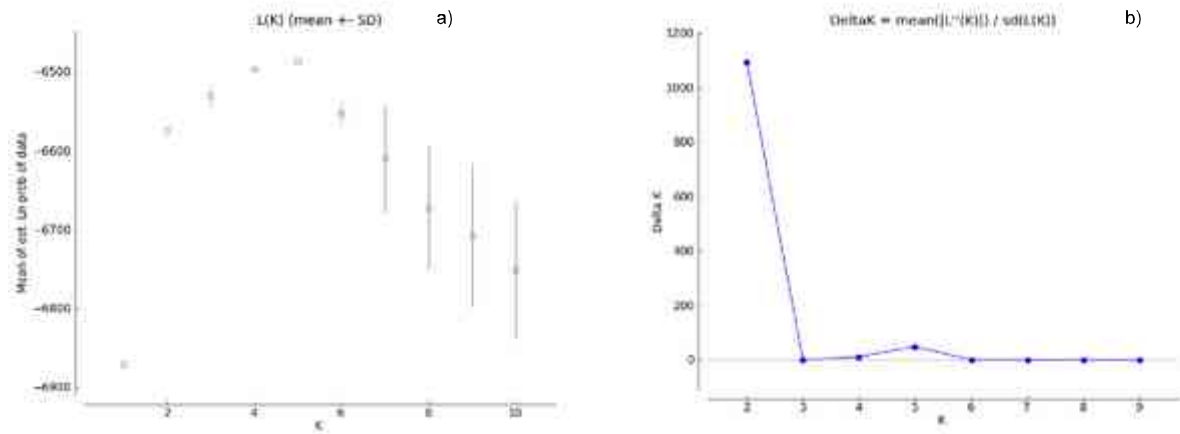
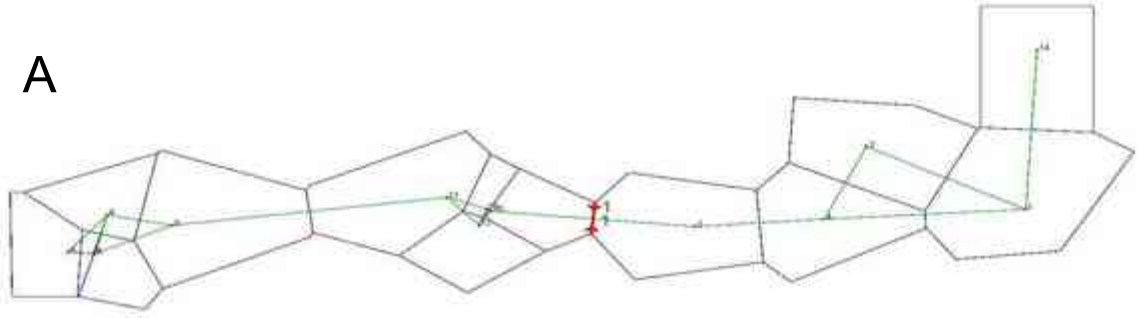
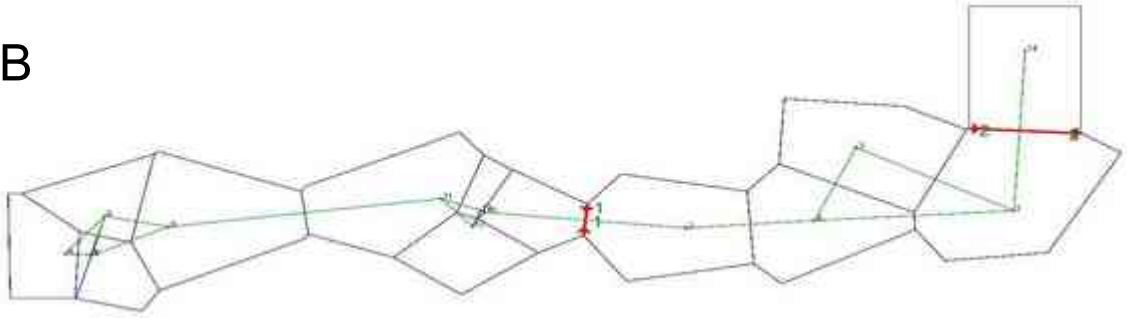


Figure S7. Report of the first five largest genetic barriers identified in *Z. v. carniolica* across the Alps. Labels of the populations used in the analysis (for more details on the populations, see Table S1b): 1, Treviso; 2, Ampola; 3, Palù Longa; 4, Costa; 5, Monte Barone; 6, Valle Strona; 7, Val Sessera; 8, Monte Croce; 9, Val Dolca; 10, Valgoglio; 11, Averara; 12, Serina; 13, Roncobello; 14, Rasun.

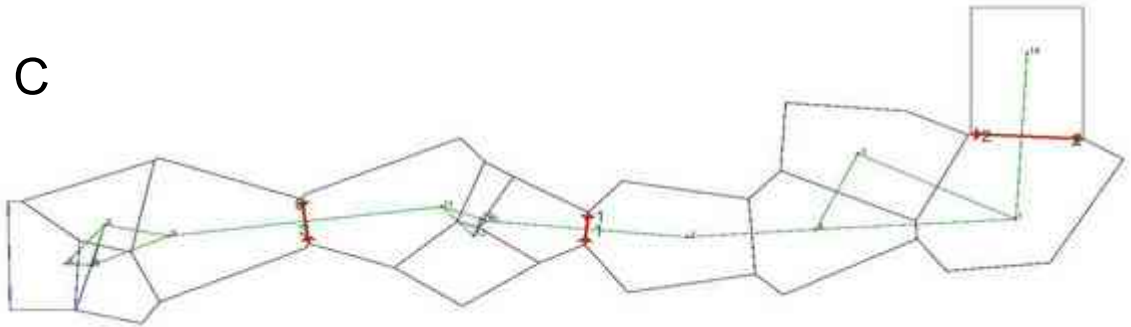
A



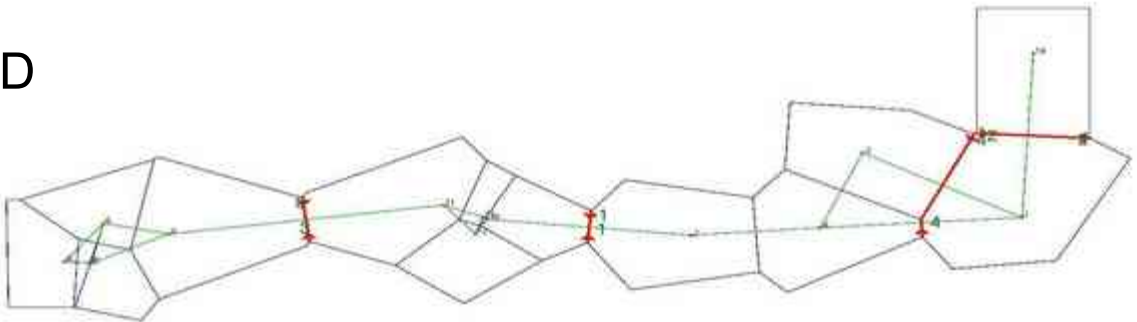
B



C



D



E

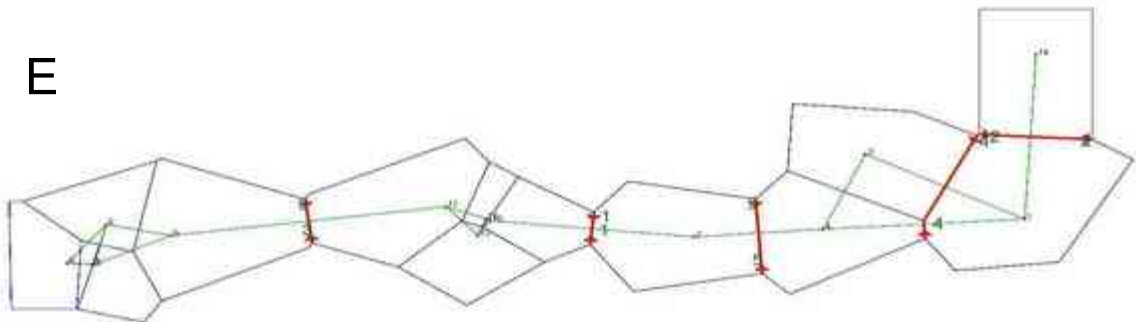
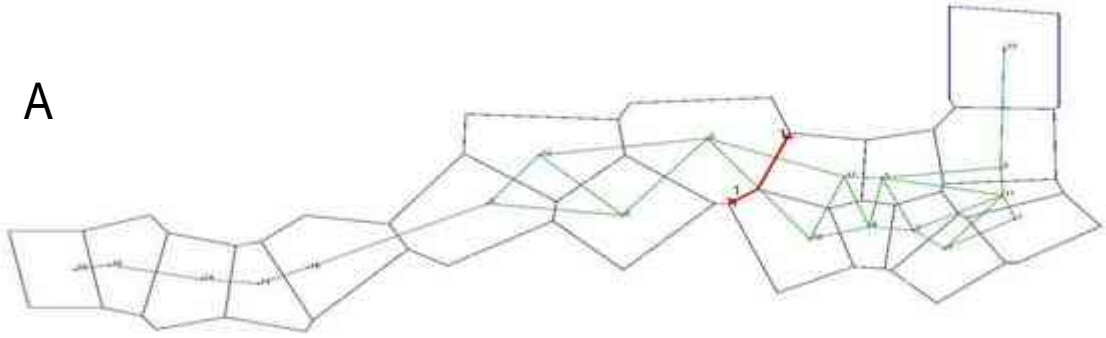
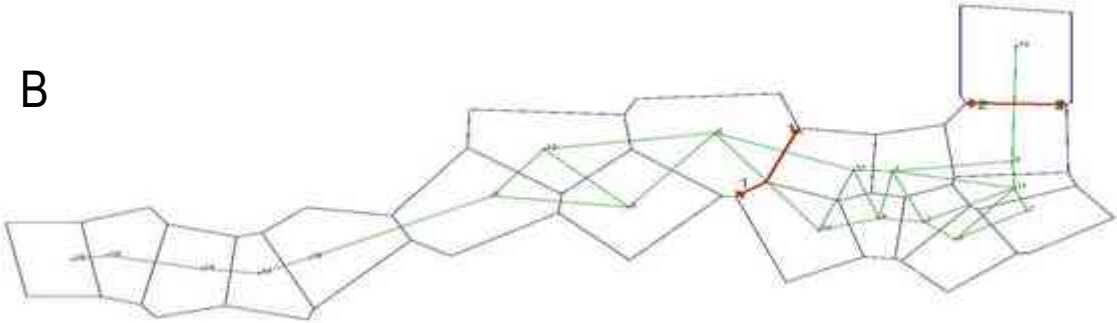


Figure S8. Report of the first five largest genetic barriers identified in *Z. v. vivipara* across the Alps. Labels of the populations used in the analysis (for more details on the populations, see Table S1a): 1, Passo Lavazè; 2, Lasteati; 3, Masi Carretta; 4, Passo Manghen; 5, Passo Tonale; 6, Palù Tremole; 7, Lago Calaita; 8, Campo Carlo Magno; 9, Passo San Pellegrino; 10, Passo Redebus; 11, Passo Rolle; 12, Lago Lama; 13, Vilminore; 14, Gandellino; 15, S.Brigida; 16, Schilpario; 17, Redagno; 18, Valleve; 19, Alpe Luson.

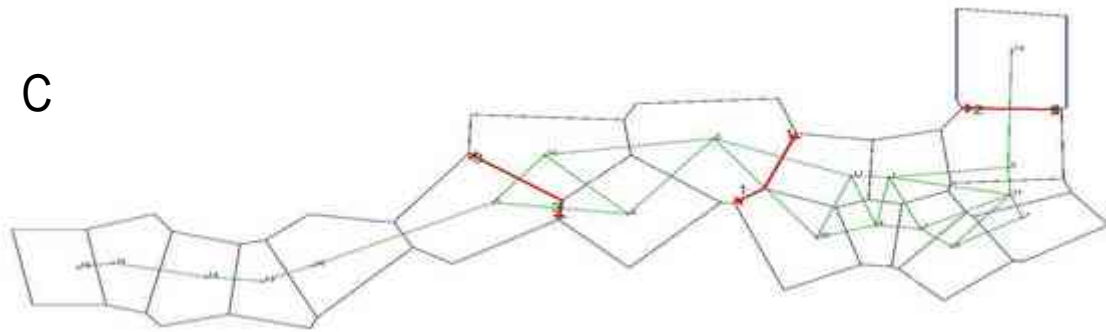
A



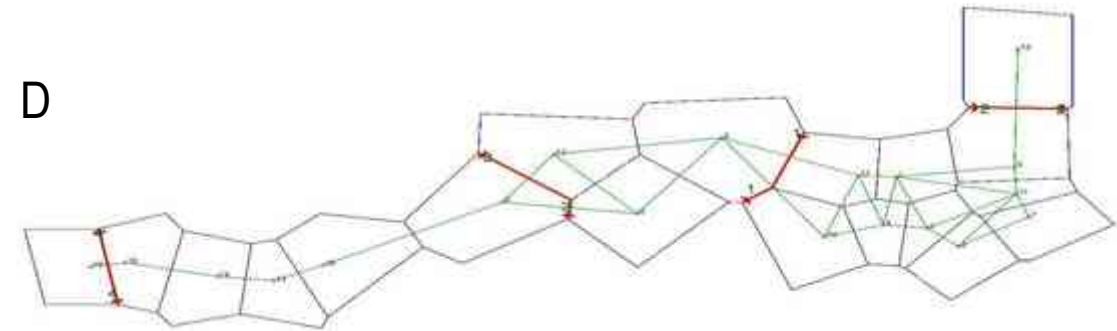
B



C



D



E

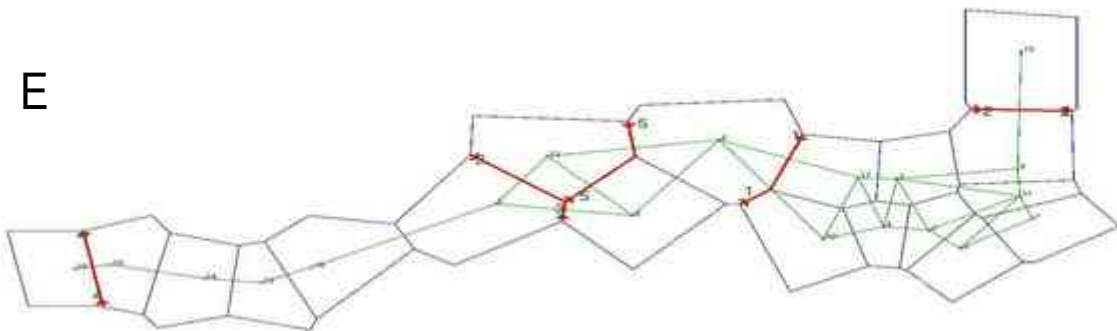
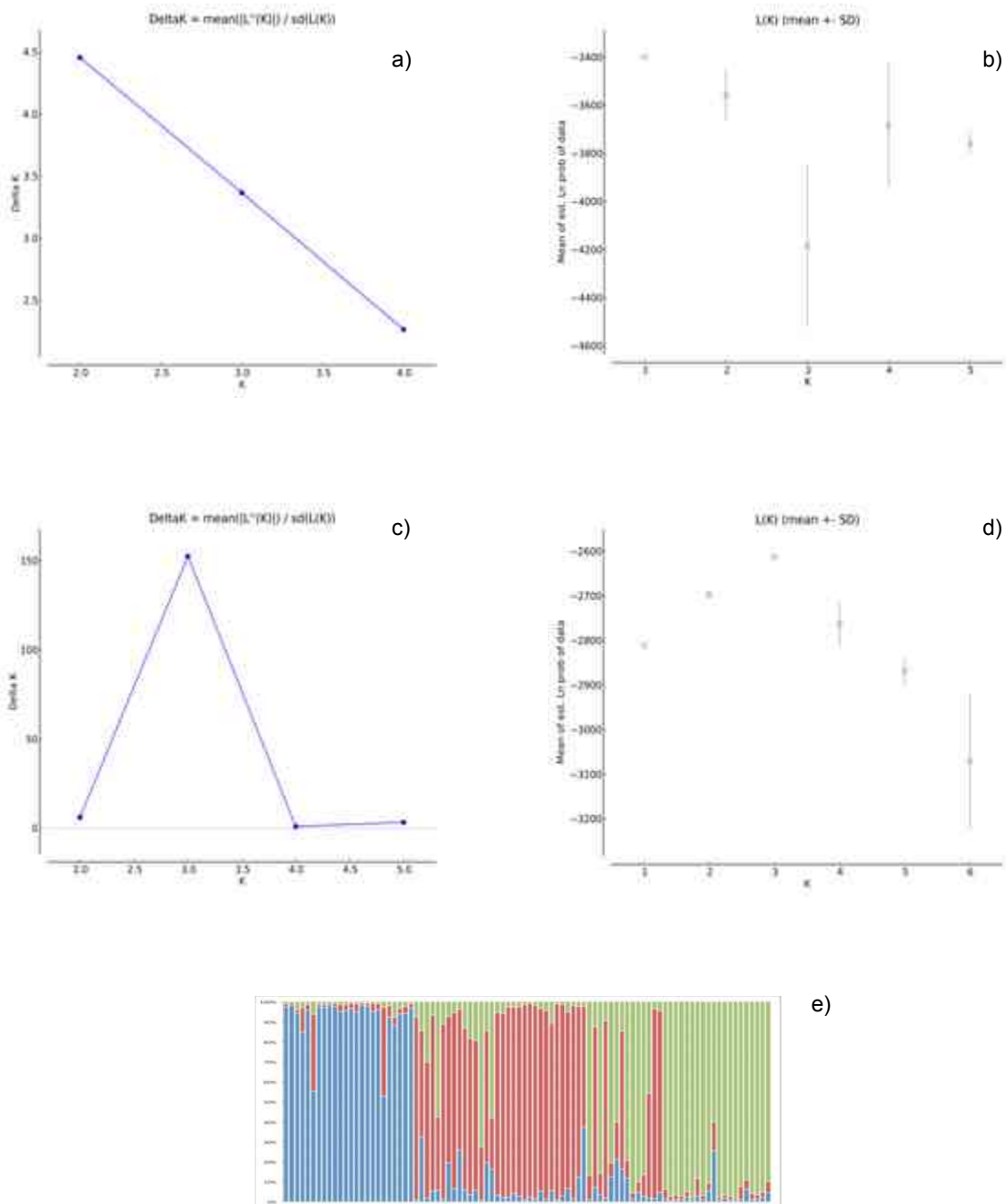


Figure S9. Estimates of best (K) number of genetically homogeneous groups in *Z. v. vivipara* Eastern clade according to the methods by (a) Evanno *et al.* (2005) and (b) Pritchard *et al.* (2000). Estimates of K in *Z. v. vivipara* Western clade according to the methods by (c) Evanno *et al.* (2005) and (d) Pritchard *et al.* (2000). STRUCTURE plot relative to Western clade using K=3 and with individuals ordered by longitude (from West to East, e).



Supplementary tables

Table S1. Sampling site details across the European Alps. Number of samples collected for each location (N), GPS Coordinates, altitude and mtDNA cytb haplotype with numbers in brackets referring to the frequency of each haplotype for *Z. v. vivipara* (a) and *Z. v. carniolica* (b).

a)

Location	N	GPS Coordinates		Altitude (m a.s.l.)	MtDNA haplotype (frequency)
		East	North		
Passo Tonale	20	10.5731	46.2553	1850	VB1 (19), VT_23 (1)
Lasteati	6	11.5580	46.1676	2080	VB1 (4), VB11 (2)
Masi Carretta	12	11.6311	46.1067	1305	VB1 (2), VB11 (10)
Palù Longia	6	11.0819	46.4717	1565	VB1 (6)
Palù Tremole	14	11.0753	46.4789	1720	VB1 (13), VB11 (1)
Lago Calaita	7	11.7939	46.2058	1660	VB1 (6), VB11 (1)
Campo Carlo Magno	7	10.8851	46.2171	1650	VB1 (7)
Passo San Pellegrino	6	11.7608	46.3754	1795	VB11 (6)
Passo Lavazè (peatbog)	14	11.4869	46.3404	1565	VB11 (14)
Passo Lavazè (lake)	5	11.4930	46.3562	1805	VB11 (5)
Passo Redebus	3	11.3191	46.1382	1435	VB1 (1), VB11 (2)
Passo Rolle	3	11.7661	46.2833	1915	VB11 (3)
Passo Manghen	28	11.4516	46.1777	2050	VB1 (21), VB11 (5), VT_25 (1), VT_24 (1)
Lago Lama	4	10.6923	46.4165	2275	VB1 (4)
Vilminore	5	10.0417	45.9906	1640	VB1 (5)
Gandellino	4	9.9119	46.0050	1720	VB1 (4)
Valbondione	4	10.0108	46.0225	1290	VB1 (4)
Biotopo 'I Mughì'	4	11.6106	46.0978	1220	VB1 (2), VT_24 (1), VT_25 (1)
Santa Brigida	3	9.6167	46.0375	1600	VL_26 (3)
Averara	6	9.6244	46.0369	1780	VL_26 (6)

Cusio	1	9.5961	46.0119	1810	VL_26 (1)
Valmora	6	9.6217	46.0342	1575	VB1 (2), VL_26 (4)
Mezzoldo	2	9.6272	46.0283	1720	VB1 (1), VL_26 (1)
Schilpario	6	10.1589	46.0378	1800	VB1 (6)
Redagno	2	11.3986	46.3447	1540	VB11 (2)
Valleve	2	9.6969	46.0517	1830	VB1 (2)
Lago Morasco	1	8.4028	46.4236	1790	VB1 (1)
Ridanna	1	11.2500	46.9417	1715	VB1 (1)
Vallmigalm	1	11.3000	46.9472	1815	VB11 (1)
Passo Brocon	1	11.6861	46.1202	1670	VB11 (1)
Siror	1	11.7719	46.2714	1680	VB11 (1)
Passo Valles	1	11.7994	46.3394	2030	VB11 (1)
Lago di Campo	1	10.4934	46.0387	1975	VB1 (1)
Alpe Luson	3	11.7689	46.7692	1900	VB11 (3)
Total	190				

b)

Location	N	GPS Coordinates		Altitude	MtDNA haplotype (frequency)
		East	North	(m a.s.l.)	
Segusino	4	12.0286	45.9631	1240	OS8 (4)
Lago d'Ampola	17	10.6553	45.8719	735	OS6 (17)
Palù Longa	21	11.3671	46.2955	1435	OS8 (19), OT_11 (2)
Costa	3	11.1925	45.9108	1250	OS8 (3)
Inghiaie	1	11.3103	45.9975	450	OS8 (1)
Monte Grappa	2	11.3025	45.8722	1700	OS8 (2)
Santa Colomba	1	11.1817	46.1249	950	OS8 (1)
Monte Barone	9	8.1667	45.7275	1650	OS4 (6), OS3 (2), OL_15(1)
Monte Mottarone	2	8.2197	45.9336	860	OS5 (2)
Valle Strona	4	8.2958	45.8892	1500	OS5 (4)
Alpe di Mera	1	8.0706	45.7397	1630	OS4 (1)
Val Sessera	6	8.0978	45.6658	1500	OS4 (2), OL_16 (3), OL_14 (1)
Monte Croce	2	8.4867	45.8778	1660	OS5 (2)
Bocchetta Luvera	1	8.1608	45.7292	1300	OS4 (1)
Valle Dolca	4	8.0656	45.7375	1700	OS4 (3), OL_14 (1)
Ardesio	2	9.8492	45.8667	1610	OL_11 (2)
Valgoglio	2	9.9069	45.9904	1420	OL_11 (2)
Averara	1	9.6286	46.0267	1420	OS3 (1)
Valmora	6	9.6286	46.0267	1510	OS3 (6)
Cusio	1	9.6107	45.9927	1120	OS3 (1)
Ornica	2	9.5608	45.9983	1330	OS3 (2)
Serina	4	9.7603	45.8794	1290	OS3 (4)
Oneta	1	9.7936	45.8636	1320	OL_12 (1)

Roncobello	1	9.7917	45.9653	1880	OL_11 (1)
Branzi	1	9.7850	46.0028	1790	OS3 (1)
Cascinetto di Menna	1	9.7528	45.9067	1405	OS3 (1)
Rasun	14	12.0753	46.8083	1100	OS2 (14)
Total	114				

Table S2. Thermocycling condition of microsatellites loci amplifications and genotyping. The 11 loci were amplified in 4 multiplexes with an initial incubation at 94°C for 10 min, followed by 30 cycles of 94°C for 1 min, annealing temperature (see table) for 45 s, and 65 °C for 1 min, with a final extension of 65 °C for 10 min. PCR amplifications were conducted in a final volume of 20 µl containing: 1µl of template DNA, 2 µl HotMaster™ Taq Buffer 25 mM Mg2 (Eppendorf), 100 µM dNTPs, variable concentration of each pair of primer, 1 unit of HotMaster™ Taq Polymerase (Applied) and ultra-pure water. PCR labeled products were run on a four capillary system ABI 3130 Genetic Analyzer (Applied Biosystem) and scored with an internal lane standard (ROX) using GeneMapper software.

	Amplified loci	Annealing temperature
Multiplex 1	B107, D109, B114, Lv-2-145	50°C
Multiplex 2	Lv-4-115, Lv-3-19	55°C
Multiplex 3	Lv-4-X, Lv-4-72	51°C
Multiplex 4	Lv-4-alpha, C103	52°C

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