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Conservation genetics of the yellow-bellied toad (*Bombina variegata*) and the common lizard (*Zootoca vivipara*) in the Italian Alps

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General introduction

Global change in mountain ecosystems

Mountain ecosystems are generally considered to be more vulnerable than other areas to global changes (Beniston 2003). The European Alps, the highest mountain system in Europe, is about 1000 km long, ranging from near the Mediterranean Sea on the border between France and Italy, to Slovenia, through northern Italy, Switzerland, southern Germany and Austria. The broadest section of the Alps is over 260 km wide. As many of the most important mountainous regions (e.g. Andes, Rocky Mountains, Himalayas; Rabatel *et al.* 2013, Rieman & Isaak, 2010; Tse-ring *et al.* 2010), the Alpine environment is heavily affected by global change, in terms of both climate change and land use (Cannone *et al.* 2008; Vanham *et al.* 2009; Keiler *et al.* 2010; Huggel *et al.* 2010). Temperatures in the Alps have increased in the last century twice that of the global average (Brunetti *et al.* 2009). This exceptional temperature change is leading to profound modifications of mountain ecosystems; for example, decreasing snow and glacier cover (IPCC 2007) and changing hydrological systems. In addition, projected scenarios of changing climatic conditions (frequency and intensity of precipitation, temperature, etc.) may even worsen the current scenario (EEA 2009). In the last few decades human activities have also modified the Alpine landscape by both increased exploitation of the natural environment and decreased practice of traditional agricultural activities (Chemini & Rizzoli 2003). For example, urbanization and tourism are threatening the last natural areas leading to habitat fragmentation and loss of biodiversity in many Alpine valleys and lowlands (WWF 2013). On the other hand, abandonment of mountain fields and traditional activities, such as grazing and mowing, is resulting in forest expansion, causing changes in species biology and distribution (Chemini & Rizzoli 2003). Leonelli *et al.* (2011) observed a 115-metre upward shift of the tree-line during the last century, influencing the geomorphology of high-elevation habitats. Similarly, human impact (in term of change in land use) and modified climatic conditions have favored alien plant species invasions whose distribution may even increase in the near future under current global warming scenarios (Dainese *et al.* 2013).

Climate change is also predicted to increase the duration and frequency of heat waves (Meehl & Tebaldi 2004). The effects of such record-breaking heat wave, like

that which occurred in Europe during the summer 2003, have been described as the cause of increased mortality (without sign of recovery after 4 years) of plant species living in peatlands (Bragazza 2008); and glaciers melted eight times the annual mean of the period 1960-2000 (Paul *et al.* 2005). It is expected that the ongoing increase in greenhouse gases in the atmosphere will cause more severe heat waves in the future (Meehl & Tebaldi 2004); consequently, wetlands (such as bogs, swamps, marsh) are considered among the most threatened habitats from climate change worldwide (Moore *et al.* 2002) and in particular in the Alps, where the warming effects are amplified (Hansen *et al.* 2006).

The negative impact of global change on animal species in the Alps

Undoubtedly, the effects of global warming and change in land use have also had an impact on animal life histories and distribution. For example, Tafani *et al.* (2013) showed a continuous decrease in litter size of the Alpine marmot (Tafani *et al.* 2013) in correlation with reduced snow cover over the last 20 years. Increasing temperatures have also facilitated species invasions in the Alps, such as that of the Tiger mosquito *Aedes albopictus* (Roiz *et al.* 2011) with future scenarios predicting a northwards spread for this species (Neterler *et al.* 2013). The presence and distribution of some avian species are affected by the expansion of shrubs and forests due to the abandonment of mountain fields and meadows: in fact, grassland species, such as the rock partridge *Alectoris graeca,* have decreased in numbers in the last few decades. Similarly, the capercaille *Tetrao urogallus* is now endangered in the Alpine chain because of overhunting and decrease in forest management. On the contrary, change in land use favors the increase in density and diffusion of disease vectors (such as the tick *Ixodes ricinus*) and their main hosts (such as rodents and deer; Chemini & Rizzoli 2003). These trends can also have an impact on human health since it has been demonstrated that host (i.e. deer) abundance is among the most essential factors driving the spread of zoonoses, such as the potentially devastating tick borne encephalitis (TBE; Rizzoli *et al.* 2009).

Preserving biodiversity: the role of genetic analyses

The International Union for Conservation of Nature (IUCN) recognizes that biodiversity must be preserved at ecosystem, species *and* genetic levels. Wild species survival relies on the maintenance of all these levels and nowadays, not surprisingly, a

high number of species is considered "endangered" by the IUCN. Although extinction can be considered a natural phase of evolutionary processes, in this era of global change, the planet is facing an important loss of biodiversity due, directly or indirectly, to human activities. In fact, overexploitation of natural populations (hunting, fishing, etc.), habitat deterioration and fragmentation, pollution, introduction of alien species or pathogen outbreaks, interfere with populations survival, usually leading to reduction in population size. Small populations are prone to be affected by inbreeding and loss of genetic diversity, with negative consequences on the individual fitness and on the species or population evolutionary potential to adapt to a changing environment. In fact, the effects of genetic drift, which is the change in allele frequencies due to random sampling, are stronger in small populations. The integration of environmental and genetic factors that could lead to the shrinking of a natural population is described in the extinction vortex (Figure 1). In order to preserve global biodiversity, especially in regions were risks are higher, conservation biologists should therefore study the conservation status of wild species including always the analysis of genetic variation patterns within and between species.

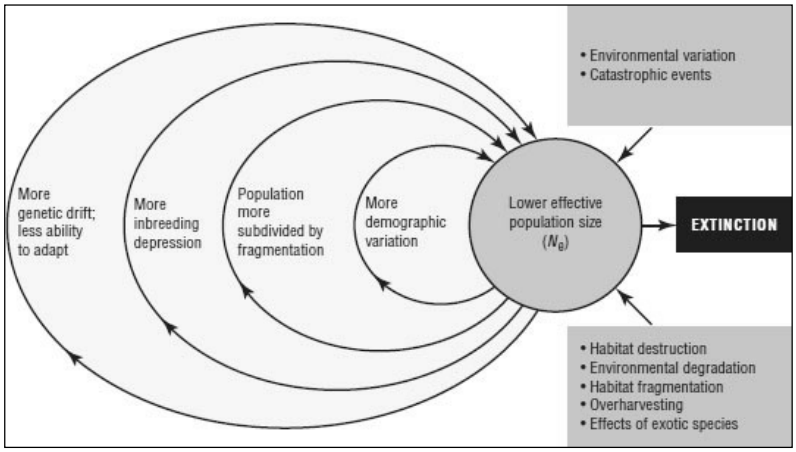


Figure 1. The extinction vortex

An emerging discipline: conservation genetics

Conservation genetics is the use of genetics to aid in the conservation of population or species (Frankham *et al.* 2002). More specifically, conservation genetics can also be described as the science that attempts to preserve, from human-mediated environmental modifications, the current genetic diversity produced by evolution during the history of life in 3.5 billion years on the Earth (Eisner *et al.* 1995). Several

scientific fields contributes to conservation genetics, the most relevant being evolution, ecology, population genetics, phylogenetics, molecular biology and mathematical modelling. And several issues are addressed by conservation genetics, such as the loss of genetic variation in small populations or the identification of genetically distinct groups within a species, but also resolving taxonomic uncertainties and using molecular tools to identify illegal hunting. Very importantly, conservation genetics helps biodiversity managers to define conservation priorities and management strategies, and can detect conservation threats (for example, gene flow barriers or low evolutionary potential in populations with large census size) earlier than traditional population biology and ecology methods (Hoban *et al.* 2013).

The approach of conservation geneticists starts from the study of genetic markers, taking advantage of standard molecular biology techniques (Polymerase Chain Reaction (PCR), sequencing, genotyping, etc.) or from recently developed Next Generation Sequencing (NGS) technologies. Genetic information can be obtained even from a few milligrams of biological samples by means of semi- or non-invasive sampling. For example, high DNA/RNA quality can be achieved from hair, faeces or feathers, from biological samples that can be obtained without handling the animals. Similarly, genetic typing is now feasible from ancient samples available in museum collections.

Three major aims of a conservation genetics analysis, relevant for this thesis, are now briefly described.

Identifying conservation units. Genetic analyses are very useful for detecting evolutionary distinct groups of animals within a species. These groups are usually identified as "Evolutionary Significant Units" (ESUs). Many proposed definitions of ESU exist, but an ESU can be operatively summarized as a population or a group of populations that deserve separate management or priority for conservation because of its high genetic and ecological distinctiveness. Only the integration of genetic and ecological data reinforces the reasons for ESU preservation.

Estimating demographic dynamics. Genetic data are useful for estimating evolutionary parameters in natural populations. In particular, the pattern of genetic variation can be used to estimate the effective population size (N_e) and its dynamic in

time. N_e is a fundamental parameter that describes the number of individuals in an ideal population having the same amount of genetic drift as in the actual population. Many important processes in evolution and conservation, such as the reduction of fitness due to inbreeding and the capability to adapt to new environments, depend on the effective, and not the census, population size. And the effective population size can be reduced by several factors even when the census size is high, such as unequal sex ratio, non-random number of progeny and fluctuating population size. Conservation geneticists are concerned by changes in N_e and particularly by the reduction in effective population size, which is defined as a bottleneck. A bottleneck is a sharp decrease in the population size that can lead to reduction of genetic diversity and effective size, promoting the stochastic, and often negative, effects of genetic drift, and thus an additional reduction in population size. A bottleneck event can be viewed as the first step in the extinction vortex (Figure 1).

Identifying genetic fragmentation. Genetic data are also useful for understanding the level of population fragmentation and estimate the divergence among demes. Habitat fragmentation, due to anthropogenic pressure and, consequently, population subdivision are risk factors contributing to extinction of natural populations. Genetic substructure is the result of the lack (or reduced amount) of gene flow, which can counteract the effects of genetic drift. In order to preserve biodiversity, conservation plans are needed for reducing the consequences of fragmentation on wild populations.

In this thesis, I analyzed two vertebrate species living in the Italian Alps, whose distribution is strictly correlated with wetland habitats. The thesis is subdivided into four chapters, corresponding to four different studies. Before describing in detail the studies, I present here the two species, the study area, and provide a short summary of the specific aims of each study.

Studied species

Bombina variegata

Subphylum Vertebrata
Class Amphibia
Order Anura
Family Bombinatoridae
Genus *Bombina*
Species *Bombina variegata*

The yellow-bellied toad, *Bombina variegata* is a small (3-6 cm in length) aquatic toad. Its name derives from its brightly coloured orange-yellow ventral surface with irregular dark markings; its dorsal surface has a wrinkled grey-brown skin. It is well known for the faint but melodic mating call of the males, which can be heard during the mating season (usually from April to August).

B. variegata is found in central-southern Europe, including northern Italy (Figure 2). Its preferred habitats are ephemeral sites, such as small ponds, puddles, river loops and even wheel-ruts, where it reproduces several times a year, usually after heavy rainfalls that fill temporary basins. Egg deposition, which consists of a clutch of 45 to 100 eggs, peaks between the end of April and the end of June, depending on the altitudinal-latitudinal distribution of the population. Larval development is rapid, taking about 40 days. *B. variegata* can live for more than 10 years in the wild, but records of even 20 years have been reported (Dino *et al.* 2010). Adults use the venomous mucus produced by their skin and bright ventral colours as warning signals to predators.

Figure 2. Current distribution of *Bombina variegata* (in yellow, from IUCN 2013).

Zootoca vivipara

Subphylum Vertebrata
Classe Reptilia
Ordine Squamata
Famiglia Lacertidae
Genere *Zootoca*
Specie *Zootoca vivipara*

The common lizard *Zootoca vivipara* (formerly *Lacerta vivipara*) has a squat body that reaches a length of 15-20 cm, including the tail. The dorsal surface is brown to grey, often with a darker streak running the entire length of the body. Small yellow-white spots are often present on its sides. The ventral surface shows sexual dimorphism: males have bright coloration, from yellow and orange to vermillion with black spots, while females have yellowish-grey unspotted belly.

Zootoca vivipara has a wide distribution throughout Europe and Asia, reaching north of the Artic Circle. In the Mediterranean area it mainly occurs in mountainous regions (Alps, Balkans and Pyrenees, Figure 3).

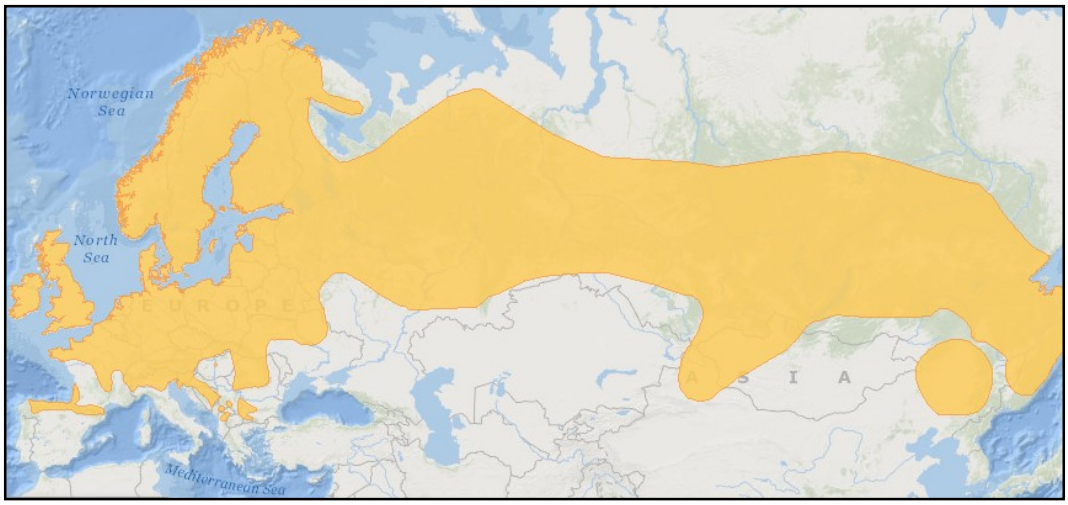


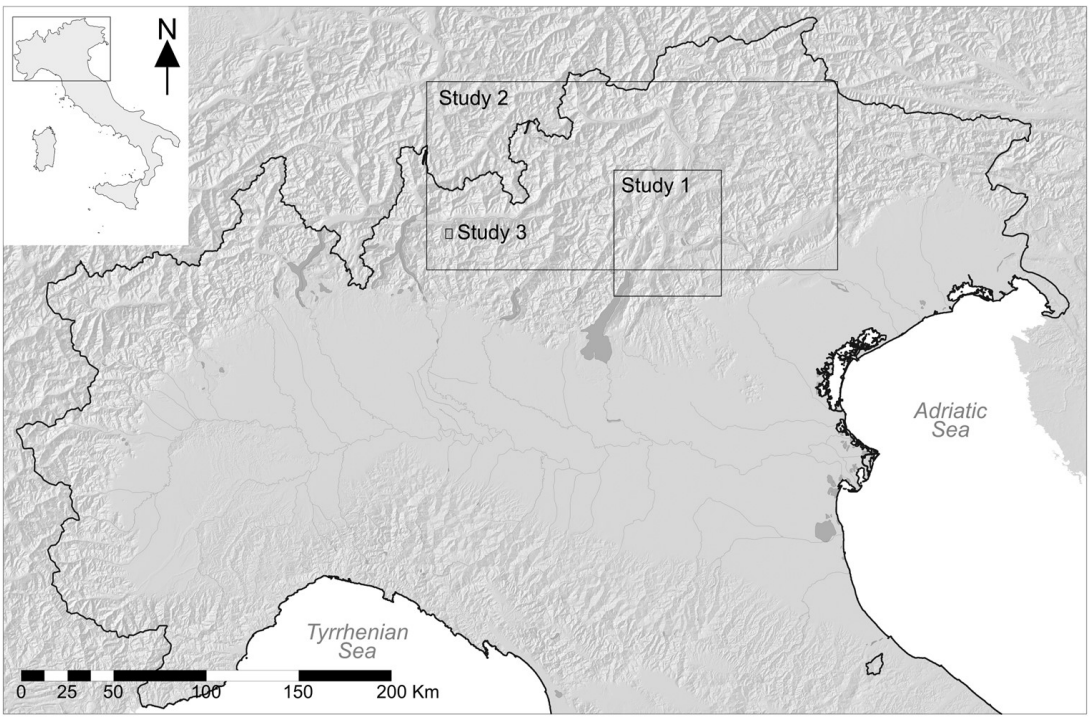
Figure 3. Current distribution of *Zootoca vivipara* (in yellow, from IUCN 2013).

It is a grassland species and principally lives in herbaceous vegetation, favouring damp meadows, swamps and peat bogs. In particular, populations at low to medium altitudes are closely associated with wet environments. Depending on altitude-latitude, *Z. vivipara* is active from March-May to September-October and mating occurs after emergence from hibernation. Most of *Z. vivipara* populations are viviparous, in fact its name derives from the ability to give birth to live offspring; however, in the southern part of the distributional range of the species, oviparous populations occur.

The study area

This thesis is focused on the central-eastern part of Alpine chain in Italy where *B. variegata* is present, and *Z. v. vivipara* and *Z. v. carniolica* overlap their distributional range. Most of the field work was conducted in the Trentino-Alto Adige region.

In the following map, the representation of the study area and the details about each study are given. The fourth study includes samples obtained from the second study and samples from the whole European distribution of *Z. vivipara*.



Major aims of each study

In this thesis, I used different types of genetic data and statistical methods for understanding the recent demographic dynamics and the conservation status of two vertebrate species across the Italian Alps.

In the *first* study, I investigated the genetic variability of the toad *B. variegata* populations to understand if the genetic pattern showed evidence of low effective size, demographic decline and fragmentation, as predicted by some field surveys.

In the *second* study, I analyzed mitochondrial and nuclear DNA phylogenies in the common lizard *Z. vivipara*. I focused on two subspecies (the oviviviparous *Z. v. carniolica* and the viviparous *Z. v. vivipara*) adding new molecular data for testing the ESU hypothesis for the endangered *Z. v. carniolica* subspecies.

In the *third* study, I analyzed a rare syntopic area between *Z. v. carniolica* and *Z. v. vivipara* that was identified during my field work in the central Alps, focusing on the level of gene flow between ovoviviparous and viviparous populations.

In the *fourth* study, I applied a Next Generation Sequencing approach (RAD-tag sequencing) for investigating the divergence between *Z. v. carniolica* and *Z. v. vivipara* subspecies at the genomic level. I also used *Z. vivipara* as model organism for studying the evolutionary transition from oviparity to viviparity in squamate reptiles.

Zootoca vivipara

Bombina variegata

Funding of the project

My PhD scolarship was funded by the Autonomous Province of Trento within the ACE-SAP project (**A**lpine ecosystems in a **C**hanging **E**nvironment: biodiversity **S**ensitivity and **A**daptive **P**otential; University and Scientific Research Service, regulation number 23, 12 June 2008, Trento). Work Package 1 (WP1) of ACE-SAP project was concerned with biological conservation and assessing the threat status of a group of species, including vertebrates, invertebrates and plants. Within WP1, I was co-responsible for the analyses of the toad *B. variegata* and the lizard *Z. vivipara*. The molecular analyses were performed at 'Fondazione Edmund Mach' in S. Michele all'Adige (Trento) in the laboratory of the Department of Biodiversity and Molecular Ecology.

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First study: **Small effective population size and fragmentation in Alpine populations of *Bombina variegata*: the combined effects of recent bottlenecks and postglacial recolonization**

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Abstract

Amphibians are experiencing population declines in all continents caused by anthropogenic and natural factors. Evidences of reduction in population size and even local extinctions have been reported for *Bombina variegata* along all its distributional range. In this study, we examined 200 samples of *B. variegata* from Northern Italy and genetic variation within 9 populations using mtDNA cytochrome b and 11 nuclear microsatellites. We investigated fine-scale population structure and tested for genetic traces of population decline using different methods. We estimated that analyzed populations showed low level of genetic diversity in comparison with other studies. Moreover, low estimates of effective population size were found for all populations. The demographic analyses support a scenario with population decline due to postglacial recolonization, but also suggest that recent anthropogenic modifications and climate changes likely shaped genetic variability of the species in this area and contributed to reduction in effective population size.

Introduction

Amphibians are among the most threatened vertebrates. Many species from all continents are experiencing a demographic decline (Stuart *et al.* 2004), likely due to anthropogenic pressure and climate change (Allentoft & O'Brien 2010). Human activities, in fact, have an important impact for example on land use, leading to habitat loss and fragmentation, on pollution levels and indirectly on the increase of UV-B irradiation (Weyrauch *et al.* 2006.). Also, climate change and especially global warming affect the distribution of amphibians, influencing breeding phenology or leading to pathogen outbreaks (Corn 2005, Rohr *et al.* 2008). Amphibians appear to be particularly sensitive to all these processes, making them good biological indicators of the environmental quality (Blaustein & Wake 1990).

The causes and the consequences of the amphibian decline, and the use of this taxon as a biological indicator, cannot be generalized at a global scale. Many factors interact, and their impact likely differs in different geographic areas and in different species (Beebee & Griffiths 2005, Tafani *et al.* 2013). Studies at regional scale, where the major factors of habitat disturbance can be identified, the demographic dynamic of a species and its genetic impact can be reconstructed, and the possible association between causes and consequences can be inferred, are therefore very useful and valuable. In this context, the Alpine environment is of particular interest.

The Alpine environment is heavily affected by global change, in term of both climate change and land use (Cannone *et al.* 2008; Vanham *et al.* 2009; Keiler *et al.* 2010; Huggel *et al.* 2010). In particular, temperatures in the European Alps increased in the last century twice as much as the global average increase (Brunetti *et al.* 2009). In this area, the effects of climate warming, such a as the upwards shift of the tree-line (Leonelli *et al.* 2011), or the change in population genetic structure, have been already demonstrated or predicted in many plants species (Jay *et al.* 2012; Moradi *et al.* 2012). However, few examples exist in animals (but see for example the decreasing litter size in the marmot, Tafani *et al.* 2013), especially in terms of genetic patterns. Here we analyzed the genetic variation pattern in an amphibian species, *Bombina variegata,* sampled at different sites in an Alpine Italian region. The main objective of our study is to estimate the genetic impact, if any, of the demographic decline occurred in this species in the recent past.

The yellow-bellied toad, *Bombina variegata,* is mainly distributed across central western Europe, from Spain to the Carpathian Mountains, where it forms a stable hybrid zone with the sister species, *B. bombina* (Fijarczyk *et al.* 2011). Breeding sites are usually ephemeral, and include small puddles in meadows and river loops and occasionally farm ponds or water-filled wheel ruts (Gollnmann *et al.* 1999; Cabela *et al.* 2001; Di Cerbo & Ferri, 2001). Although the species is globally considered of Least Concern by the IUCN (IUCN, 2013), extinctions or demographic reductions have been reported in the last decades across the distributional range. In particular, severe declines are documented in Romania, the Netherlands, and Italy (Covaciu *et al.* 2010; Goverse *et al.* 2007; Barbieri *et al.* 2004). Only one population is now described in Luxembourg, and the species is probably extinct in Belgium and highly fragmented in France (Kuzmin *et al.* 2009). Urbanization and consequent loss of suitable habitat (e.g. abandonment of pastures, heavy use of unpaved forestry roads and drainage of natural breeding sites) are considered as the major factors reducing the population sizes and increasing the fragmentation in this species. As in many other amphibians in natural conditions, *B. variegata* has small effective population size (Funk et al. 1999; Beebee & Griffiths 2005) and low dispersal ability (Blaustein *et al.* 1994; Kraaijeveld *et al.* 2005), making the genetic and non genetic risks associated to small numbers of highly isolated individuals even higher.

In Italy, *B. variegata* was common in the last century (De Betta 1857; Giacomelli 1887; Vandoni 1914), but it is significantly declining in many areas (Stagni *et al.* 2004). Anthropization of natural habitats, pollution and use of pesticides lead to a regular decline in the last decades (Barbieri *et al.* 2004), fragmentation and local extinction events (Di Cerbo & Ferri 2000). In a recent study, it has been estimated using simulations under various models of climate change, environmental alteration and solar irradiation, that the yellow-bellied toad in Italy will lose between 13 and 75% of its suitable natural habitat in the next 50 years (D'Amen *et al.* 2011).

Here we study the pattern of genetic variation at the mitochondrial cytochrome b gene and at 11 microsatellite markers in a restricted area in the Italian Alps, where local extinctions and population declines have been previously documented (Caldonazzi *et al.* 2002). We typed 200 individuals from 9 different populations to address the following two main questions: 1) Is the genetic pattern showing evidence of low effective population size, demographic decline and/or fragmentation? 2) Can we directly infer that recent human-related factors are responsible for the genetic

pattern? We will address these questions using a set of statistical methods suitable to estimate effective population sizes, times of population size changes, population structure, individual genomic compositions and isolation by distance, and to probabilistically compare alternative demographic models. We believe that our results have important implications for evaluating the conservation status in this species and developing conservation plans, and in general for the understanding of the genetic effects of recent and ancient demographic declines.

Materials and Methods

Samples collection and DNA extraction

Two hundred samples of *B. variegata* were collected from nine different localities in the Province of Trento (Northern Italy) from 2009 to 2011. Sampling sites and their abbreviation used throughout this paper are reported in Figure 1. Different ecosystems were sampled: samples from Spiz (SPI) and Monte Baldo (MBA) came from isolated mountain areas (about 1500 m asl); samples from Zambana (ZAM) and Mezzolombardo (MEZ) were collected in the main valley of the Region (the Adige valley), close to areas devoted to agriculture (about 210 m asl); samples from Nago (NAG) and Loppio (LOP) came from sites close the touristic area of Garda Lake (160 and 250 m asl, respectively); samples from Verla (VER), Pozzolago (POZ) and Prà (PRA) were collected from scarcely urbanized areas along the Avisio river (from 450 to 620 m asl), and in particular from agricultural ponds (VER) and river loops (POZ and PRA). Individual GPS coordinates of each sample were recorded. Toe clips were obtained and stored in 95% ethanol; about 20 mg of tissue were used to perform DNA extraction using the protocol of the DNeasy Tissue kit (QIAGEN Inc, Hilden, Germany). All sampling procedures were approved by the Italian Ministry of Environment and Wildlife Committee of the Autonomous Province of Trento (DPN/2D/2003/2267 and 4940-57/B-09-U265-LS-fd).

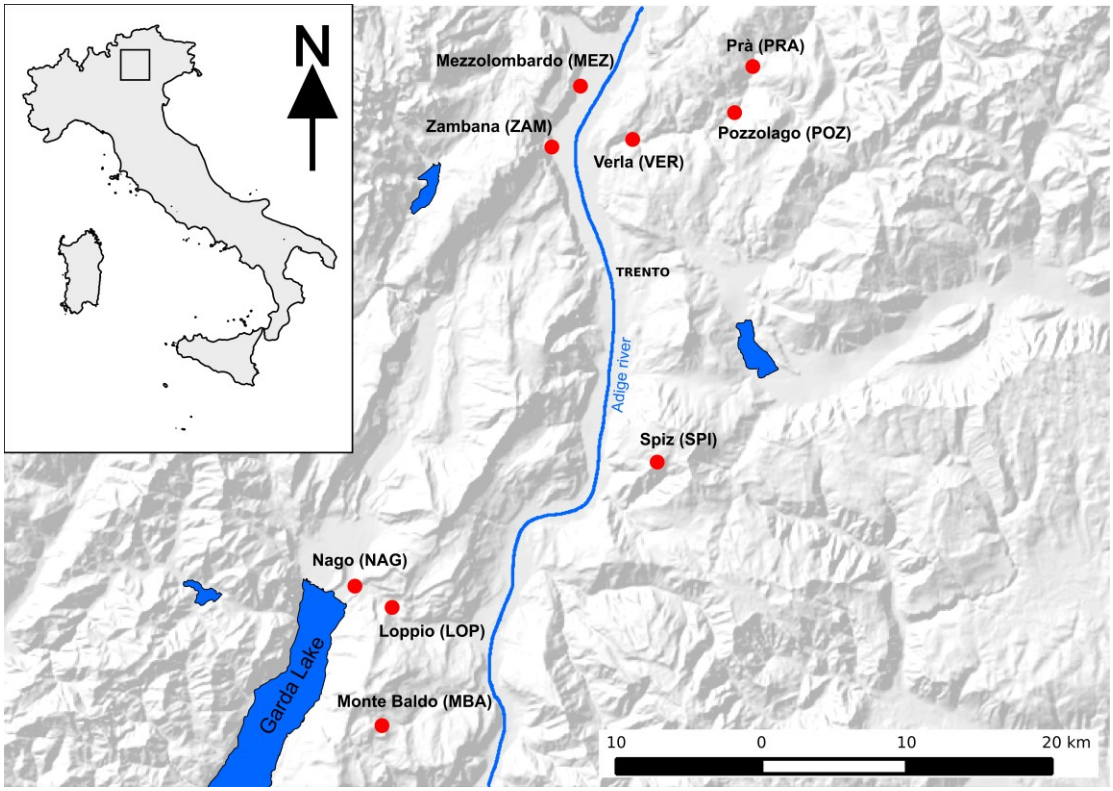


Figure 1.1 Map of the nine sampling sites (indicated by red dots) in the Alpine region of Trentino Alto-Adige.

Genetic typing

We initially sequenced a fragment of the mitochondrial DNA (mtDNA) cytochrome b gene to verify the haplotypic affiliation of the samples, with respect to the known maternal phylogeographic pattern in Europe. We used the primer pairs L14850 and H 15410 according to Tanaka *et al.* (1994). PCR amplifications were conducted in 20 ul (containing 1ul of template DNA 2 ul of buffer, 1 ul of each pair of primers, 1 unit of Hot Master Taq polymerase and ultra pure water) under the following conditions: 10 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 45 sec at 52°C, 60 sec at 65°C, and a final extension step for 10 min at 65°C. Sequences were edited using Finch TV 1.4.0 (an open source application developed by Geospiza Research Team), assembled with Sequencer v.4.7 and aligned using ClustalX (Thompson *et al.* 1997) using default parameters.

The genetic variation level and structure at the micro-geographic scale was then investigated typing 11 autosomal microsatellites. Nine of them were originally developed for the sister species *B. bombina* (Nurnberger *et al.* 2003; Hauswaldt *et al.* 2007; Stuckas & Tiedemann 2006). PCR amplifications were conducted in four

different multiplex reactions in a final volume of 20 ul containing: 1ul of template DNA, 2 ul of buffer, 0,5 ul of each pair of primers, 1 unit of Hot Master Taq polymerase (Applied) and ultra-pure water. The amplification protocol consisted of an initial denaturation step at 94°C for 10 minutes, followed by 30 cycles of the series: 94°C for 30 seconds, annealing temperature (Ta: 53°C for Bv11 and Bv32; Ta: 56°C for 1A, 10F and F22; Ta: 45°C for B13 and 8A; Ta: 52°C for 5F, 9H, 12F and B14) for 30 seconds, 65°C for 45 seconds; then, a final extension step at 65°C for 10 minutes. PCR labeled products were run on a four capillary system ABI 3130 Genetic Analyzer (Applied Biosystem) and scored with an internal lane standard (LIZ) using GeneMapper software.

Statistical analysis

Mitochondrial DNA

A phylogenetic tree was built using the maximum-likelihood algorithm implemented in MEGA5 (Tamura *et al.* 2011), using the Kimura two-parameter model (selected as the best model by JModelTest (Posada 2008)) and 1000 bootstrap replicates. This analysis included the haplotypes from our study, the sequences available in Genbank for *B. variegata* (EF212448-EF212809), and two sequences used as outgroups from *B. bombina* and *B. orientalis* (JF898352, EU531278).

Microsatellites

Microsatellites were tested for the presence of null alleles, allele drop-out and scoring errors using MicroChecker (Van Oosterhout *et al.* 2004). We used GENEPOP 3.4 (Raymond & Rousset 1995) to test for deviations from Hardy–Weinberg equilibrium for each locus and globally. We also tested genotypic Linkage Disequilibrium (*LD*) for each pair of loci. To evaluate overall genetic variation, expected and observed heterozigosity (H_e and H_o) and number of alleles (N_a) within each population were calculated using Arlequin v3.5 (Excoffier & Lischer 2010); FSTAT software (Goudet 1995) was used to calculate allelic richness (A_r). In addition, pairwise F_{st} values between populations were computed with Arlequin v3.5 and the corresponding triangular matrix of distances was visualized using Principal Coordinates analysis (PCoA) implemented in GenAlex v6.5 (Peakall & Smouse 2012).

Bayesian clustering analyses

STRUCTURE v2.3.4 (Pritchard *et al.* 2000; Hubisz *et al.* 2009) was used to detect the most plausible number K of genetically homogeneous groups and to estimate the genetic composition of each individual. We applied the LOCPRIOR with admixture model, which assumes that sampling locations are informative and allows for mixed ancestry of individuals. This model is more powerful in detecting weak genetic structure and reduces misassignments (Hubisz *et al.* 2009). Each run of STRUCTURE consisted of 1000000 iterations after a burn-in period of 250000, and 10 runs were analysed for all K values between 1 and 9. The most probable K was selected comparing the likelihood at different K values and using the approach of Evanno *et al.* (2005) based on the rate of change of the likelihood.

Genetic vs. geographic distances

The correlation between genetic similarity (or dissimilarity) and geographic distance was evaluated separately at the individual and at the population levels. At the individual level, we estimated with the software SPAGeDi (Hardy & Vekemans 2002) the kinship coefficient derived by Loiselle *et al.* (1995) for all pairs of individuals. These coefficients were then pooled in classes with similar number of comparisons, corresponding to different geographic distances. At the population level, we analysed the relationship between the linearized F_{st} based distance ($F_{st}/(1-F_{st})$) and the logarithm of the linear geographic distance. The statistical significance of this relationship was evaluated using the Mantel's test.

Recent effective population size

Two methods were used to estimate the recent effective population size (N_e) of each population: LDNe (Waples & DO 2008) and ONeSAMP (Tallmon *et al.* 2004). LDNe is based on the linkage disequilibrium among unlinked loci created by random drift, and the estimated N_e reflects the population size in the last few generations (Hare *et al.* 2011). As suggested by the authors (Waples & DO 2008), we excluded the alleles with frequencies smaller than 0.02 to avoid the bias related to rare alleles. ONeSAMP implements an Approximate Bayesian Computation analysis (Beaumont *et al.* 2002; Bertorelle *et al.* 2010). Eight summary statistics are used by ONeSAMP to compare observed and simulated data sets, but the inclusion of linkage disequilibrium among these statistics makes this method particularly sensitive to recent population sizes

(Skrbinsek *et al.* 2012). The lower and upper limits of the uniform prior distribution of N_e were set to 2 and 5000, respectively.

Demographic dynamic

We analysed the demographic dynamic of each population using four different methods: 1) the M-ratio test (Garza & Williamson 2001); 2) the heterozygosity excess test implemented in the software BOTTLENECK 1.2.02 (Piry *et al.* 1999), 3) a Bayesian analysis based on the coalescent framework and able to estimate the posterior distributions of the parameters of a contraction/expansion demographic model, as implemented in the software MSVAR v1.3 (Beaumont 1999, 2004); 4) a model comparison based on the Approximate Bayesian Computation approach (Beaumont *et al.* 2002; Bertorelle *et al.* 2010), as implemented in the software in DIYABC v 1.0.4.46b (Cornuet *et al.* 2008, 2010). These methods have different statistical properties, which differently depend on the number of markers, the specific feature of the bottleneck (like age, initial population size, intensity, presence or not of recovery) and the possible violations of the model they assume (e.g., migration events among populations). Therefore, no one can be considered better than the others in all conditions (e.g., Swatdipong *et al.* 2010; Chikhi *et al.* 2010; Peery *et al.* 2012; Hoban *et al.* 2013). We briefly describe here these methods, and we will come back on their properties in the discussion.

The M-ratio test is based on the frequency distribution of allelic sizes, which is expected to have gaps after a bottleneck due to stochastic loss of rare alleles. Statistical significance was established comparing the observed values with the empirical null distribution obtained simulating 10,000 times the genealogy expected under demographic stability with M_P_VAL (Garza & Williamson 2001). Simulations assume the two-phase mutation model, and require three parameters: the population- mutation parameter, $\theta = 4N_e\mu$, the mean size of multi-repeat mutations, δ_g, and the proportion of multistep events, p_s. Different values of θ were tested, i.e. 1, 2, and 5 (corresponding to pre-bottleneck N_e of 500, 1000 and 2500 individuals, respectively, assuming a standard rate $\mu = 5x10^{-4}$); δ_g and p_s were fixed to 3.1 and 0.22 as estimated in a recent review by Peery *et al.* (2012).

The heterozygosity excess test is based on the comparison between heterozygosity and number of alleles, which is predicted to deviate from the

expectation after a bottleneck because the former decreases more slowly than the latter. Statistical significance (one tail) is computed using the Wilcoxon's signed ranked test to compare observed and expected heterozygosities (Cornuet & Luikart 1996), where expected values are computed by simulations assuming again a two phase mutation model, a variance among multiple steps equal to 12 (corresponding to δ_g = 3.1, see Peery *et al.* 2012) and p_s = 0.22.

The method implemented in MSVAR assumes that an ancestral population with effective size N_1, increased or decreased (linearly or exponentially) to its current size N_0, starting T generations ago. The estimation algorithm is based on Markov Chain Monte Carlo simulations, and the simple Single-step Mutation Model (SMM) is assumed. Simulations were run for 4 x 10^8 iterations; convergence and posterior distributions of the parameters were evaluated with Tracer v1.5 (Rambaut & Drummond 2007), after discarding the first 10% of the chains (burn-in). For each population, three independent runs were performed assuming an exponential demographic change. The possible effect of this choice was tested assuming a linear change in an additional run of the program. Priors means for the ancestral and current population sizes were set equal to a log-10 transformed value of 3 (1000 individuals), with a standard deviation equal to 1. The prior distributions are log-normal, and this setting allows the testing of population sizes from few tens to hundred of thousand of individuals. Three different prior distributions of the time since the demographic change were tested, with means equal to 2, 3, and 4, respectively (corresponding to 100, 1000, and 10000 years) and standard deviations equal to 1. All the other prior settings in the hierarchical model implemented in MSVAR are reported in Table S1.1 and follow standard choices used in other studies (e.g., Storz *et al.* 2002). Time estimates are transformed in years assuming a generation time of 3 years (Szymura 1998; Gollmann & Gollman 2002).

Finally, the demographic dynamic was also analysed comparing three alternative scenarios with the ABC (Approximate Bayesian Computation) approach as implemented in DIYABC (Cornuet *et al.* 2010): constant effective population size, ancient bottleneck, recent bottleneck. The models assuming ancient or recent reductions were simulated to mimic the demographic effects possibly related to the post-glacial founding of the Alps populations and the human-mediated processes affecting amphibians in the last century, respectively. Hereafter, we call these models

Const (constant population size), GlaD (post-glacial decline) and HumD (human-related decline). Different settings and prior distributions were tested to check the robustness of the results (Table S1.2).

Results

Mitochondrial sequences

Three polymorphic sites, and an average pairwise divergence of 0,043% among individuals, were found in the 420 bp alignment of the cytb gene. Four different haplotypes were detected, three of which had never been observed before in this species. The ML phylogenetic tree (Figure S1.1) indicates that the samples we analyzed belong to the previously described "Balkano-Western" clade of the nominal form, *Bombina variegata variegata* (Hoffman *et al.* 2007).

Microsatellite markers

All the 200 samples from 9 populations were successfully genotyped at all the 11 amplified loci. MicroChecker results did not suggest any significant presence of null alleles, scoring errors or allelic drop-out. Systematic deviation form Hardy-Weinberg and linkage equilibrium can be excluded: only 5 out of 99 (11 loci x 9 populations) Hardy-Weinberg tests were significant with $P<0.05$, and only 2 out of 55 locus pairs showed significant genotypic linkage with $P<0.05$. All loci were polymorphic and the number of alleles per locus ranged from 2 for F22 to 11 for Bv32 (Table S1.3). Genetic variation is relatively low in all populations. Heterozygosity values are around 0.50, with very low values in in SPI (H_e = 0.41) and NAG (H_e = 0.34). The allelic richness per locus is between 3 and 4 for most populations, again with SPI and NAG showing the lowest values (2.5 and 2.4, respectively).

Population	Label	N	N_a	A_r	H_o	H_e	N_e (LDNe)	N_e (OneSamp)
Zambana	ZAM	29	4.2	3.7	0.58	0.54	51.9 (23.9-552.1)	26.17 (20.32-49.93)
Mezzolombardo	MEZ	10	3.4	3.4	0.52	0.49	31.1 (6.9-inf)	12.24 (9.41-19.44)
Nago	NAG	23	2.5	2.4	0.36	0.34	6.5 (2.0-25.7)	17.85 (13.45-29.99)
Monte Baldo	MBA	25	3.5	3.2	0.47	0.51	61.5 (22.7-inf)	23.15 (17.12-39.64)
Prà	PRA	17	3.6	3.2	0.49	0.48	10.0 (3.2-32.3)	17.58(14.07-27.75)
Pozzolago	POZ	25	4.0	3.5	0.53	0.51	55.7 (17.1-inf)	25.47 (19.69-41.21)
Verla	VER	24	3.9	3.3	0.54	0.48	50.8 (17.4-inf)	19.10 (15.29-27.59)
Loppio	LOP	32	3.6	3.1	0.50	0.50	166.6 (29.1-inf)	26.62 (20.55-41.09)
Spiz	SPI	15	2.5	2.5	0.42	0.41	65.8 (12.7-inf)	13.08 (10.86-18.59)
Mean			3.5	3.1	0.49	0.47		

Table 1.1. Genetic diversity and effective population size estimates of 9 populations of *B. variegata.* Sampling localities, including the corresponding acronym, number of samples collected (*N*), number of alleles (N_a), allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosity, and estimates of effective population size by linkage-disequilibrium method (N_e (LDNe)) and by Bayesian method (N_e (OneSamp)). Intervals in brackets are 95% confidence intervals (LDNe) and 95% credible limits for the posterior distribution (OneSamp).

Population differentiation

Significant genetic differentiation (after following the Benjamini & Hochberg (1995) approach for multiple testing) was found in 34 out 36 pairwise F_{st} comparisons. The only exceptions are the comparisons between two pairs of geographically adjacent populations (ZAM vs. MEZ and PRA vs. POZ). F_{st} values (see Table S1.4) ranged between 5% and 15% in most cases, with higher values (up to 32%) when the NAG site was involved. The matrix of distances is graphically visualized in Figure 1.2 using the Principal Coordinate Analysis (PCoA).

Bayesian clustering analyses

The inspection of the likelihood plot for different K values (Figure S1.2a), and the plot based on the rate of change of the likelihoods (Figure S1.2b), clearly suggests that the most relevant partition of the data are those with 2 and with 5 inferred groups. We present therefore these results.

For K=2, the inferred groups are predominant in central/northern and southern locations (Figure S1.3), respectively. All the individuals in 4 populations can be entirely or almost entirely assigned to the central/northern (PRA, POZ and SPI) or the

southern (NAG) groups. Individuals in the other 5 populations show admixed composition with very similar fractions of the two inferred groups within the same locality, suggesting shared ancestry rather than recent admixture (e.g., Jarvis *et al.* 2012)

For K=5, the groups inferred by STRUCTURE roughly correspond to the groups graphically identified by the PCoA analysis (Figure 1.2). In the southern area, NAG is genetically distinct form MBA and LOP, but with a clear portion of shared ancestry with these neighboring localities. Some individuals in NAG also appears as recent hybrids, with ancestors both in NAG and in MBA or LOP. In the central/northern group, PRA and SPI are well differentiated, POZ is very similar to the adjacent PRA, and all VER individuals have a majority of their genetic composition shared with the adjacent PRA and POZ, but also a similar and relatively large affinity with the southern localities of MBA and LOP.

Genetic vs. geographic distances

The relationship between linearized F_{st} and the logarithm of geographic distance is positive, weak (R^2 = 0,07, Figure S4), and statistically significant (Mantel test, P= 0.04). Estimated kinship coefficients are relatively high (as among first cousins) only when individuals from localities separated by 5 kilometers or less are compared, and very low otherwise (Figure S1.5).

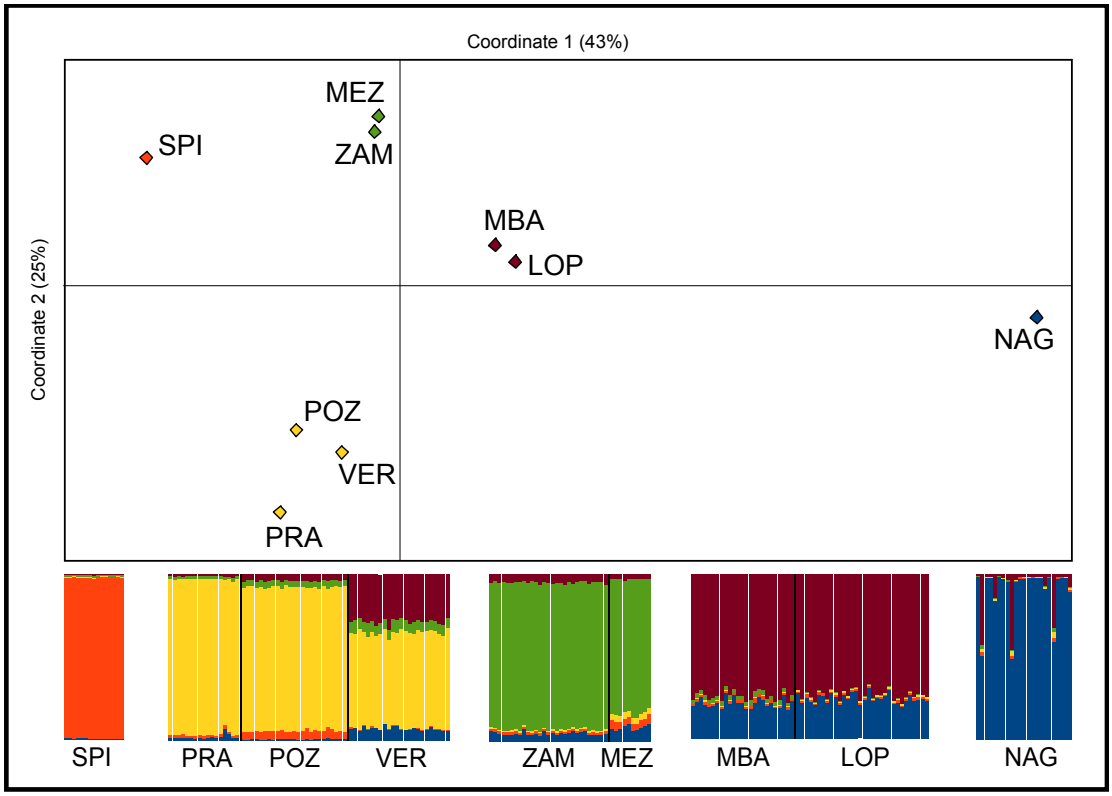


Figure 1.2. Principal Coordinate Analysis of pairwise *Fst* distances among populations and plots of proportion of ancestry of each sampled individual for five genetic clusters inferred using STRUCTURE.

Recent effective population sizes

Point estimates of recent effective population sizes are low or very low (Table 1.1). The maximum value is around 170 individuals for the Loppio population using the LDNe method, but for the same population the estimated size is less than 30 when the OneSamp method is applied. All the other values range approximately between 10 and 50, with LDNe producing in most cases larger estimates than OneSamp. The confidence intervals have large upper limits in most LDNe estimates, but the posterior distributions of Ne produced by OneSamp have very small probabilities for N_e>50.

Demographic dynamic

All the populations have M-ratio values (see Table 1.2) below the 0.68 threshold usually taken as evidence for a bottleneck (Garza & Williamson 2001). When M-ratios are formally tested controlling for false positives (Benjamini & Hochberg 1995), significant support of the bottleneck (P<0.05) is found in all populations, the only exception being ZAM and MEZ when the largest values of $\theta = 5$ is assumed. The

heterozygosity excess test indicates that heterozygosities are higher than predicted from the number of alleles, as expected after a bottleneck, but this difference is significant only for SPI.

The posterior distributions of ancestral and current population sizes, as estimated by MSVAR, are shifted from their equal prior distributions, clearly suggesting a demographic decline in all populations (Figure 1.3). The distributions in different populations are largely overlapped, but considering the point estimates we note that the ratio between ancestral and current median sizes varies approximately between 7 and 70. NAG, MBA, LOP, and SPI show the most extreme reduction (>25 fold), and a less extreme decline is estimated for the other populations (<15 fold). Ancient sizes distributions have peaks at around 2000-4000 individuals, and current sizes estimates vary between 70 to 300 animals in different populations. The most supported age of the decline varies in different populations between 500 and 3000 years BP (see Figure 4a). Given the evident overlap between prior and posterior distributions, we checked the influence of the former on the latter. The posterior distributions support a decline age between few hundred and few thousand years also when the prior mean was decreased or increased by a factor of 10 (see Figure 4b and 4c). These general results of MSVAR are consistent across runs, assuming either an exponential or linear decline, and pooling samples from pairs of populations not genetically differentiated (see all the point estimates and 95% credible intervals in Table S5).

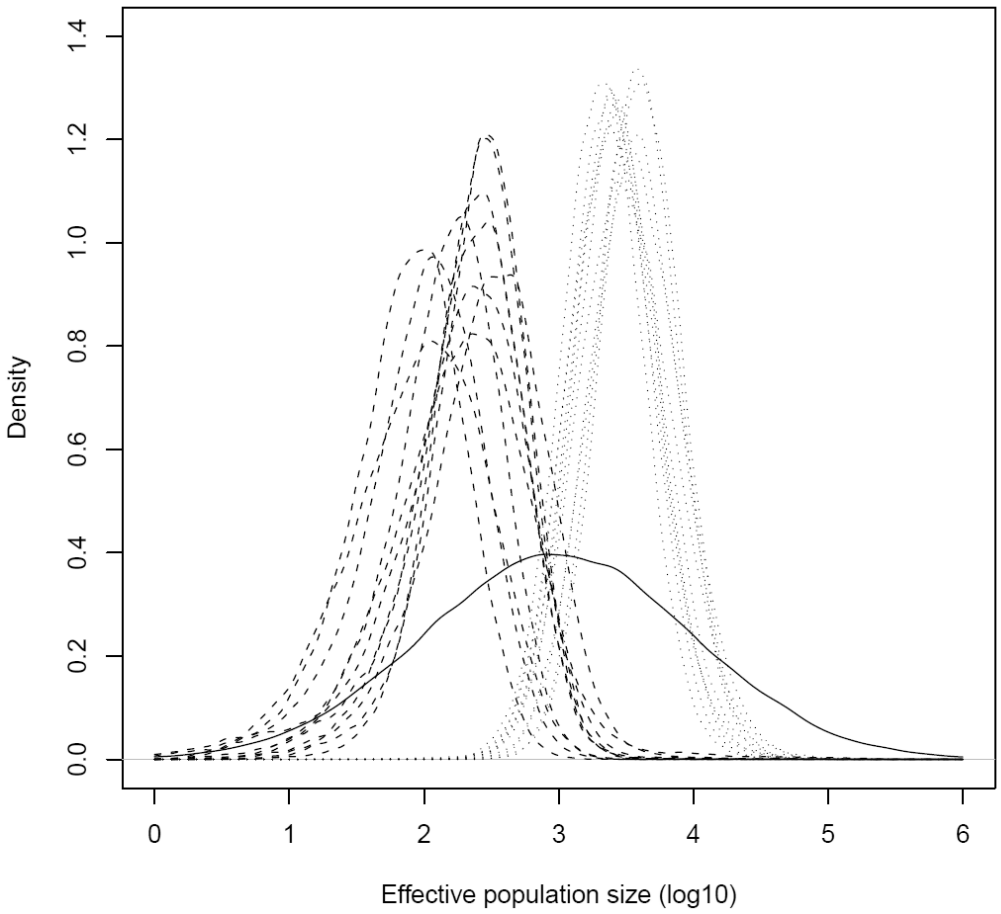


Figure 1.3. Posterior distribution of the effective population sizes (in log 10 units) for each population obtained with MSVAR (assuming the exponential demographic change). Dashed lines represent current *Ne*, while dotted lines represent pre-bottleneck *Ne.* The solid line is the prior distribution for both current and ancient population sizes.

The ABC analysis confirms that the genetic variation pattern is compatible with a demographic decline (Table 1.2). In particular, the model GlaD (post-glacial decline) obtains in all population a posterior probability at least double than Const (constant size) or HumD (human-related decline). When different prior distributions are tested, these general results are confirmed, though in some specific setting and only for NAG, MBA, LOP and SPI, the posterior probability of HumD becomes similar to the posterior probability of GlaD (Table 1.2 and Figure S1.6).

Population	Label	N	M-ratio	P value (M-ratio) θ=1	θ=2	θ=5	H excess (p-value)	Scenario 1 Const	Scenario 2 HumD	Scenario 3 GlaD
Zambana	ZAM	29	0.63	0.010	0.029	0.062	0.052	0.04	0.06	0.89
Mezzolombardo	MEZ	10	0.60	0.003	0.011	0.072	0.216	0.06	0.07	0.87
Nago	NAG	23	0.48	0.001	0.001	0.001	0.326	0.31	0.07	0.62
Monte Baldo	MBA	25	0.51	0.001	0.001	0.001	0.042	0.10	0.13	0.76
Prà	PRA	17	0.53	0.001	0.001	0.001	0.080	0.07	0.02	0.90
Pozzolago	POZ	25	0.61	0.002	0.003	0.008	0.350	0.07	0.01	0.92
Verla	VER	24	0.61	0.001	0.003	0.010	0.382	0.36	0.02	0.62
Loppio	LOP	32	0.53	0.001	0.001	0.001	0.042	0.11	0.08	0.81
Spiz	SPI	15	0.60	0.005	0.016	0.008	0.002	0.06	0.21	0.73

Table 1.2. Tests of demographic bottleneck. The heterozygosity excess is tested using the Wilcoxon approach implemented in the software BOTTLENECK. Significant P values (α=0.05) for the M-ratio and the heterozygosity excess tests, after controlling (separately for each test) for multiple testing, are underlined. The last three columns report the posterior probabilities of three different demographic scenarios tested with the ABC approach. Const = constant population size; HumD = recent, human related, decline; GlaD = ancient decline associated to the post-glacial colonization of the Alps. Prior distributions for all the model parameters are reported in Table S1.2 (in italics). The posterior probabilities of each model in each population are only slightly affected by the choice of prior distributions (see details of all the analyses with different priors in Table S1.2).

Discussion

Phylogeography and genetic diversity

The mitochondrial phylogenetic analysis showed that all samples included in this study fall into Balkan-Western clade (Figure S1.1). The level of variation at this marker was very low, with 89% of the individuals sharing the same mtDNA sequence, and only 4 haplotypes in total. This result is compatible with the hypothesis by Hofman *et al.* (2007) and Fijarczyk *et al.* (2011), who suggested that the populations characterized by this clade originated in a Balkan refugium and expanded northwestward after the last glaciation, losing genetic variation during the colonization process. Additional analyses at this marker in the Alpine populations here considered are prevented by the very low level of polymorphism.

The microsatellite markers allowed a more detailed genetic analysis even in a geographically restricted area in the Italian Alps, a biodiversity hot-spot where declines and local extinctions of *B. variegata* have been previously documented. First of all, the populations of yellow-bellied toad in the Alps show low to moderate level

of genetic variation when compared to other populations of this species or the sister species *B. bombina* sampled in Northern Europe (where the drift effect associated to the post-glacial colonization is expected to be higher). In particular, when samples sizes are adjusted by resampling, and only overlapping loci are considered, the average number of alleles was about 27% and 40% lower in the Alps than in a *B. variegata* and *B. bombina* population in Northern Germany, respectively (Hauswaldt *et al.* 2007). The number of alleles and the heterozygosities observed in the Italian Alps are similar to the values found in endangered frog or toad species (Wang 2012; Igawa *et al.* 2013, Morgan *et al.* 2008; Beauclerc *et al.* 2010).

When the microsatellite variation pattern was used to estimate the contemporary effective population size, most populations showed values smaller than 50 individuals, and some of them values smaller than 20. These values are lower than those estimated in other ranid species (Wilkinson *et al.* 2007; Phillipsen *et al.* 2011), and similar to the estimates obtained in endangered anuran species (Ficetola *et al.* 2010; Wang 2012).

Considering the environmental modifications predicted in the future, and that small populations showing low genetic variation have reduced capacity to adapt to global changes (Willi *et al.* 2006), we should take the levels of microsatellite variation within the populations and the estimated contemporary effective population sizes as an early warning of genetic risks and a motivation for implementing specific conservation measures in this species.

Habitat fragmentation

Gene flow, which could counteract the negative consequences of genetic drift and inbreeding, is unlikely to occur in a fragmented landscape and especially in species with reduced movement capabilities such as frogs (e.g., Dolgener *et al.* 2012; Igawa *et al.* 2013). This expectation was met in our study: despite the fine geographic scale, a clear evidence of genetic substructure was found, and most populations were genetically differentiated from the others. Five major genetic groups were identified, with two of them corresponding to two single and highly divergent populations, and the others associated to geographically homogenous areas. Genetic data also showed that kinship levels are high only at very short distances, supporting very small local population sizes and previous mark-recapture field studies that indicated travel

distances of single adult individuals rarely exceed 500 meters (Smith & Green 2005 and Hartel 2008).

The population of NAG showed the highest values of F_{st} against all the other (from 0.15 to 0.32). In this case, although the population of LOP is very close, gene flow is probably prevented because of habitat discontinuity due to urbanization in the touristic area of Garda Lake. Interestingly, NAG is also the only population where clear signals of recent hybridization with the neighboring populations were found in some individuals. Future investigations should be performed to test the hypothesis of human-mediated translocation events.

All in all, the high level of genetic structure among populations indicates that gene flow is very limited, and rapidly declines as the geographic distance increases. In terms of conservation, this result suggests that, as in other species with limited mobility (Walker *et al.* 2008), the risk of inbreeding due to low population sizes will be enhanced.

Bottleneck inferences

Are the low contemporary population sizes we estimated related to the population declines recently documented in *B. variegata* populations in Northern Italy (Caldonazzi *et al.* 2002)? Here, we applied several statistical methods to address this question. More specifically, we aimed at detecting if the genetic variation data support the bottleneck hypothesis and, if so, for estimating the properties of this decline.

Clear genetic traces of extreme bottleneck events emerged according to all the statistical approaches in most of the populations. Declines from some thousand to few hundred of individuals, or less, were estimated by the full-likelihood Bayesian method MSVAR, and the starting date of the decline estimated by MSVAR and the ABC approach was mostly compatible with a postglacial (model GlaD) rather than a recent human-related (model HumD) decline. However, the four populations showing the most extreme (>25fold) decline, NAG, MBA, LOP, and SPI, also showed an equivalent support for the GlaD and the HumD models under the ABC approach. In addition, MBA, LOP, and SPI were the only populations where a significant or marginally significant support was found by the Wilcoxon test, which is a statistical test that detects the transitory excess of heterozygosity over a very short period of time (Luikart & Cornuet 1998). Considering only these last three populations, we note

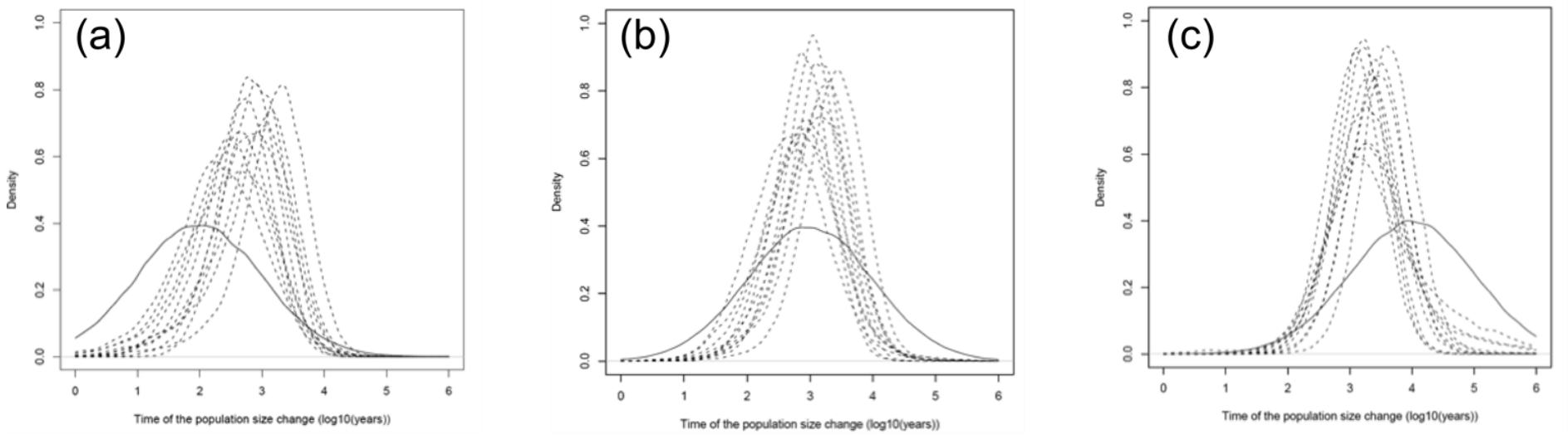
that a genetic impact due to recent human-related processes appears likely. In 1956 the Loppio Lake, where we collected samples for LOP population, was totally drained because of construction of a tunnel under the lake, leading to drastic changes of the habitat and dangerous consequences for the species living there. On the other hand, MBA and SPI are the most elevated populations in our study (and also higher than the mean altitudinal distribution of the species), and the negative effects due to global warming are expected to be high at these altitudes. The timing of seasonal activities, including hibernation and breeding, are in fact tightly related to climatic conditions. Blaustein *et al.* (2001), for example, showed a trend of earlier breeding activity for 9 amphibian species in response to increasing temperature, but the occurrence of late frosts, increased by climate change, can have fatal consequences on spawn especially for early breeding species (Henle *et al.* 2008). Moreover, the decreasing in depth of winter snow cover (IPPC 2007) may have an impact on amphibian survival during hibernation, making them more vulnerable to cold waves. This pattern, amplified for populations with low N_e, such as MBA and SPI, could have led to population decrease and genetic depletion.

In summary, therefore, we suggest that at least one demographic decline occurred and left a genetic signature in all populations, but it is difficult to safely discriminate whether the bottleneck occurred during the post-glacial colonization of the Alps, in the last century, or in both time intervals. This uncertainty is due to the relatively small number of markers we analyzed, but also to the not well-known statistical properties of some of methods we applied. For example, it is not clear if, in case of two successive bottlenecks, the single decline model implemented in the method MSVAR would identify the earliest, the latest, or would rather estimate a decline age of intermediate age. We can however conclude that current effective sizes are much smaller than in the past, that some demographic process occurring in *B. variegata* and likely related to the colonization of the Alps reduced the genetic variation, and that, at least in some areas, recent anthropogenic modifications and climate changes are likely to have contributed to the reduction in effective population size.

Finally, we note that the interaction between low genetic variation and effects of climate change has been considered one of the main cause for the outbreak of the fungus *Batrachochytrium dendrobatidis*, which is driving the worldwide declines and extinctions of amphibians (Allentoft & O'Brien 2010 and references therein).

Infections of this fungus were largely recorded in the endangered Apennine yellow-bellied toad *B. pachypus* (Canestrelli *et al.* 2013). In the light of the genetic results obtained here and the predicted effects of climate change, northern Italian populations of *B. variegata* might be also at risk of chytridiomycosis.

Figure 4. Posterior distributions in different populations (dashed lines) of the time since the change in effective population size estimated by MSVAR assuming the exponential change. Three different means of the prior distribution (solid lines) were tested: a) 100 years (log10 transformed value = 2); b) 1,000 years (log10 transformed value = 3); c) 10,000 years (log10 transformed value = 4).



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Supplementary materials

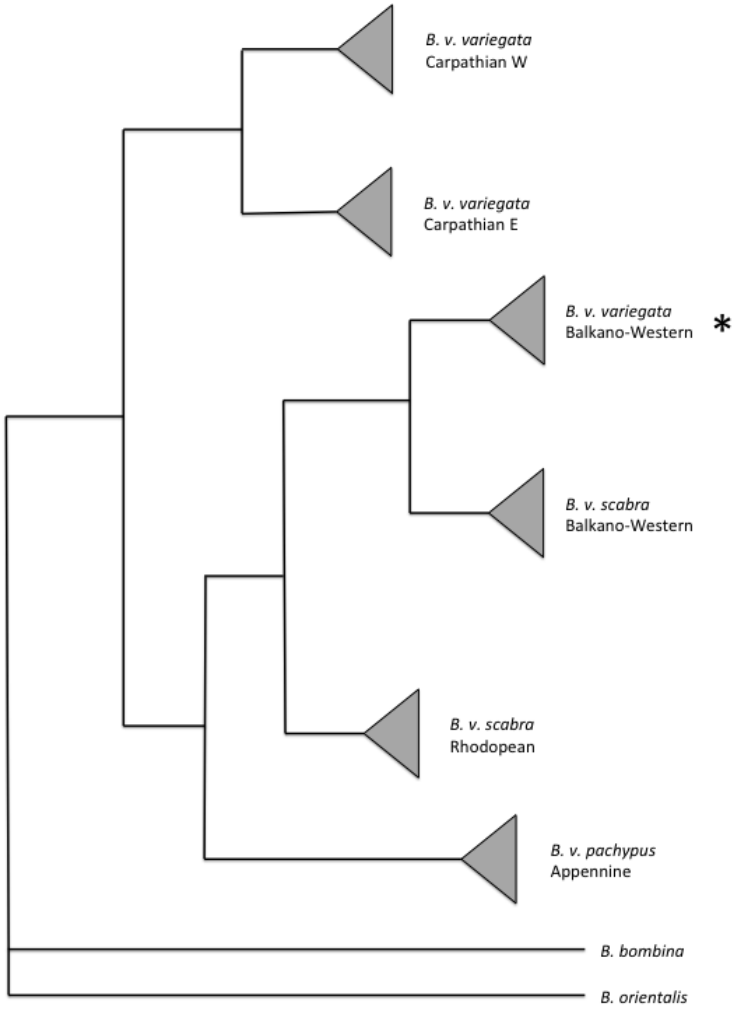

Figure S1.1. ML phylogenetic tree from partial cytb (mtDNA) haplotypes obtained in this study and deposited sequences of *B. variegata*. *B. bombina* and *B. orientalis* were used as outgroups. All the haplotypes from this study belong to the clade indicated by asterisk.

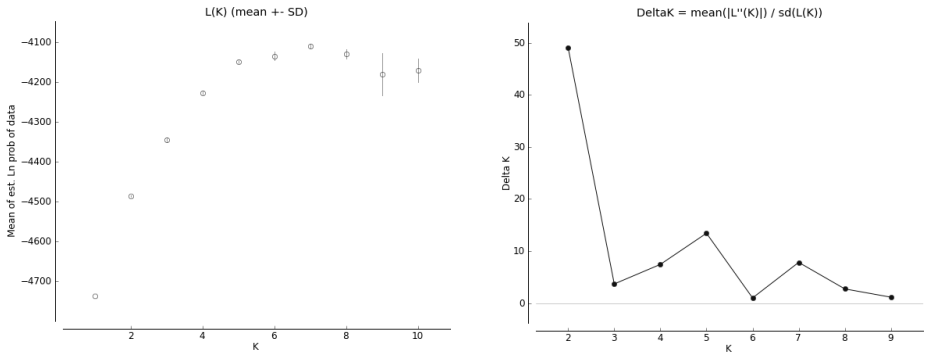

Figure S1.2. Estimates of best (K) number of genetically homogeneous groups according to the methods by (a) Pritchard *et al.*, (2000) and by (b) Evanno *et al.*, (2005).

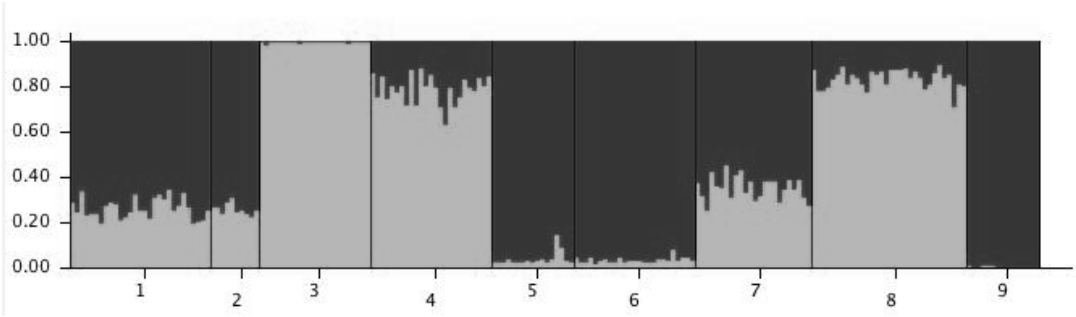

Figure S1.3. STRUCTURE plot describing the population structure using K=2 as most probable number of genetic groups. Each bar represents an individual and its proportional membership to one of the two clusters. Individuals are ordered by sampling locations, numbered as follow: 1: ZAM, 2: MEZ, 3: NAG, 4: MBA, 5: PRA, 6: POZ, 7: VER, 8: LOP, 9: SPI.

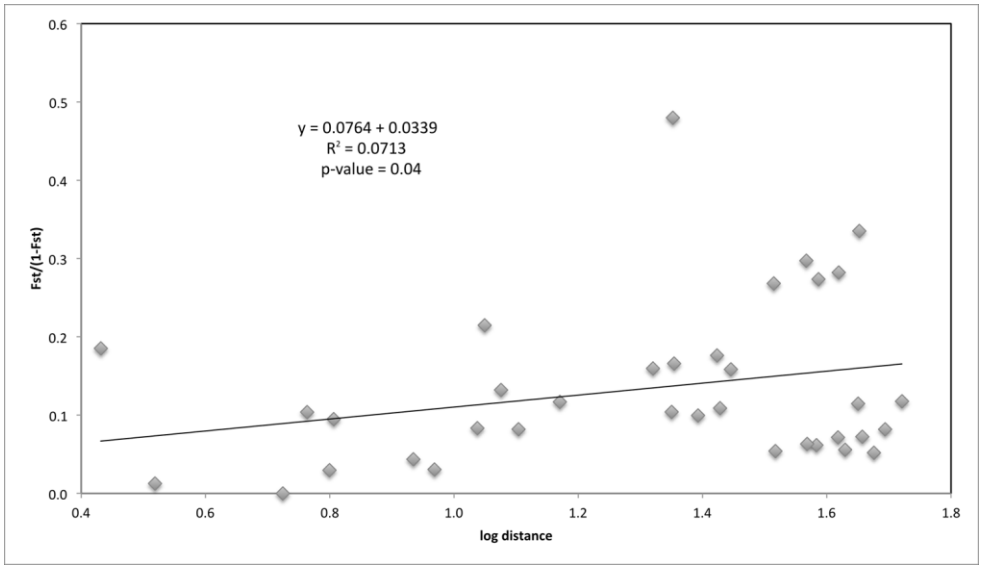

Figure S4. Isolation by distance analysis. Pairwise genetic (*Fst*/(1-*Fst*)) vs geographic (log) distances between the nine populations of *B. variegata* analysed.

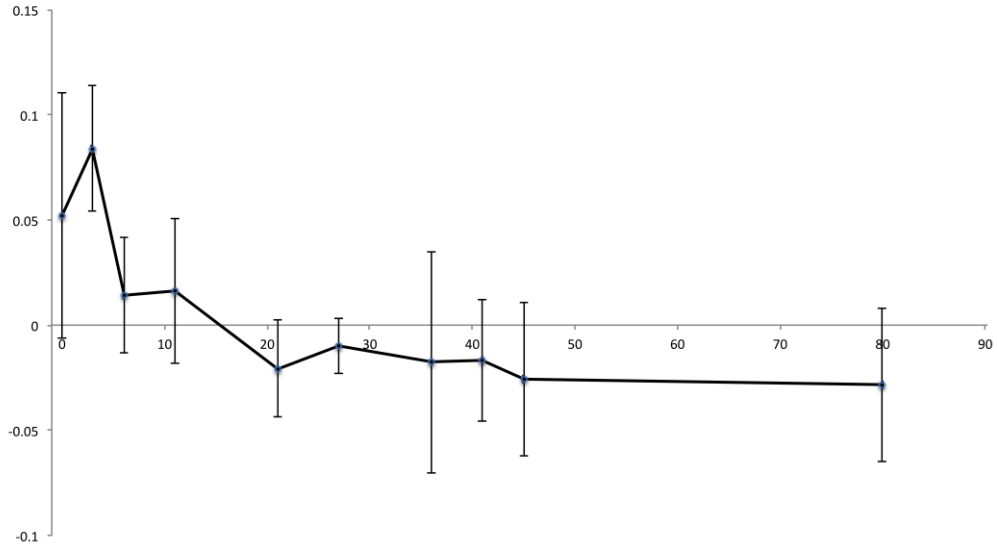

Figure S1.5. Spatial autocorrelation of genetic similarity (kinship) and geographic distance (km) within distance classes with similar numbers of individual pairwise comparisons. Error bars indicate standard errors.

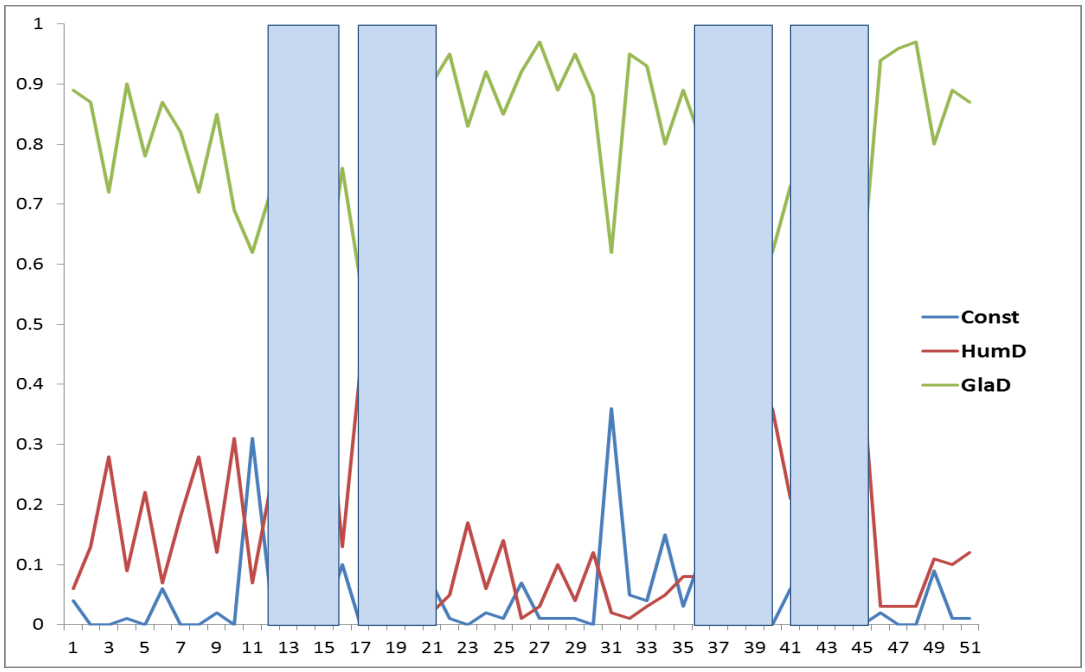

Figure S1.6. Simple representation of the posterior probability (Y axis) of three demographic models computed using the ABC approach in different analysis and different populations (X axis, see Table S2 for the analysis number). The grey bands refer to the analyses in NAG, MBA, LOP, and SPI, respectively

Table S1.1. Parameters setting for the prior distributions used in MSVAR. All values are log-10 transformed, and the age is in years. Three values were tested for the mean of the time since the population change.

	α	σ	β	τ
Current size	3	1	0	0.05
Ancestral size	3	1	0	0.05
Age of the population change	2 or 3 or 4	1	0	0.05
Mutation rate	-3.3	0.25	0	1

Table S1.2. Posterior probability of different demographic models (last three columns) tested with DIYABC under different priors settings. Ages are in years. Population sizes are in individuals. Nanc = ancient population size. Ncurr = current population size. In all these analyses, the prior distributions were uniform. The lower and upper limits of the prior distribution of the mutation rate were 0.001 and 0.00001 per locus per generation, respectively, and the parameter P refers to the geometric distribution of the increase or decrease of the number of repeated units when a mutation occurs at a microsatellite locus. The lower and upper limits of the prior distribution of the population size in the constant population size model (Const) were 10 and 5000, respectively. Ages are in years. Population sizes are in individuals. Nanc = ancient population size. Ncurr = current population size. HumD = demographic model that assumes a recent, human related decline. GlaD = demographic model that assumes an ancient model associated to the post-glacial colonization of the Alps.

Analysis	Sample	Prior limits of the parameter P	Prior limits of the age of the population decline age in the HumD model	Prior limits of the age of the population decline age in the GlaD model	Prior limits of the current population size	Prior limits of the ancient population size	Constrains in the parameters setting	Probability of the models		
								Const	HumD	GlaD
1	*ZAM*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.04*	*0.06*	*0.89*
2		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.00	0.13	0.87
3		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.00	0.28	0.72
4		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.01	0.09	0.90
5		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.22	0.78

6	*MEZ*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.06*	*0.07*	*0.87*
7		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.00	0.18	0.82
8		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.00	0.28	0.72
9		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.02	0.12	0.85
10		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.31	0.69
11	*NAG*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.31*	*0.07*	*0.62*
12		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.05	0.23	0.72
13		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.02	0.50	0.48
14		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.10	0.15	0.75
15		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.01	0.44	0.55
16	*MBA*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.10*	*0.13*	*0.76*
17		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.00	0.43	0.57
18		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.00	0.50	0.50
19		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.04	0.15	0.81
20		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.42	0.57
21	*PRA*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.07*	*0.02*	*0.90*
22		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.01	0.05	0.95
23		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.00	0.17	0.83
24		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.02	0.06	0.92
25		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.01	0.14	0.85
26	*POZ*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.07*	*0.01*	*0.92*
27		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.01	0.03	0.97
28		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.01	0.10	0.89
29		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.01	0.04	0.95
30		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.12	0.88
31	*VER*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.36*	*0.02*	*0.62*

32		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.05	0.01	0.95
33		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.04	0.03	0.93
34		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.15	0.05	0.80
35		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.03	0.08	0.89
36	*LOP*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.11*	*0.08*	*0.81*
37		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.01	0.16	0.84
38		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.01	0.32	0.67
39		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.05	0.13	0.82
40		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.36	0.62
41	*SPI*	*0.1 - 0.9*	*1-200*	*4000-15000*	*10-5000*	*5001-50000*	-	*0.06*	*0.21*	*0.73*
42		0.1 - 0.3	1-200	4000-15000	10-5000	5001-50000	-	0.01	0.35	0.64
43		0.1 - 0.3	1-600	601-15000	10-5000	5001-50000	-	0.00	0.49	0.51
44		0.1 - 0.3	1-200	4000-15000	10-50000	10-50000	Nanc>Ncurr	0.03	0.12	0.85
45		0.1 - 0.3	1-600	601-15000	10-50000	10-50000	Nanc>Ncurr	0.00	0.41	0.59

Additional analyses of the prior impact on two populations

46	ZAM	As in analysis 1, but with gamma distributed mutation rates (shape parameter: 3.2, prior limits: 0,00001-0,001)	0.02	0.03	0.94
47	ZAM	As in analysis 1, but with prior limits of the population size in the Const model set to 1-50000	0.00	0.03	0.96
48	ZAM	As in analysis 1, but including both changes introduced in 46 and 47	0.00	0.03	0.97
59	LOP	As in analysis 1, but with gamma distributed mutation rates (shape parameter: 3.2, prior limits: 0,00001-0,001)	0.09	0.11	0.80
50	LOP	As in analysis 1, but with prior limits of the population size in the Const model set to 1-50000	0.01	0.10	0.89
51	LOP	As in analysis 1, but including both changes introduced in 46 and 47	0.01	0.12	0.87

Table S1.3. Characterization of 11 microsatellite loci used in this study. Repeat motifs, size range (bp), number of alleles (N_a) and source.

Locus	Repeat motif	Range	*Na*	Source
1A	$(GATA)_{12}$	322-326	2	Hauswaldt et al., 2007
10F	$(GATA)_{12}$	206-230	7	Hauswaldt et al., 2007
8A	$(AGAT)_7AAAGAGAT(GATA)_9$	291-331	6	Hauswaldt et al., 2007
5F	$(GACA)_{13}GGCA(GACA)_7(GATA)_{14}$	116-148	3	Hauswaldt et al., 2007
9H	$(GATA)_9TAAA(GATA)_2GAAA(GATA)_6$	156-176	6	Hauswaldt et al., 2007
12F	$(GATA)_9$	219-247	8	Hauswaldt et al., 2007
F22	$(GA)_{30}$	142-148	2	Stuckas & Tiedmann, 2006
B13	$(GA)_{22}$	114-134	3	Stuckas & Tiedmann, 2006
B14	$(TC)_4T(TC)_7T(TC)_9GC(TC)_7$	164-172	5	Stuckas & Tiedmann, 2006
Bv11.7	$(GT)_{18}$	99-199	11	Nurnberger et al., 2003
Bv32.7	$(TA)_{22}$	168-210	10	Nurnberger et al., 2003

Table S1.4. Pairwise *Fst* genetic divergence among populations of *B. variegata*. Statistically significant values are in bold

	ZAM	MEZ	NAG	MBA	PRA	POZ	VER	LOP	SPI
ZAM	0.00000								
MEZ	-0.01226	0.00000							
NAG	**0.21152**	**0.21485**	0.00000						
MBA	**0.05290**	**0.04952**	**0.17688**	0.00000					
PRA	**0.10480**	**0.11676**	**0.25113**	**0.10543**	0.00000				
POZ	**0.07589**	**0.07701**	**0.22011**	**0.07576**	0.01261	0.00000			
VER	**0.08673**	**0.09419**	**0.22921**	**0.06763**	**0.04192**	**0.02886**	0.00000		
LOP	**0.05145**	**0.05813**	**0.15643**	**0.02976**	**0.10300**	**0.06687**	**0.05959**	0.00000	
SPI	**0.09433**	**0.09831**	**0.32426**	**0.14995**	**0.13690**	**0.09070**	**0.14243**	**0.13770**	0.00000

Table S1.5. Estimated demographic parameters for each population (median, lower and upper 95% credible interval) obtained with MSVAR. Three independent analyses were replicated with the exponential demographic change model and one run with the linear model. Current and ancestral population sizes are expressed in thousands of individuals and the time of the reduction in thousand of year. The prior mean of the time since the demographic change in these analysis is equal to 1,000 (3 in log10 units) years. The last eight analyses refer to two datasets obtained by pooling two pair of samples that are geographically close and genetically very similar (non-significant F_{st}).

Sample	Msvar	Demographic	Current Ne ($x10^3$)			Ancestral Ne ($x10^3$)			Time (kya)		
	Run	Change	CI lower	Median	CI upper	CI lower	Median	CI upper	CI lower	Median	CI upper
ZAM	1	exponential	0.02	0.22	1.29	0.53	2.11	8.52	0.03	0.46	8.79
	2	exponential	0.02	0.25	1.68	0.52	2.22	9.25	0.02	0.51	12.43
	3	exponential	0.03	0.26	1.58	0.50	2.01	7.90	0.03	0.53	11.90
	4	linear	0.01	0.20	1.57	0.50	2.12	8.77	0.06	0.92	17.75
MEZ	1	exponential	0.02	0.21	1.94	0.63	2.64	11.07	0.04	0.73	12.48
	2	exponential	0.01	0.20	1.72	0.64	2.69	11.45	0.03	0.69	15.56
	3	exponential	0.02	0.21	2.23	0.60	2.65	11.42	0.03	0.69	14.51
	4	linear	0.01	0.13	1.43	0.64	2.56	10.53	0.10	1.33	18.34
NAG	1	exponential	0.01	0.08	0.40	0.85	3.59	15.96	0.10	1.03	6.01
	2	exponential	0.01	0.08	0.42	0.86	3.62	16.02	0.12	1.06	6.71
	3	exponential	0.01	0.07	0.40	0.85	3.63	16.14	0.09	0.89	5.84
	4	linear	0.00	0.05	0.32	0.77	3.20	14.51	0.52	3.14	21.67
MBA	1	exponential	0.02	0.11	0.56	0.53	3.85	14.84	0.03	0.75	5.17
	2	exponential	0.02	0.10	0.56	0.52	4.03	15.76	0.02	0.74	5.03
	3	exponential	0.03	0.11	0.58	0.50	3.94	15.40	0.03	0.78	5.46
	4	linear	0.01	0.07	0.47	0.50	3.59	13.96	0.06	2.26	12.74
PRA	1	exponential	0.05	0.27	1.14	0.66	3.10	18.24	0.20	2.50	17.89
	2	exponential	0.04	0.25	1.09	0.66	3.08	17.17	0.16	2.44	16.85
	3	exponential	0.06	0.27	1.14	0.64	3.45	25.79	0.27	3.00	19.88
	4	linear	0.04	0.22	0.99	0.67	3.00	18.20	0.43	5.05	56.17
POZ	1	exponential	0.02	0.23	1.06	0.65	2.58	11.99	0.07	1.24	12.80
	2	exponential	0.02	0.19	0.97	0.64	2.60	10.99	0.07	0.99	10.00
	3	exponential	0.03	0.24	1.13	0.63	2.54	11.60	0.08	1.29	11.93
	4	linear	0.01	0.14	0.80	0.63	2.52	11.07	0.20	1.74	19.47
VER	1	exponential	0.03	0.23	1.08	0.58	2.47	11.69	0.09	1.40	13.81

	2	exponential	0.03	0.23	1.02	0.61	2.53	11.15	0.10	1.44	12.37
	3	exponential	0.04	0.26	1.13	0.60	2.50	11.75	0.11	1.69	15.43
	4	linear	0.01	0.16	0.83	0.60	2.32	9.71	0.21	2.13	20.57
LOP	1	exponential	0.01	0.15	0.70	1.06	4.09	16.25	0.08	1.26	8.04
	2	exponential	0.01	0.13	0.64	1.09	4.21	16.86	0.09	1.06	7.00
	3	exponential	0.01	0.15	0.71	1.01	3.88	16.18	0.09	1.23	7.81
	4	linear	0.00	0.10	0.58	0.94	3.60	14.34	0.45	2.76	19.55
SPI	1	exponential	0.01	0.10	0.66	0.81	3.70	20.22	0.08	0.89	11.45
	2	exponential	0.01	0.10	0.60	0.81	3.55	18.79	0.08	0.86	9.77
	3	exponential	0.01	0.09	0.62	0.83	3.57	17.65	0.07	0.79	9.19
	4	linear	0.00	0.06	0.51	0.77	3.47	19.47	0.38	2.78	36.11
PRA+POZ	1	exponential	0.04	0.27	1.09	0.69	2.83	14.00	0.08	1.63	12.52
	2	exponential	0.05	0.29	1.25	0.69	2.90	13.68	0.15	1.93	14.64
	3	exponential	0.05	0.31	1.26	0.68	2.81	12.94	0.16	1.87	13.81
	4	linear	0.03	0.22	1.04	0.64	2.64	11.67	0.28	2.58	25.85
ZAM+MEZ	1	exponential	0.03	0.32	1.84	0.57	2.38	9.71	0.03	0.71	14.03
	2	exponential	0.02	0.27	1.55	0.65	2.53	9.83	0.02	0.54	8.40
	3	exponential	0.04	0.32	1.76	0.63	2.55	10.31	0.04	0.79	11.74
	4	linear	0.02	0.31	1.91	0.59	2.40	10.96	0.06	1.32	30.12

Table S1.6. Parameter estimation under the ABC approach implemented in DIYABC. The demographic model assumed a demographic decline occurring between 1 and 15000 years ago (uniform prior). Current (Ncurr) and ancient (Nanc) population sizes had uniform priors between 10 and 50000 individuals, with Nanc always larger than Ncurr. The prior distribution of the mutation rate was a gamma distribution with shape parameter = 3.2, and constrained between 0,00001 and 0,001 per locus per generation. The parameter P (that refers to the geometric distribution of the increase or decrease of the number of repeated units when a mutation occurs at a microsatellite locus) had a uniform prior distribution constrained between 0.1 and 0.3.

Population	Label	Ncurr ($x10^3$)			Nanc ($x10^3$)			Age of the decline (kya)		
		CI lower	Median	CI upper	CI lower	Median	CI upper	CI lower	Median	CI upper
Zambana	ZAM	0.32	0.94	2.64	11.8	35.7	49.3	0.30	1.63	4.58
Loppio	LOP	0.29	0.87	2.52	8.00	32.4	49.0	2.52	1.48	4.64

Second study: **Mitochondrial and nuclear DNA survey of *Zootoca vivipara* across the eastern Italian Alps: evolutionary relationships, historical demography and conservation implications**

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Abstract

The European common lizard *Zootoca vivipara* exhibits reproductive bimodality, with populations being either viviparous or oviparous. In the central-eastern Italian Alps oviparous populations (*Z. v. carniolica*) and viviparous populations (*Z. v. vivipara*) partly overlap geographically. Studying the evolutionary relationship between these taxa presents an interesting opportunity to gain insight into the evolution of this trait. We aim to: i) test whether *Z. v. carniolica*, which is endangered, constitutes an ESU (Evolutionary Significant Unity); ii) infer mtDNA divergence time between the *Z. v. carniolica* clade and all the other *Z. vivipara* subspecies with the aid of an external calibration point; iii) describe the phylogeographical and demographic scenarios in the area. To do so we sequenced about 200 individuals for mitochondrial variation; 64 of them were also analysed for three nuclear genes. Furthermore, we analysed the same nuclear markers in 17 individuals from the other oviparous subspecies *Z. v. louislantzi* and 11 individuals of *Z. v. vivipara* from widespread geographical origins.

The mtDNA and nDNA loci that we examined supported the monophyly of *Z. v. carniolica*. The mtDNA-based estimate of divergence time between *Z. v. carniolica* and all the other subspecies indicated a separation at 4.5 Mya (95 % CI 6.1-2.6), with about 5% of sequence divergence. Considering that *Z. v. carniolica* harbours higher genetic diversity, while *Z. v. vivipara* from central-eastern Alps shows a signature of recent population and spatial expansion, we argue that *Z. v. carniolica* represents a distinct evolutionary unit, with a presumably long-term evolutionary history of separation. *Z. v. carniolica* populations, occurring at higher latitudes and altitudes than insofar supposed, live in peat bogs, a seriously threatened habitat: taking into account also its evolutionary distinctness, specific conservation measures should be considered.

Introduction

The Eurasian common lizard, *Zootoca vivipara* (Jacquin, 1787), is among the few squamate reptiles displaying reproductive bimodality at the intraspecific level.

Viviparous (or better 'lecithotrophic viviparity', i.e. live-bearing with nutrition from the yolk, Blackburn 1994) populations, ascribed to the nominotypical subspecies *Zootoca vivipara vivipara*, are widely distributed from the British Isles and central France to Scandinavia and north-eastern Asia as far as Japan (Dely and Bohme 1984). Oviparous populations are restricted to the southern edges of the range, in two disjunct areas: southern France-northern Spain and northern Italy-southern Austria-Slovenia-Croatia. The French-Spanish oviparous populations have been recently attributed to the subspecies *Z. v. louislantzi* (Arribas 2009), whose range is clearly geographically separated from viviparous *Z. v. vivipara* populations (Arribas 2009, Brana and Bea 1987, Heulin 1988, Heulin 1993). All the other oviparous populations are included in the subspecies *Z. v. carniolica* (Surget-Groba *et al.* 2006): in this case, the range has been described as parapatric to *Z. v. vivipara* (Mayer *et al.* 2000, Heulin *et al.* 2000, Surget-Groba *et al.* 2002, Figure 2.1). However, a contact zone between *Z. v. vivipara* and *Z. v. carniolica* has been recently found in Carinthia, Austria (Lindtke *et al.* 2010).

Using karyotype (Odierna *et al.* 2001) and mitochondrial DNA (mtDNA) sequence variation (Mayer *et al.* 2000, Surget-Groba *et al.* 2001, Surget-Groba *et al.* 2006), several studies have addressed the phylogenetic relationships between the different subspecies. The scenario can be summarised as follows: the two oviparous subspecies, *Z. v. louislantzi* and *Z. v. carniolica* are not reciprocally monophyletic. Considering the most comprehensive mtDNA survey (Surget-Groba *et al.* 2006), it appears that *Z. v. carniolica* is sister to all the other subspecies, namely *Z. v. vivipara*, *Z. v. louislantzi* and *Z. v. pannonica* (Lac & Kluch 1968: in this study the term used was still the former, *Lacerta vivipara pannonica*). Therefore, neither

the oviparous (*Z. v. carniolica* and *Z. v. louislantzi*) nor viviparous subspecies (*Z. v. vivipara* and *Z .v. pannonica*) form monophyletic groups, making a single transition from oviparity to viviparity unlikely.

Z. v. carniolica has been considered as an Evolutionarily Significant Unit (ESU) *sensu* Moritz (1994). However, none of the aforementioned studies included nuclear DNA sequence variation. Proving the ESU status for this subspecies would significantly support the conclusions of earlier studies pointing out that *Z. v. carniolica* would deserve specific conservation measures (Surget-Groba *et al.* 2002).

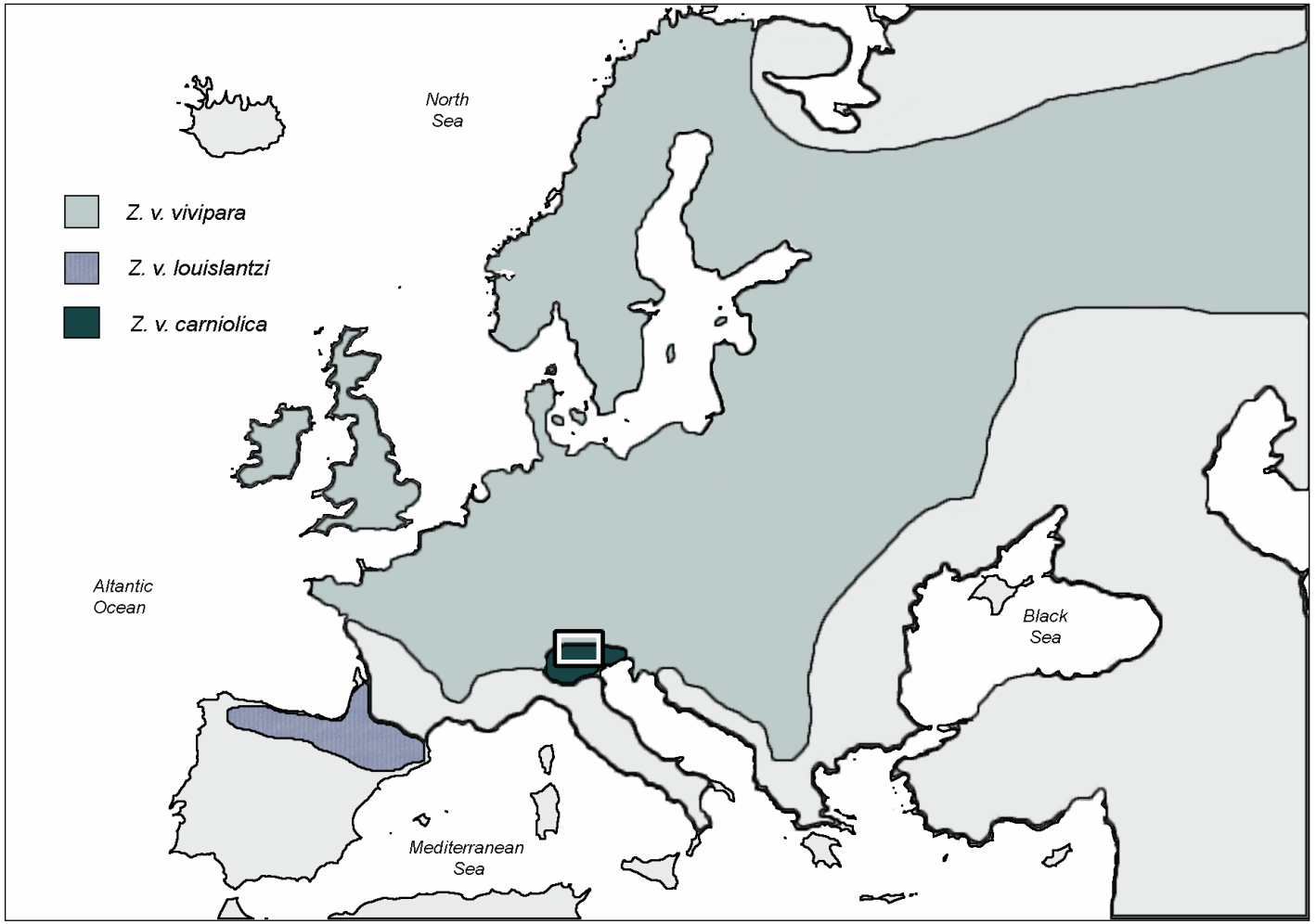

Figure 2.1. Distribution of *Zootoca* subspecies. Highlighted rectangle represents the area of the study

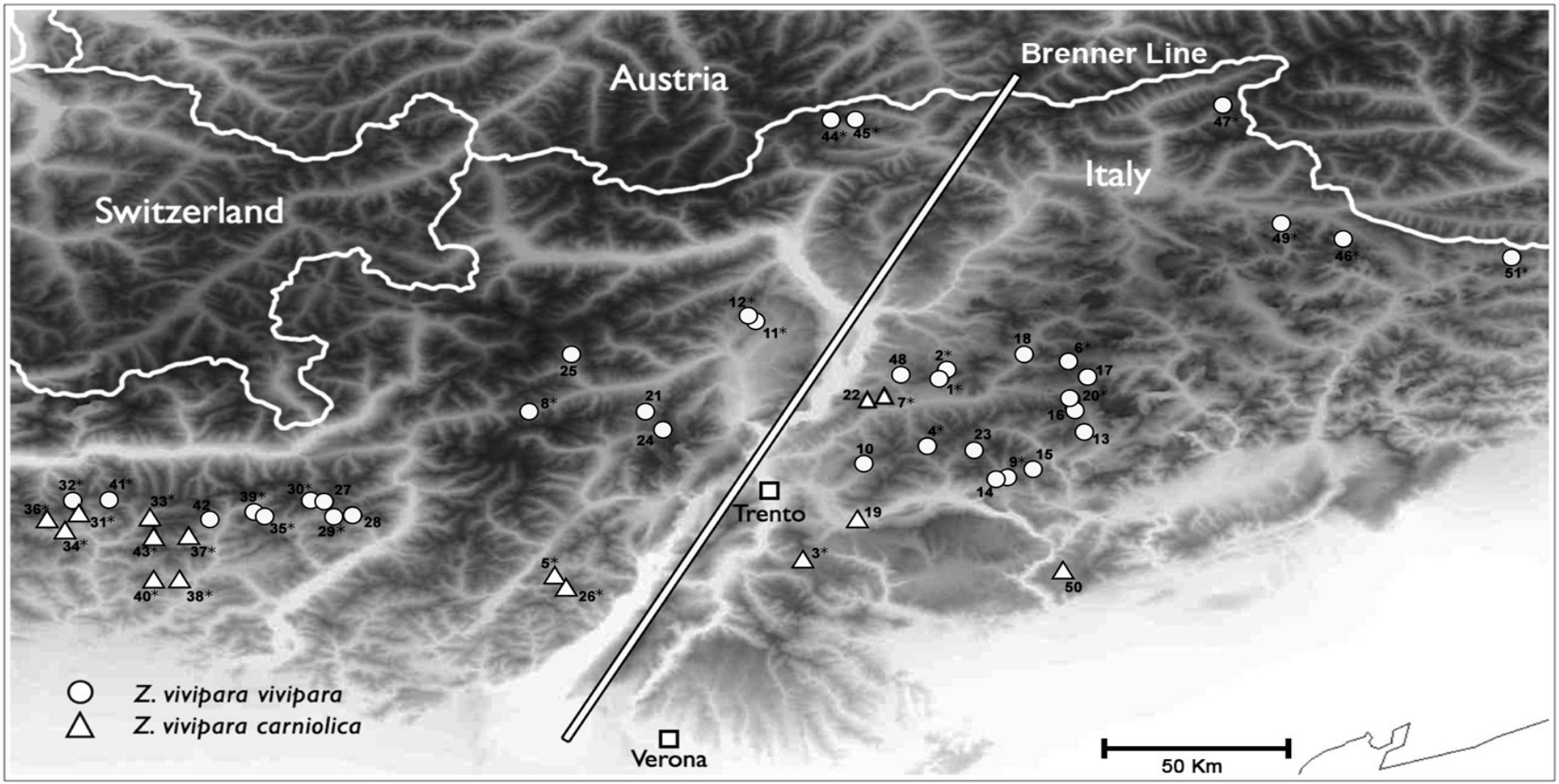

Figure 2.2. Sampling sites of *Zootoca vivipara* sp. in Northern Italy. Label, site names and coordinates are listed in Table S2.1. Circles and triangles indicate locations where we found *Z. v. vivipara* and *Z. v. carniolica,* respectively, according to cytb results. The white line represents the Brenner line. Asterisks indicate locations for which at least one sample was analysed for nuclear genes.

Z. v. carniolica has been found from Piedmont via northern Italy and Austrian Carnian Alps to Slovenia and north-west Croatia, and its northern limit appears to correspond with the Italian Prealps, while the southern limit is represented by a few wetland areas in the Po Valley. The high degree of fragmentation of these low-mid-altitude wetland areas, affected by both climate change and human activities, might pose a serious threat to *Z. v. carniolica* persistence, as highlighted by some local extinction documented in the Po Valley area (Giovine 1989, Mazzoni & Stagni 1993).

The main aim of this work was to test the ESU hypothesis for *Z. v. carniolica* by contrasting patterns of DNA sequence variation at nuclear and mitochondrial markers in 92 and 230 individuals, respectively, of *Z. vivipara* spp. from different European regions. Moreover, we specifically focused on the evolutionary history of *Z. v. carniolica* at both macro- and micro-scale by: a) inferring mtDNA divergence time between the *Z. v. carniolica* clade and all the other *Z. vivipara* subspecies with the aid of an external calibration point; b) describing the phylogeographical and demographic scenarios in an area of partial distribution overlap - central-eastern Italian Alps- between the oviparous populations of *Z. v. carniolica* and the viviparous populations of *Z. v. vivipara*.

Materials and methods

Ethics statement

All conducted experiments complied with the current laws of Italy. The Italian Ministry of Environment and the Environmental Unit of the Autonomous Province of Trento approved capture, handling, and tissue sampling (DPN/2D/2003/2267 and 4940-57/B-09-U265-LS-fd). In this study we did not apply laboratory techniques on living animals, therefore authorization from the Italian Ministry of Health was not required.

Sampling

Approximately 1 cm of tail was collected from 191 specimens of *Z. vivipara* coming from 51 locations throughout the central eastern Alps and Prealps (Figure 2.2 and Table S2.1). All animals were released in their own habitat after pouring liquid sterilizer on the tail. Tissue samples were preserved in 95% ethanol and then stored at – 80 °C until molecular analyses were performed. To have a good representation of the whole

geographic distribution and of the known subspecies within the *Zootoca* genus, 39 additional specimens were included in the nuclear marker analyses: their geographical origin and the subspecies they belong to is reported in Table S2.2 along with their mtDNA haplogroup (previously determined in Surget-Groba *et al.* 2006 and Heulin *et al.* 2011).

DNA extraction, amplification and sequencing

DNA was extracted with the commercial QIAGEN DNeasy Tissue Kit (QIAGEN Inc., Hilden, Germany) according to manufacturer's protocol. A 385 base pair (bp) fragment of mtDNA cytb gene was amplified using MVZ04 and MVZ05 primers (Smith & Patton 1991). The PCR amplification was carried out in a 20 µl reaction mix containing: 1 µl template DNA, HotMaster™ Taq Buffer 25 mM Mg^2 (Eppendorf), 100 µM dNTPs, 10 µM of each primer, 0.5 mg/ml BSA and 1 unit of HotMaster™ Taq. The thermocycling regime consisted of incubation at 94°C for 10 min, followed by 35 cycles of 94°C for 1 min, 59 °C for 45 s, and 65 °C for 1 min, with a final extension of 65 °C for 10 min. Moreover, three nuclear genes were investigated. A 572 bp fragment of oocyte maturation factor (C-mos) coding gene, a 447 bp fragment of acetylcholinergic receptor M4 (ACM4) gene and a portion of 579 bp of melanocortin receptor 1 (Mc1r) gene were amplified. All these nuclear sequences have been already used as phylogenetic markers in lacertid species (Mayer & Pavlicev 2007, Barata *et al.* 2012). The amplification protocol consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles of the series: 94°C for 1 min, annealing temperature (57°C for Hcmos3 and L-1zmos (C-mos, Mayer & Pavlicev 2007); 59°C for MC1RF and MC1RR (Mc1r, Pinho *et al.* 2010); 59°C for tg-F and tg-R (ACM4, Gamble *et al.* 2008) for 45 s and 65°C for 1 min; then, a final extension step at 65°C for 10 min. For all amplifications, contamination was rigorously checked by means of blank samples in both extraction and PCR. Before sequencing, the excess primers and dNTPs were removed using ExoSAP-IT (USB Corporation, Cleveland, OH). Sequencing of double-stranded DNA was performed in both directions using a Big Dye Terminator cycle sequencing kit (Applied Biosystems) following manufacturer's instructions; the sequencing reaction products were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The resulting sequences were edited with FinchTV version 1.4.0 (open source application developed by Geospiza Research Team), sequence fragments were assembled using Sequencher version 4.7 (Gene Codes. Corporation, USA), aligned

using Clustal X (Thompson *et al.* 1997) and checked by eye. All sequences have been deposited in GenBank database under Accession No. KF886538-KF886566.

Phylogenetic analysis and estimation of divergence time

We inferred phylogenetic relationships and divergence-times using a relaxed Bayesian molecular clock with an Uncorrelated Lognormal model (BEAST version 1.6; Drummond & Rambault 2007) on a cytb dataset comprising 96 unique haplotypes (*Z. vivipara* of our study plus deposited sequences of *Z. vivipara* spp., *Podarcis peloponnesiaca, Podarcis cretensis* and *Lacerta viridis* as outgroup (GenBank accession numbers: *Z. vivipara*: AY714882-AY714929, *Podarcis peloponnesiaca:* AY896117-AY896123*, Podarcis cretensis*: AF486191-AF4861220 and *Lacerta viridis*: EU116514). JModelTest version 1.0.1 (Posada 2009) was used to select the appropriate model of evolution for cytb gene under the Akaike Information Criterion AIC (Posada & Buckley 2004). The GTR model of nucleotide substitution with gamma rate heterogeneity among sites and, as a prior, a Yule pure birth model of speciation to estimate the time of divergence between *Z. v. carniolica* and all the others *Z. vivipara* subspecies were used. The analysis was calibrated by setting an age prior on a single node: the divergence of *Podarcis peloponnesiaca* from *Podarcis cretensis* (Lymberakis *et al.* 2008). This divergence can be approximately posed during the Messinian geological events that occurred in Mediterranean Sea at around 5.2 +- 0.1 Mya, when Crete became isolated from Peloponnese (Beerli *et al.* 1996). We adopted the vicariant event as the most likely explanation for biogeography of Mediterranean Isles as outlined by a previous study (Runemark *et al.* 2012). Posterior distributions for each parameter were obtained using a Monte Carlo Markov Chain (MCMC), which was run for 100 million generations, and sampled every 10000 generations. Inspection of the results using Tracer version 1.5 (Rambaut & Drummond 2007) confirmed that stationarity was achieved in all cases and that effective sample sizes (ESS) were adequate (all higher than 200). Trees were summarized as maximum clade credibility trees using the TreeAnnotator program which forms part of the BEAST package, and visualized using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). In each case, the first 10% of samples was discarded to avoid sampling the burn-in phase. A Bayesian Skyline Plot was also constructed with the software BEAST version 1.6 (Drummond & Rambaut 2007) using the GTR + G evolutionary model, a log-normal relaxed molecular clock with a mean substitution rate of 7.8 x 10e-9 per site per year and visualized with

Tracer version 1.5 (Rambaut & Drummond 2007). The evolutionary rate was calculated on the basis of the *P. peloponnesiaca* and *P. cretensis* divergence time. This analysis was run multiple times to check for convergence with 50 million iterations and samples drawn every 5000 MCMC steps, after a discarded burn-in of 5 million steps.

To confirm BEAST results and to get Bayesian posterior probability values of the tree we also applied MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). We ran the same dataset with 10 million generations (after this number of generations the standard deviation of split frequencies had reduced to less than 1%) with a sampling frequency of 1000, to be sure that a good sample of the posterior distribution had been obtained. The first 2500 sampled trees were discarded as 'burn-in' and posterior probabilities were calculated and reported on a 50% majority rule consensus tree of the remaining 7501 trees in the sample. The GTR + G evolutionary model was used. Moreover, a Maximum Likelihood analysis was performed with PAUP* version 4.0 (Swofford 2003) using tree-bisection-reconnection (TBR) branch swapping with 1000 rearrangements and 100 bootstrap replicates.

Additionally, we performed phylogenetic analyses based on three nuclear genes (C-mos, ACM4, Mc1r) on a subset of 92 samples of the entire dataset (64 samples from eastern Alps and 28 from the whole distributional range of the species), selected to include all the major cytb mtDNA clades. We performed phylogenetic tree reconstructions for each single nuclear gene and for a concatenated sequence of 1598 bp. We first applied PartitionFinder version 1.1.1 (Lanfer *et al.* 2012) in order to test the best partition scheme for codon positions and different single gene models of molecular evolution using the Bayesian information criterion (BIC). Four partitions were identified in nuclear sequences: ACM4 and C-mos 1st position (GTR+I+G), C-mos 2nd position (JC), C-mos 3rd position and Mc1r 3rd position (GTR) and Mc1r 1st and 2nd positions (HKY+I). These partitions and models were applied for performing phylogenetic reconstruction for each single nuclear gene and for the concatenated sequence using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003) for Bayesian analyses with the same settings as for mtDNA; for Maximum Likelihood analyses we used RAxML (version 7.4.2, Stamatakis 2006) and each partition was run under GTR+G model. For nuclear phylogenetic reconstructions, we used *Atlantolacerta andreanskyi* as outgroup (accession numbers: JX485363, JX462052, JX461870), being the phylogenetically closest lacertid lizard with all three nuclear genes available (Barata *et al.* 2012). We chosen not to concatenate mtDNA and nuclear genes, since we focused

cytb analysis on the estimation of divergence time between *Z. v. carniolica* and *Z. v. vivipara* taking advantage of a calibrated external node dated on the divergence time between *P. peloponnesiaca* and *P. cretensis*. The three nuclear genes we analysed were not available for the latter species, therefore we ran nuclear analyses separately.

Using the median-joining algorithm in the Network version 4.5.1.0 software (Bandelt *et al.* 1999) we inferred a cytb mtDNA haplotype network, combining our sequences with all those already deposited in public repositories. After phasing nuclear genes with PHASE version 2.1 (Stephens & Donnelly 2003), Network version 4.5.1.0 (Bandelt *et al.* 1999) was also used to obtain haplotype networks of the three nuclear genes. Net nucleotide divergence (Da, Nei 1987), defined as distance between cytb clades, was calculated with MEGA version 4 (Kumar *et al.* 2008). Standard and molecular diversity indices, neutrality tests and mismatch distribution were calculated using ARLEQUIN version 3.11 (Excoffier *et al.* 2005). Specific analyses on C-mos sequences for estimating the ratio, ω, between the rate of non-synonymous, dN, and synonymous, dS, substitutions were performed with DNAsp version 5 (Librado & Rozas 2009). The gametic phase of nuclear markers was not considered in phylogenetic analyses.

Results

Cytb

A 385 bp portion of the mtDNA cytb gene was examined in our 191 Italian samples. A total of 28 polymorphic sites, all of which were parsimony-informative, and 11 haplotypes were identified. Five of these were new haplotypes, since no match was found with any previous published haplotype (Table S2.3). No deletions or insertions were observed in our dataset. Mean nucleotide percentage composition was T, 35.5; C, 25.3; A, 26.7; G, 12.4; the estimated transition/transversion ratio was 8.36. We reconstructed a phylogenetic tree using Italian samples from this study and deposited sequences from different clades and origins. The phylogenetic tree obtained using BEAST, ML and Bayesian inference (Figure 2.3), with *Lacerta viridis* as the outgroup, was topologically similar to others reported in the literature (Surget-Groba *et al.* 2001, Surget-Groba *et al.* 2006). Indeed, analyses showed clear separation between the clade A, including only *Z. v. carniolica* haplotypes and the remaining clades B, C, D, E and F,

comprising all the other European *Z. vivipara* subspecies, which was well supported by bootstrap and posterior probability values. All Italian specimens clustered in two distinct clades: 42 were grouped in clade A, while 149 were placed in clade E, the western European viviparous clade in which only *Z. v. vivipara* individuals have been insofar included.

Estimation of the divergence time between the *Z. v. carniolica* clade A and the clades (B, C, D, E and F) comprising all the other subspecies, namely *Z. v. vivipara*, *Z v. louislantzi*, and *Z. v. pannonica* was obtained by adding a prior of 5.2 +- 0.1 Mya on the node separating *P. cretensis* from *P. peloponnessiaca.* Using this calibration, the divergence time between A and all the other clades was found to be 4.5 Mya with a 95 % credibility interval between 6.1 and 2.6 Mya (Figure 2.3).

The median joining network (Figure 2.4) confirmed the clear separation in different clades. As before, 42 individuals of our data set were grouped into clade A, formed by *Z. v. carniolica* haplotypes from Slovenia, Italy and southern Austria (Surget-Groba *et al.* 2006), while the other 149 were included in clade E, consisting of haplotypes of *Z. v. vivipara* from northern and western Europe. This viviparous clade E showed a distinctive "star-shape" topology, suggesting that populations of this clade might have experienced a recent demographic expansion. Assuming neutrality, population expansion gives rise to an increase in the number of rarer haplotypes in the population under examination (star-shaped network), which also leads to a unimodal mismatch distribution. To further confirm this demographic scenario, we calculated the mismatch distribution (Figure 2.5) and the values of the D (Tajima 1989) and Fs (Fu 1997) statistics, both of which were significantly negative: Tajima's D =-2.207 and Fs = -24.700 ($p < 0.01$). In the absence of selection, significantly negative values for both statistics are usually interpreted as a signature of population expansion events. Moreover, a confirmation of this demographic expansion was gained through the Bayesian coalescent-based skyline plot (Figure 2.5, inset a), showing a clear pattern of effective population size (Ne) increase in the last tens thousands years. In contrast to clade E, clade A (corresponding to subspecies *Z. v. carniolica*) did not present a star-shape topology (Figure 2.4) and both Tajima's D and Fu's Fs were not significantly different than 0 (data not shown), thus not showing any departure from neutrality. In addition, clade A showed a multimodal mismatch distribution (Figure 2.5) and no evidence of expansion through Bayesian skyline plot (Figure 2.5, inset c).

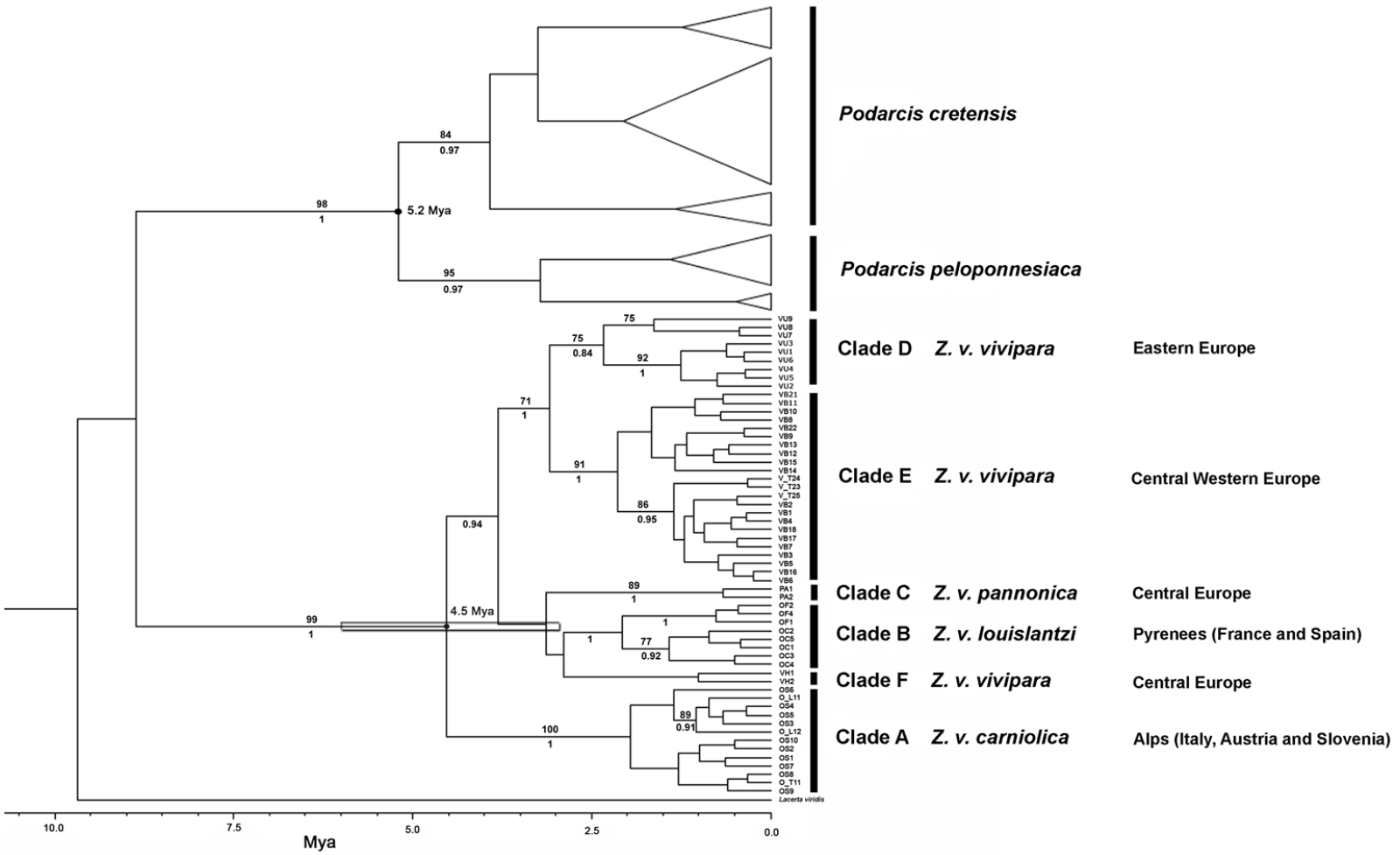

Figure 2.3. Maximum clade credibility tree from Bayesian analysis of mitochondrial cyt b with chronogram. Bar around the 4.5 Mya divergence time estimate of *Z. vivipara* sp. vs *Z. v. carniolica* shows the 95 % Credibility Interval. Bootstrap support values of Maximum Likelihood analysis > 70% are shown above the branches, while posterior probability values of Bayesian Inference > 0.7 are shown below the branches. Clade names as in Surget-Groba *et al.* (2002).

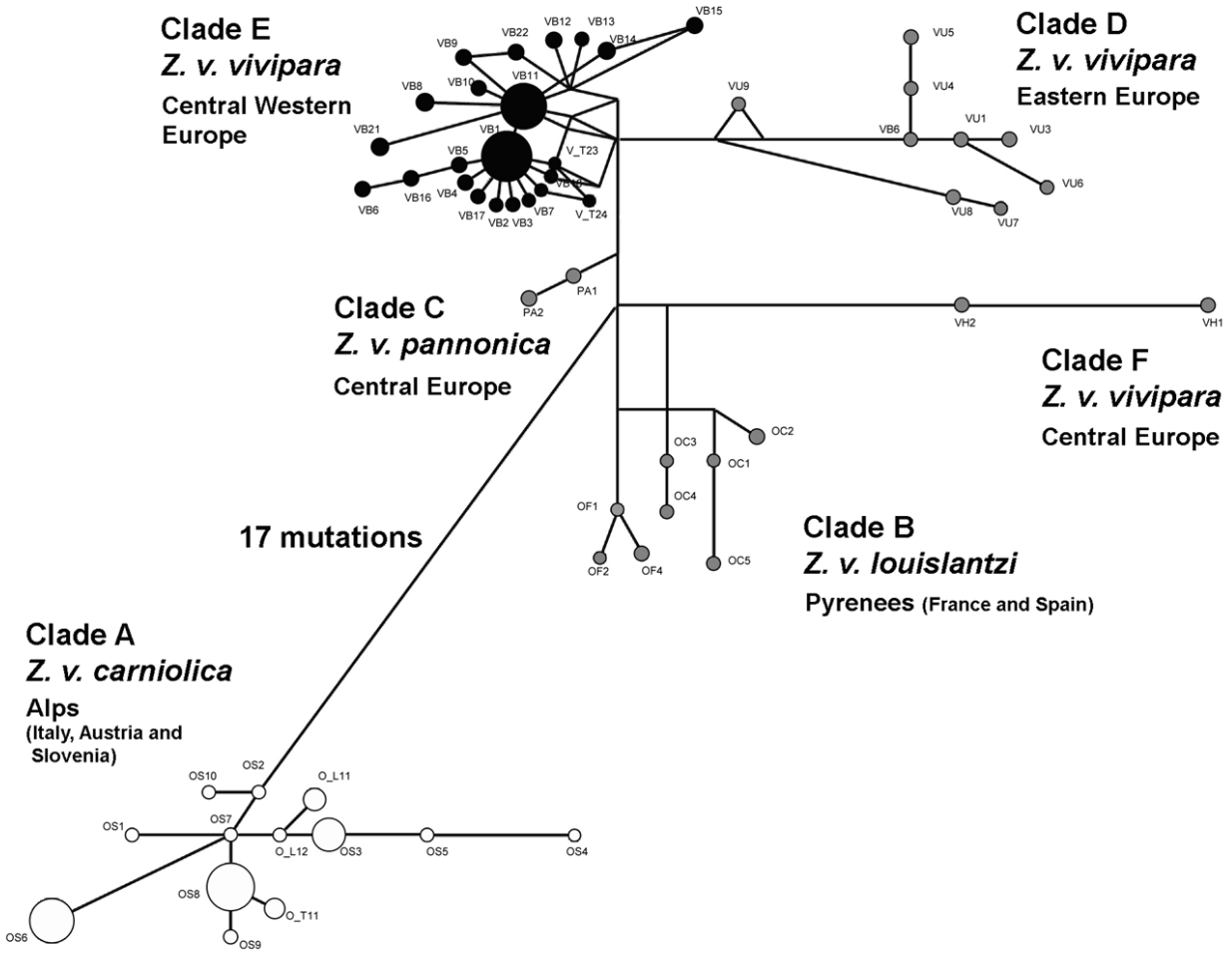

Figure 2.4. Median joining network of mtDNA cty b haplotypes. Circles represent haplotypes, area is proportional to frequency and colour indicates the subspecies (black, *Z. v. vivipara*; white, *Z. v. carniolica*; grey, other subspecies or European populations).

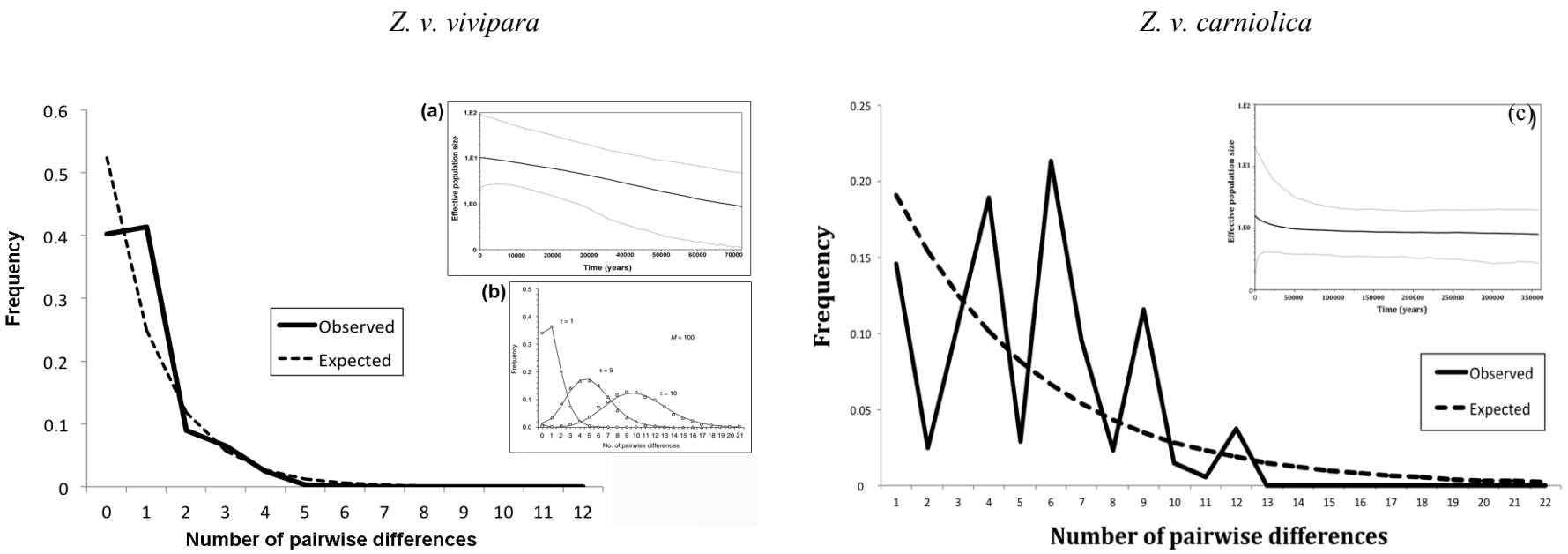

Figure 2.5. *Z. v. vivipara* (left, clade E) and *Z. v. carniolica* (right, clade A) cyt b mismatch distribution. The number of nucleotide site differences between pair of individuals and the frequency of observation, are reported on the x- and y-axis respectively. Dashed and thick lines represent observed and expected (under sudden expansion model) distribution, respectively. In the insets: a) and c) Bayesian skyline plot with median value and 95 % Credibility Interval for *Z. v. vivipara* and *Z. v. carniolica*, respectively; b) mismatch distribution under demographic and spatial expansion model as in Excoffier (2004).

Finally, we estimated the average number of nucleotide differences between the two groups (clade A and clade E) in which all our Italian individuals were divided: it was equal to 20.801 +/- 3.974 (SD), hence 0.054 per site. The number of net nucleotide substitutions per site between groups, Da, was 0.049, indicating an average difference of about 5%. The two groups did not share any substitution. The results, reported in Table 2.1, indicate that despite the lower number of individuals analysed, there was much higher genetic variation within *Z. v. carniolica* clade than in *Z. v. vivipara* clade E.

		n[a]	k[b]	s[c]	n transitions	n transvertions	π[d]	MPD[e]	H[f]
mtDNA cyt b	*Z. v. vivipara*	149	5	3	3	-	0.001 ± 0.001	0.533 ± 0.434	0.480 ± 0.029
	Z. v. carniolica	42	6	9	9	-	0.008 ± 0.005	3.120 ± 1.673	0.721 ± 0.044
nuDNA C-mos	*Z. v. vivipara*	33	1	-	-	-	-	-	-
	Z. v. carniolica	31	5	4	2	2	0.002 ± 0.002	1.361 ± 0.860	0.569 ± 0.080
nuDNA Mc1r	*Z. v. vivipara*	33	6	6	5	1	0.003 ± 0.002	2.000 ± 1.240	0.889 ± 0.091
	Z. v. carniolica	31	5	4	4	-	0.002 ± 0.001	1.036 ± 0.745	0.709 ± 0.136
nuDNA ACM4	*Z. v vivipara*	33	3	2	1	1	0.003 ± 0.002	1.333 ± 0.910	0.667 ± 0.131
	Z. v. carniolica	31	2	1	1	-	0.001 ± 0.001	0.436 ± 0.421	0.436 ± 0.133

Table 2.1. Indices of genetic variability for the two subspecies at cyt b and nuclear markers. [a]n, sample size , [b]k, number of haplotypes, [c]s, number of polymorphic sites, [d]π, nucleotide diversity, [e]MPD, mean pairwise differences, [f]H, gene diversity

Nuclear genes

We successfully analysed 92 samples (30 individuals belonging to clade A, 17 to clade B, 4 to clade D, 39 to clade E and 2 to clade F) with three different nuclear genes: C-mos, ACM4 and Mc1r.

In some Lacertid species the presence of several functional and non-functional copies of the C-mos gene has been reported (Pavlicev & Mayer 2006). Before any phylogenetic analysis, it is therefore important to verify that only orthologous C-mos sequences are used for comparisons. None of our sequences presented deletion, insertion or internal stop codons. The ratio ω (dN/dS) was significantly higher than 1, thus rejecting the hypothesis of neutrally evolving sequences, as expected in case of non-functional copies. We concluded that all our C-mos sequences were functional copies of the C-mos gene, being therefore orthologous.

The median joining network of the phased alleles of C-mos and Mc1r, showed that individuals belonging to mtDNA clade A (*Z. v. carniolica*) do not share any allele with individuals from other clades (Figure 2.6). In contrast, two out of five ACM4 alleles (ACM4_4 and ACM4_6) are shared among individuals of *Z. v. carniolica* and individuals of clades B (*Z. v. louislantzi*), D (*Z. v. vivipara*), E (*Z. v. vivipara*) and F (*Z. v. vivipara*). Similarly, phylogenetic trees obtained from each single nuclear gene suggested a highly supported (bootstrap and Bayesian posterior probability higher than 97% and 0.97, respectively) monophyly of *Z. v. carniolica* in C-mos and Mc1r (Figure 2.7), but not in ACM4. Phased alleles along with their accession numbers were listed in Table S2.3.

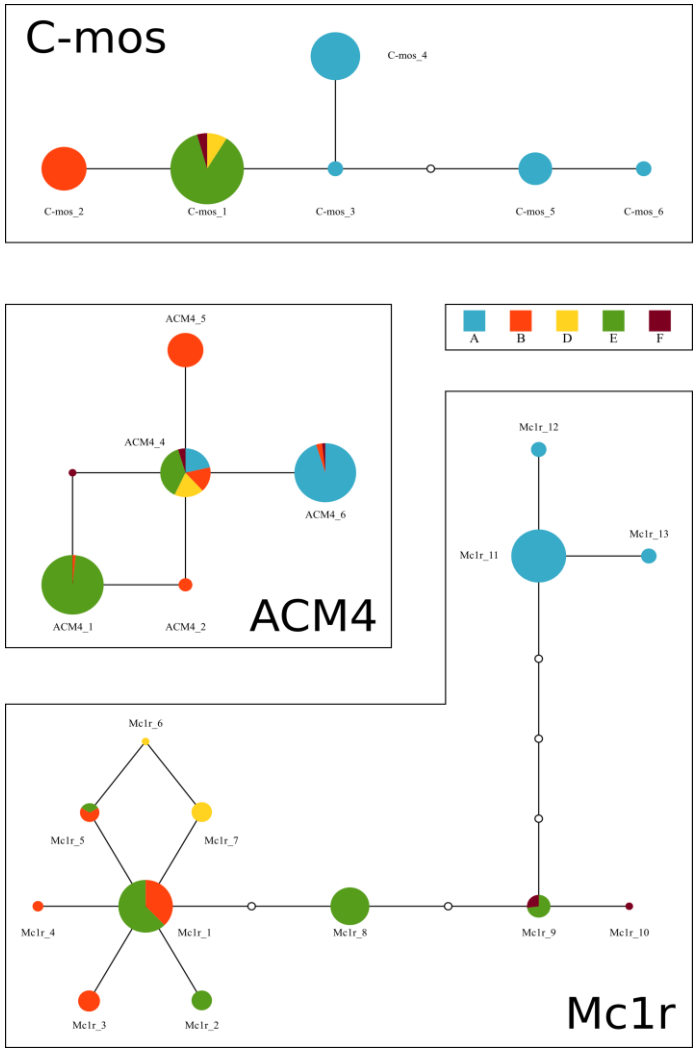

Figure 2.6. Median joining network of three nuclear genes. Circles represent phased alleles, area is proportional to frequency and colour indicates the mtDNA clade (see legend).

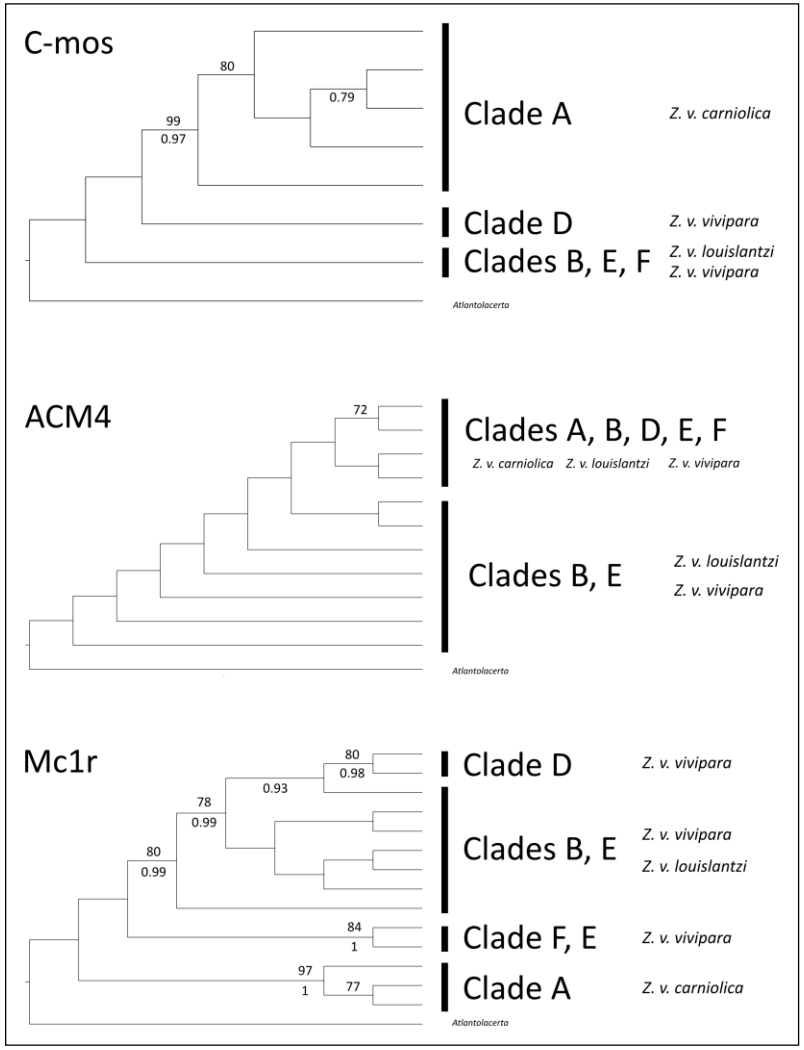

Figure 2.7. Maximum clade credibility trees of Bayesian analyses of three nuclear genes. Bootstrap support values of Maximum Likelihood analysis > 70% are shown above the branches, while posterior probability values of Bayesian Inference > 0.7 are shown below the branches. *Atlantolacerta andreanskyi* was used as outgroup.

After concatenating the three genes, we obtained a 1598 bp sequence. Thirty-two variants out of the 92 sequences were identified. The tree topology obtained with different methods was concordant so that only the Maximum Clade Credibility Tree is presented in Figure 2.8. The tree showed two well supported clades. The first composed only of individuals belonging to mtDNA clade A, that is the *Z. v. carniolica* clade (90 % and 0.84, bootstrap and Bayesian posterior probability, respectively), whilst the second consisted of individuals with mtDNA belonging to all the other clades, namely B (*Z. v. louislantzi*), D (*Z. v. vivipara*), E (*Z. v. vivipara*) and F (*Z. v. vivipara*). We did not get any reliable nuclear sequences from the only two individuals of clade C (*Z. v. pannonica*) at our disposal. Analysis of these nuclear markers, thus, confirmed the monophyly of *Z. v. carniolica*. At the same time, it confirmed that the other oviparous subspecies, *Z. v. louislantzi*, is much more closely related to the viviparous subspecies. Nuclear markers, therefore, indicated a likely reversal from viviparity to oviparity, as originally hypothesised by mtDNA results (Surget-Groba *et al.* 2006).

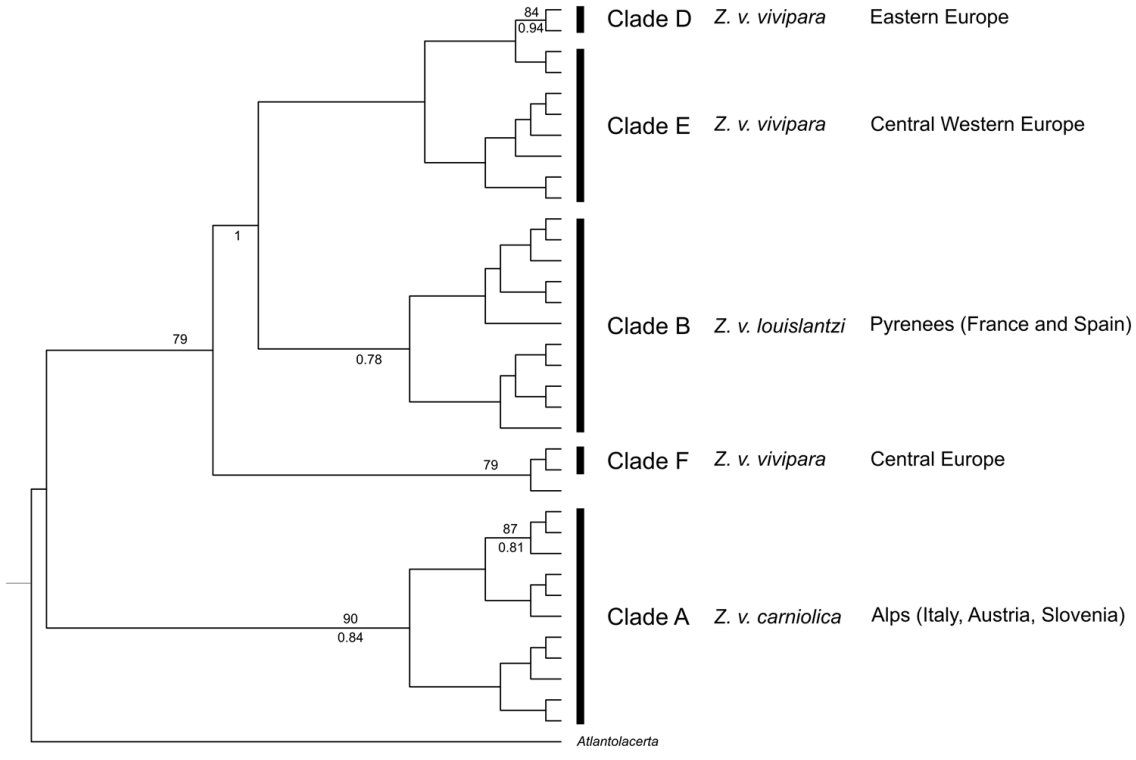

Figure 2.8. Maximum clade credibility tree of Bayesian analysis of three concatenated nuclear genes variants (C-mos, ACM4 and Mc1r). Bootstrap support values of Maximum Likelihood analysis > 70% are shown above the branches, while posterior probability values of Bayesian Inference > 0.7 are shown below the branches. *Atlantolacerta andreanskyi* was used as outgroup.

Discussion

ESU status

Considering not only the reproductive mode but also mtDNA and karyological features, Surget-Groba *et al.* (2002) proposed to consider *Z. v. carniolica* populations from Slovenia and northwestern/northeastern Italy as an Evolutionarily Significant Unit (ESU) following Moritz (1994). According to this definition, ESU status is evaluated by taking into account both mtDNA and nuclear loci: two populations would be considered ESUs if reciprocally monophyletic at mtDNA alleles and showing significant divergence of allele frequencies at nuclear loci. Although Moritz's ESU definition has been debated (e.g. Crandall *et al.* 2000), it is nonetheless widely used in the conservation field. In particular, contrasting the patterns of mtDNA and nuclear variation is routine for testing the distinctiveness of natural populations (see Frankham *et al.* 2002). Due to differences in effective population size and mutation rate, nuclear DNA loci attain monophyly at a considerably slower pace than mtDNA haplotypes. Instances of concomitant monophyly at mtDNA and nuclear loci imply, therefore, a long-term history of evolutionary separation. While it is arguable whether such a separation is sufficient for the recognition of different taxonomic units (e.g., under the genealogical species concept, Baum & Shaw, 1995), its importance from an evolutionary and conservation perspective cannot be neglected.

In this survey we considered all the subspecies of the *Zootoca* genus: the results showed that *Z. v. carniolica* monophyly is strongly supported by both mtDNA (cytb; Fig. 2) and nuclear markers (C-mos, ACM4 and Mc1r; Figure 5) trees. In the concatenated nuclear markers tree, the other known oviparous subspecies, *Z. v. louislantzi* (mtDNA clade B), clustered within the same clade as *Z. v. vivipara*, (mtDNA clades D, E and F) from different origins within its distribution range. We could not incorporate any individual from the *Z. v. pannonica* subspecies (mtDNA clade C) due to poor DNA quality. However, if we consider the concordance between the mitochondrial- and nuclear-based phylogeny and the previously demonstrated inclusion of *Z. v. pannonica* in the same mtDNA clade as *Z. v. vivipara* and *Z. v. louislantzi* (Surget-Groba *et al.* 2006), this omission would not be expected to alter the overall scenario. To the best of our knowledge, this is the first phylogenetic inference based on nuclear DNA in *Zootoca* genus. The nuclear and mitochondrial DNA tree topologies

support *Z. v. carniolica* monophyly, which is also essentially confirmed by the extent of nuclear allele sharing. *Z. v. carniolica* individuals do not share any allele with individuals from other subspecies at C-mos and Mc1r genes; just two out of the five alleles of ACM4, on the other hand, are shared among individuals of *Z. v. carniolica*, *Z v. louislantzi* and *Z. v. vivipara*, most likely due to the retention of ancestral polymorphism. Finally, the concordance between mitochondrial and nuclear markers confirms the reliability of mtDNA-based discrimination of the different subspecies. This can help to assess the geographic occurrence of *Z. vivipara* subspecies that, otherwise, might be problematic if based only on morphology.

Evolutionary, demographic and phylogeographical scenarios

Adopting the mtDNA clades definition of Surget-Groba *et al.* (2006), our investigation has been concentrated on clade A (eastern oviparous) and clade E (western viviparous) in a specific area (central-eastern Italian Alps) where distributions partially overlap.

The results of mtDNA and C-mos, in particular, showed much greater genetic variability in populations of *Z. v. carniolica* than in those of *Z. v. vivipara* (see Table 2.1), even though a far lower number of individuals of the latter have been analysed. Gene diversity was significantly higher in *carniolica* than in *vivipara* (z-test, $p < 0.05$), in both the cytb and the C-mos. Greater genetic diversity can be related to a longer evolutionary history (see Hartl & Clark 2006). In this case, the molecular data would confirm the phenotypic data with respect to reproductive mode, with oviparity being the ancestral condition. The reduced genetic variation of clade E (*Z. v. vivipara*) compared with clade A (*Z. v. carniolica)* could be associated to a small effective population size during the divergence from an oviparous form, followed by a more recent demographic expansion (see below) after the retreat of the ice from western central Europe.

From the phylogenetic tree (Figure 2.3) it emerges that oviparous clade A and viviparous clade E are not sister clades. It is, however, worth noting that the overall cytb phylogeny of the different oviparous (A and B) and viviparous (C, D, E and F) clades clearly shows that clade A is the sister clade to all the others. Our inference places the divergence of clade A at approximately 4.5 Mya (95% CI 6.1-2.6 Mya) that is during the Pliocene. This estimation should not be over-emphasized or, even worse, taken at face value. We obtained this estimated divergence time after setting, as geological calibration on the node separating *P. cretensis* from *P. peloponnesiaca*, the date the island of Crete presumably separated from the Peloponnese, according to geological

evidence (Beerli *et al.* 1996), that is 5.2 Mya before the present day. While having a more refined calibration would improve our estimate of the divergence time (see Yang and Yoder, 2003), it nonetheless can be said that the sister oviparous clade A had a long evolutionary history since the original split from all the other clades of the species.

Another indication of the deep evolutionary distance between clade A and E stems from the number of differences in the cytb fragment. The average number of nucleotide differences between the two clades was 20.801 +/- 3.974 (hence 0.05403 per site), with a net nucleotide divergence of approximately 5% (Da = 0.049), which, for the mitochondrial cytb gene, indicates a rather large divergence. These figures are similar to observations between two distinct species belonging to a genus "close" to *Zootoca: P. peloponnesiaca* and *P. cretensis.* They showed an average number of nucleotide differences of 17.589 +/- 3.292 and Da of 0.047 for the same cytb marker (Slatkin & Hudson 1991).

The haplotype cytb network (Figure 2.4) shows another striking difference between the two subspecies: all our *Z. v. vivipara* individuals harbour haplotypes clustering in clade E, that is, according to its original definition (Surget-Groba *et al.* 2001), the western viviparous group. This clade is characterized by a "star-shape" topology, suggesting recent population expansion (Rogers & Harpending 1992). In contrast, all our *Z. v. carniolica* individuals have haplotypes belonging to clade A (Surget-Groba *et al.* 2001), and whose network does not present any particular topology. The indication of a demographic expansion in clade E can be further evaluated through the mismatch distribution graph (Figure 2.5). This graph shows a unimodal trend, which is again considered a signature of a recent demographic expansion (Excoffier 2004). Our distribution is not in contradiction with the theoretical model of Excoffier (2004), describing an instantaneous range expansion in a two-dimensional stepping-stone model, with large migration rates and recent expansion (Figure 2.5, inset b). A plausible scenario would thus imply that, with the retreat of the ice in the post-Pleistocene era, populations of *Z. v. vivipara* belonging to clade E were able to spread into northwestern Europe (see Heulin *et al.* 1993), leading to a simultaneous population and spatial expansion. The signature of this demographic expansion appears robust as it is also supported by the results of the neutrality tests (both Tajima's D and Fu's Fs, statistically significant and negative) and of the coalescent-based skyline plot that shows a pattern of a relatively recent increase of Ne within clade E (Figure 2.5, inset a).

All the aforementioned demographic inferences are based on the assumption of neutrality for mtDNA cytb. Although the mitochondrial cytb marker was considered neutral in most of the previous studies on the phylogeography of *Z. vivipara* (Surget-Groba *et al.* 2001, 2002, 2006), a recent paper, focussed on a contact zone of two mtDNA *Z. v. louislantzi* lineages (Heulin *et al.* 2011), questioned this assumption. The authors hypothesised a kind of thermal-related selection for explaining the differential survival of two subadults' cytb haplogroups living in syntopy, in a secondary contact zone. It appears at least advisable to wait for new evidence (comparison of survivorship over a longer period) supporting this hypothesis. Moreover, we think that even if there is selection acting locally on mtDNA at a contact zone, it does not mean that we cannot recover a historical pattern (e.g. expansion) from other wider regions and for other lineages with allopatric distributions. Thus, we think that cytb can still be used for general demographic and phylogeographical inferences, especially if they are confirmed by other markers, like in our study.

Biogeographical distribution

According to our results, 149 out of 191 individuals of our Italian samples were assigned to the subspecies *Z. v. vivipara* and 42 to the subspecies *Z. v. carniolica*. This allowed clarification of the situation surrounding the distribution of the two subspecies in the 51 sites sampled. In 15 sites only the subspecies *Z. v. carniolica* was found, while in 36 sites only the subspecies *Z. v. vivipara* was present (Figure 2.2).

According to our study, in the central-eastern Italian Alps *Z. v. vivipara,* on average, tends to live at higher altitudes (mean 1701 m) than *Z. v. carniolica* (mean 1210 m). *Z. v. carniolica* populations can be found at higher altitudes than initially thought: at sites above 1400 m in Trentino (Tremalzo 1545 m, Lago Nero 1625 m, Palù Longa 1435 m), in Veneto (Monte Grappa 1700 m), and in Lombardy (Branzi 1800 m, Ardesio 1600 m, Roncobello 1880 m). A high altitude (1900 m) population of *Z. v. carniolica* was also identified in Piedmont (northwestern Italy) by Ghielmi *et al.* (2001, 2006). *Z .v. carniolica* and *Z. v. vivipara* exhibit an indisputable overlap of their altitudinal distributions in the Italian Alps, similarly to other areas such as Carinthia, Austria, where the two subspecies have even been found in syntopy in a site at 1575 m (Lindtke *et al.* 2010).

The geographical distribution of the different haplotypes (Figure 2.4) corresponds to a biogeographical limit called the 'Brenner line' (i.e. a longitudinal line

from the Adige Valley up to the Brenner Pass, Figure 2.2). This line has been recognized as delimiting eastern and western distributions of many plant species since the 19th century (Kerner 1870 and see other examples below). All populations of *Z. v. vivipara* on the east of this line have VB11 (or derived haplotypes), while all population on the west have VB1 (or derived haplotypes). The only exception is one sample in population 12, which is on the west but shows VB11. The same pattern of east-west division by the Brenner line seems to hold for our *Z. v. carniolica* haplotypes of clade A. In this case, haplotypes OS8 and OT_11 belong only to individuals from sites east of this line. These two haplotypes cluster together with OS9, a haplotype described by Surget-Groba *et al.* (2006) and found in individuals from the Italian province of Udine that is located far east of the Brenner line.

The nuclear marker phylogenetic tree does not present the same biogeographical pattern: it is likely that the slow mutation rate of these markers limits their phylogeographical informativeness.

Further research with markers better suited for fine-scale population genetics analyses, such as microsatellites, could confirm this preliminary indication of a possible east-west differentiation along the Brenner line. This pattern would be in line with what have been already found in the high-altitude butterfly, *Erebia euryale* (Haubrich & Smith 2007), in an Alpine form of rampion, *Phyteuma globulariifolium,* in the alpine speedwell*, Veronica alpina,* (Schonswetter *et al.* 2002, Albach *et al.* 2006) and in many other plant species (Thiel-Egenter *et al.* 2009).

Implications for conservation

Considering the evidence from karyotype to cytb variation, and the results of our study, the distinction between *Z. v. vivipara* and *Z. v. carniolica* can be regarded as evolutionarily substantial. While it is arguable whether such distinction deserves a taxonomical revision, we think that nonetheless it has some important consequences for conservation. In our view, proposing specific conservation action for *Z. v. carniolica* is further strengthened by a number of important aspects.

The low and medium altitude peatland habitats that *Z. v. carniolica* prefers are already thought to be at high risk of extinction (Moore 2002). Indeed, the European Habitats Directive 43/92/CEE has classified active raised bogs (Natura Code 2000: 7110), transition and quaking bogs (7140), and alkaline fens (7230) as either threatened (7140, 7230) or even seriously threatened (7110). In particular, peatlands across the

Alps are suffering from a reduction in both the surface area of individual peatlands and their total number.

Moreover, a study on the peat bogs of Italian Alps (Bragazza 2008) revealed that heat waves, like that of 2003, affected the survival of organisms such as peat mosses (genus *Sphagnum*), which play a crucial role in maintaining bog functionality (i.e. carbon storage). Such a drastic change in mountain peat bogs due to just a single summer of higher temperatures and reduced rainfall represents a major concern with respect to the conservation status of this habitat. Exceptionally hot European summers, like that of 2003, may occur more frequently given recent climatic changes, bringing perilous consequences for mountain peatlands and their associated flora and fauna like *Z. v. carniolica*.

Conclusions

The main conclusion of our study is that the reciprocal monophyly between the oviparous subspecies *Z. v. carniolica* and all the other *Z. vivipara* subspecies has been proved for the first time using nuclear DNA markers. This now makes it possible to properly consider *Z. v. carniolica* as an ESU. The macro- and micro-scale analysis of the evolutionary history of *Z. v. carniolica* allowed us to reach the following conclusions: i) according to an external fossil calibration, the divergence time between *Z. v. carniolica* and all the other subspecies took place at least 2.6 millions years before the present day, thus corresponding to a relatively long time of evolutionary separation; ii) also in terms of demographic history, there is a remarkable difference: *Z. v. carniolica* does not show any signature of expansion as it occurs in the most widespread *Z. v. vivipara* clade (clade E of central-northern Europe); iii) the genetic evidence of this study, together with the vulnerability of *Z. v. carniolica* most suitable habitats (i.e. low-mid altitudes peat bog), suggests specific action tailored to this subspecies.

While future studies could better address the recent findings of sintopy and of possible hybridization between *Z. v. carniolica* and *Z. v. vivipara* (Lindke *et al.* 2010), a clear evolutionarily and demographic distinction has now been demonstrated, much likely prompting a taxonomical revision.

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Supplementary materials

	Sites	N	GPS coordinates		Altitude	mtDNA	cyt b haplotypes	Cmos	ACM4	Mc1r
			North	East	(m asl)	clade				
1	P. Lavazè (Peat bog)	11	46°20'25"	11°29'13"	1565	E	VB11(11)	C-mos_1(2)	ACM4_4(2)	Mc1r_9(2)
2	P. Lavazè (Lake)	5	46°21'22"	11°29'33"	1805	E	VB11(5)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
3	Costa	3	45°54'42"	11°11'38"	1250	A	OS8(3)	C-mos_4(2)	ACM4_6(1), ACM4_4(1)	Mc1r_11(2)
4	Passo Manghen	21	46°10'39"	11°27'12"	2060	E	VB11(2), VT_24(1), VB1(18)	C-mos_1(8)	ACM4_1(5), ACM4_4(3)	Mc1r_1(4), Mc1r_2(4)
5	Lago d' Ampola	13	45°52'19"	10°39'16"	735	A	OS6(13)	C-mos_6(4), C-mos_5(2), C-mos_4(14)	ACM4_6(18), ACM4_4(2)	Mc1r_12(1), Mc1r_11(15), Mc1r_13(4)
6	Passo San Pellegrino	4	46°22'29"	11°45'32"	1795	E	VB11(4)	C-mos_1(4)	ACM4_1(4)	Mc1r_1(4)
7	Palù Longa	12	46°17'41"	11°21'58"	1435	A	OT_11(2), OS8(10)	C-mos_4(20)	ACM4_6,ACM4_4	Mc1r_11(20)
8	Passo Tonale	10	46°15'31"	10°35'52"	1850	E	VB1(9), VT_23(1)	C-mos_1(12)	ACM4_1(12)	Mc1r_8(4), Mc1r_1(5), Mc1r_2(3)
9	Masi Carretta	12	46°06'24"	11°37'51"	1305	E	VB1(2), VB11(10)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
10	Passo Redebus	3	46°08'20"	11°19'04"	1435	E	VB1(2), VB11(1)	NA	NA	NA
11	Palù Longia	6	46°28'20"	11°04'58"	1565	E	VB1(6)	C-mos_1(2)	ACM4_1(2)	Mc1r_5(2)
12	Palù Tremole	14	46°28'44"	11°04'30"	1720	E	VB1(13), VB11(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
13	Lago Calaita	7	46°12'21"	11°47'38"	1660	E	VB1(6), VB11(1)	NA	NA	NA
14	Biotopo "I Mughi"	5	46°05'52"	11°36'38"	1220	E	VB1(3),VT_24(2)	NA	NA	NA
15	Passo Brocon	1	46°07'13"	11°41'10"	1670	E	VB11(1)	NA	NA	NA
16	Malga Ces, Siror	1	46°16'17"	11°46'19"	1680	E	VB11(1)	NA	NA	NA
17	Passo Valles	1	46°20'22"	11°47'58"	2030	E	VB11(1)	NA	NA	NA
18	Soraga	2	46°23'25"	11°40'00"	1205	E	VB11(2)	NA	NA	NA
19	Inghiaie	1	45°59'51"	11°18'37"	450	A	OS8(1)	NA	NA	NA
20	Laghi di Colbricon	3	46°16'59"	11°45'59"	1915	E	VB11(3)	C-mos_1(2)	ACM4_1(2)	Mc1r_2(2)
21	Campo Carlo Magno	5	46°15'30"	10°50'50"	1650	E	VB1(5)	NA	NA	NA
22	Lago Nero	1	46°16'53"	11°19'39"	1625	A	OS8(1)	NA	NA	NA
23	Laghetti di Lasteati	6	46°10'04"	11°33'30"	2080	E	VB1(4), VB11(2)	NA	NA	NA
24	Croda Rossa	2	46°13'01"	10°53'06"	2160	E	VB1(2)	NA	NA	NA
25	Val de la Mare	2	46°23'32"	10°41'29"	1400	E	VB1(2)	NA	NA	NA
26	Tremalzo	1	45°50'25"	10°40'56"	1545	A	OS6(1)	C-mos_4(2)	ACM4_6(2)	Mc1r_11(2)

27	Valle Vernecolo	2	46°02'36"	10°09'26"	1800	E	VB1(2)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
28	Malga Lifretto	1	46°01'05"	10°12'58"	1400	E	VB1(1)	NA	NA	NA
29	Campelli	1	46°00'45"	10°10'40"	1160	E	VB1(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
30	Valle del Vò	2	46°02'57"	10°07'46"	1810	E	VB1(2)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
31	Branzi	1	46°00'10"	9°47'06"	1800	A	OS3(1)	C-mos_5(2)	ACM4_6(2)	Mc1r_11(2)
32	Mezzoldo	1	46°02'33"	9°37'03"	1800	E	VB1(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
33	Averara	1	46°01'36"	9°37'43"	1415	A	OS3(1)	C-mos_3(2)	ACM4_6(1), ACM4_4(1)	Mc1r_11(1), Mc1r_13(1)
34	Cusio	1	45°59'02"	9°36'06"	1125	A	OS3(1)	C-mos_5(2)	ACM4_6(1), ACM4_4(1)	Mc1r_11(1), Mc1r_13(1)
35	Vilminore	5	46°00'35"	10°01'55"	1640	E	VB1(5)	C-mos_1(2)	ACM4_1(2)	Mc1r_8(2)
36	Ornica	1	45°59'54"	9°33'39"	1330	A	OS3(1)	C-mos_5(2)	ACM4_6(2)	Mc1r_11(2)
37	Valgoglio	2	45°57'58"	9°52'06"	1420	A	OL_11(2)	C-mos_5(2)	ACM4_6(2)	Mc1r_11(2)
38	Ardesio	1	45°52'00"	9°50'57"	1600	A	OL_11(1)	C-mos_5(2)	ACM4_6(2)	Mc1r_11(2)
39	Val Bondione	3	46°01'21"	10°00'39"	1290	E	VB1(3)	C-mos_1(4)	ACM4_1(4)	Mc1r_1(4)
40	Oneta	1	45°51'49"	9°47'37"	1320	A	OL_12(1)	C-mos_4(2)	ACM4_6(2)	Mc1r_11(2)
41	Valleve	1	46°03'06"	9°41'49"	1830	E	VB1(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_11(2)
42	Gandellino	4	46°00'18"	9°54'43"	1720	E	VB1(4)	C-mos_1(4)	ACM4_1(4)	Mc1r_8(2), Mc1r_1(2)
43	Roncobello	1	45°57'55"	9°47'30"	1880	A	OL_11(1)	C-mos_3(2)	ACM4_6(2)	Mc1r_11(2)
44	Ridanna	1	46°56'30"	11°15'00"	1715	E	VB1(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_1(2)
45	Vipiteno	1	46°56'50"	11°18'00"	1815	E	VB1(1)	C-mos_1(2)	ACM4_1(2)	Mc1r_1(2)
46	Sesto	1	46°39'43"	12°21'07"	1470	E	VB11(1)	C-mos_1(2)	ACM4_4(2)	Mc1r_9(2)
47	Campo Tures	1	46°58'47"	12°05'39"	1860	E	VB11(1)	C-mos_1(2)	ACM4_4(1), ACM4_1(1)	Mc1r_1(2)
48	Redagno	2	46°20'41"	11°23'55"	1540	E	VB11(2)	NA	NA	NA
49	Dobbiaco	1	46°42'10"	12°13'17"	1250	E	VB11(1)	C-mos_1(2)	ACM4_4(2)	Mc1r_1(2)
50	Monte Grappa	2	45°52'20"	11°18'09"	1700	A	OS8(2)	NA	NA	NA
51	Sappada	1	46°37'15"	12°42'49"	1830	E	VB11(1)	C-mos_1(2)	ACM4_4(2)	Mc1r_9(2)
	Total	**191**					**191**			

Table S2.1. Sampling sites details across Italian Alps (see Figure 2.2). Number of samples collected for each site (N), GPS Coordinates, Altitude and mtDNA cyt b haplotype and alleles observed for each nuclear gene. Numbers in brackets refer to allele frequencies.

Sites	N	Country	Subspecies	mtDNA clade	C-mos	ACM4	Mc1r
Pinet-Bélesta	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_4 (2)	Mc1r_1 (2)
Clamondé	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_2 (2)	Mc1r_1 (2)
Etang de Lers	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_4 (2)	Mc1r_1 (2)
Clarens	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_5 (1), ACM4_1 (1)	Mc1r_5 (2)
Louvie	2	France	*Z. v. louislantzi*	B*	C-mos_2 (4)	ACM4_5 (4)	Mc1r_1 (2), Mc1r_5 (2)
Pourtalet	2	Spain	*Z. v. louislantzi*	B*	C-mos_2 (4)	ACM4_5 (4)	Mc1r_1, (2) Mc1r_3 (2)
Iraty	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_4 (2)	Mc1r_3 (2)
La Rhune	1	France	*Z. v. louislantzi*	B*	C-mos_2 (2)	ACM4_5 (2)	Mc1r_3 (2)
Gabas	7	France	*Z. v. louislantzi*	B*	C-mos_2 (14)	ACM4_5 (10), ACM4_4 (1), ACM4_6 (2), ACM4_2 (1)	Mc1r_4 (2), Mc1r_1 (10), Mc1r_3 (2)
Szklarska Poreba	1	Poland	*Z. v. vivipara*	E§	C-mos_1 (2)	ACM4_1 (2)	Mc1r_1 (2)
Ustrzyki Gorne	1	Poland	*Z. v. vivipara*	E§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_9 (2)
Paimpont	1	France	*Z. v. vivipara*	E§	C-mos_1 (2)	ACM4_1 (2)	Mc1r_8 (2)
Tarpa	1	Hungary	*Z. v. vivipara*	E§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_1 (2)
Krutyn	1	Bulgaria	*Z. v. vivipara*	E§	C-mos_1 (2)	ACM4_1 (2)	Mc1r_8 (2)
Turukchanskii Krai	1	Russia	*Z. v. vivipara*	D§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_7 (1), Mc1r_6 (1)
Sakhaline	1	Russia	*Z. v. vivipara*	D§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_7 (2)
Grossevitchi	1	Russia	*Z. v. vivipara*	D§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_7 (2)
Kara-Khol	1	Russia	*Z. v. vivipara*	D§	C-mos_1 (2)	ACM4_4 (2)	Mc1r_7 (2)
Godingberg	1	Austria	*Z. v. vivipara*	F§	C-mos_1 (2)	ACM4_4 (1), ACM4_6 (1)	Mc1r_9 (2)
Emberger Alm	1	Austria	*Z. v. vivipara*	F§	C-mos_1 (2)	ACM4_3 (1), ACM4_4 (1)	Mc1r_9 (1), Mc1r_10 (1)

Table S2.2. Sampling sites details across Europe. Number of samples for each site (N), Country, subspecies, mtDNA clade and alleles observed for each nuclear gene. Numbers in brackets refer to haplotypes (mtDNA) and allele frequencies (nuclear genes). * Heulin *et al.* (2011) § Surget-Groba *et al.* (2006).

	Sequence	Accession number
Phased nuclear alleles	C-mos_1	EF632292
	C-mos_2	KF886547
	C-mos_3	KF886546
	C-mos_4	KF886545
	C-mos_5	KF886543
	C-mos_6	KF886544
	ACM4_1	KF886565
	ACM4_2	KF886564
	ACM4_3	KF886566
	ACM4_4	KF886563
	ACM4_5	KF886562
	ACM4_6	KF886561
	Mc1r_1	KF886555
	Mc1r_2	KF886556
	Mc1r_3	KF886557
	Mc1r_4	KF886558
	Mc1r_5	KF886552
	Mc1r_6	KF886554
	Mc1r_7	KF886553
	Mc1r_8	KF886559
	Mc1r_9	KF886550
	Mc1r_10	KF886551
	Mc1r_11	KF886548
	Mc1r_12	KF886549
	Mc1r_13	KF886560
MtDNA haplotypes	VB11	AY714892
	VB1	AY714882
	VT_23	KF886538
	VT_24	KF886539
	OS3	AY714923
	OS6	AF444041
	OS8	AY714927
	OT_11	KF886540
	OL_11	KF886541
	OL_12	KF886542

Table S2.3. MtDNA haplotypes and phased nuclear sequences found in this study and their relative database accession number.

Third study: **Reproductive isolation is complete between oviparous and viviparous lineages of *Zootoca vivipara* in a contact zone.**

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Abstract

Species including both viviparous and oviparous lineages are excellent models for understanding relationships between speciation and changes in breeding parity mode. However, scarcity of contact zones makes it difficult a full understanding of processes occurring at early stages of divergence. *Zootoca vivipara* provides an intriguing model for the study of speciation between viviparous and oviparous lineages in squamate reptiles. Although the genetic pattern observed in contact zones could reflect the evolutionary history of differentiation, specific ecological requirements make the contact zones between viviparous and oviparous subspecies of *Z. vivipara* extremely rare. Here, we studied a recently discovered syntopic area of *Z. v. vivipara* (viviparous) and *Z. v. carniolica* (oviparous) in the central Italian Alps. For the first time, we used genetic markers for investigating the genetic structure and the level of introgression between these two subspecies in a contact zone, demonstrating that the speciation process is complete in this area, with no evidence of recent reinforcement. Patterns of genetic variability in mtDNA sequences and microsatellites and morphological data provide new insights into the role of reproductive mode in the speciation process. Phylogenetic and genotypic divergence suggests that the two subspecies have experienced long independent evolutionary histories, during which phenotypic differences evolved.

Introduction

Ongoing discussions in evolutionary biology debate the relative roles of genetic divergence in allopatry and reinforcement in secondary contact in the speciation process between two lineages (Jiggins *et al.* 1996; Coyne & Orr 2004). To resolve this issue, contact (or hybrid) zones between two or more differentiated lineages within a species are often studied (e.g. Phillips *et al.* 2004; Leache & Cole 2007; Johnson *et al.* 2013; Miraldo *et al.* 2013). The comparison of the patterns of genetic diversity observed in a series of contact (or hybrid) zones within the same species, reflecting varying stages of the speciation process (e.g. unimodal hybrid zones where hybrid genotypes predominate, and bimodal zones where hybrids are rare and parental genotypes prevail), may produce particularly relevant insights (Jiggins & Mallet 2000). However, in contact zones where hybrids are lacking (i.e. speciation is complete), either genetic and phenotypic differentiation in allopatry has precluded hybridization upon secondary contact, or pre-zygotic or post-zygotic barriers have reinforced partial reproductive isolation. In this case, detailed genetic studies may reveal the history of the speciation process and resolve this conundrum.

The Eurasian lacertid lizard, *Zootoca vivipara*, offers a unique model for studying the role of reproductive mode in speciation. Despite its scientific name, this species shows both ovoviviparous (more commonly referred as 'viviparous' in this species) and oviparous reproduction (Surget-Groba *et al.* 2001). Although there are two other species of squamate lizards with both modes of reproduction (the Australian scincid lizards *Lerista bougaunvilli* and *Saiphos equalis*; Qualls & Shine 1998; Smith *et al.* 2001), only *Z. vivipara* is known to have potentially hybridizing egg-bearing and live-bearing populations (Surget-Groba *et al.* 2002; Cornetti *et al.* 2014).

The subspecies *Z. v. vivipara* (viviparous), is found in many wetland areas from western Europe to Japan, while oviparous populations of *Z. vivipara* occupy two allopatric areas in southern Europe: one in the Pyrenees (*Z. v. louislantzi*; Arribas 2009), the other in the central eastern Alps (*Z. v. carniolica*; Mayer *et al.* 2000). The distributions of *Z. v. louislantzi* and *Z. v. vivipara* do not overlap, while *Z. v. carniolica* and *Z. v. vivipara* are sympatric in the Alpine chain. However, syntopic locations of the latter two subspecies are rare, probably due to ecological differentiation: viviparous individuals have 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). In fact, previous surveys have only identified one location where *Z. v. vivipara* and *Z. v. carniolica* are found in syntopy (Carinthia, Austria; Lindtke *et al.* 2010). In this contact zone, Lindtke *et al.* (2010) reported several putative hybrids with apparently intermediate developmental traits.

In this study we analyze in detail another potential contact zone between *Z. v. carniolica* and *Z. v. vivipara* identified during recent field surveys (Cornetti *et al*. in preparation) using a set of highly variable genetic markers and a morphological characteristic considered a secondary sexual trait, the number of femoral pores (Martin & Lopez 2000), with the aim of giving insight into the speciation process. These results have important implications for the taxonomy of the genus and, consequently, for the conservation status of relatively rare *Z. v. carniolica* populations.

Materials and Methods

During recent alpine-wide field surveys, some of us (LC, FB, GS, GG) identified a relatively small area (0.72 km^2) of potential overlap between *Z. v. vivipara* and *Z. v. carniolica* in the alpine valley Valmora (central northern Italy, 46°02'15"-46°02'36" N; 9°37'09"-9°38'01" E; 1400-1600 m above sea level; Figure 3.1). Subsequently, during the summers of 2012 and 2013, 60 lizards were captured by hand within this area over 27 non-consecutive days by one to four surveyors. In order to confirm that there was adequate sampling coverage, mixture models for open populations were used to estimate the local abundance of lizards for four sites of the study area (Royle 2004; Kéry *et al.* 2009; see Figure 3.1), assuming that detection probability may have been affected by the date, air temperature, precipitation, solar radiation and number of surveyors. We used the Akaike's Information Criterion, corrected for small sample size, to identify the combination of predictors best explaining detection probability (Richards *et al.* 2011); we assumed negative binomial error for the abundance component of models. Models were run using the package 'unmarked' in R (Fiske *et al.* 2011). An empirical Bayes algorithm was used to estimate lizard abundance in the

four sites and 95% CI. The results suggest that the 60 captured individuals represented at least 50% of the resident lizard population (see Table 3.1).

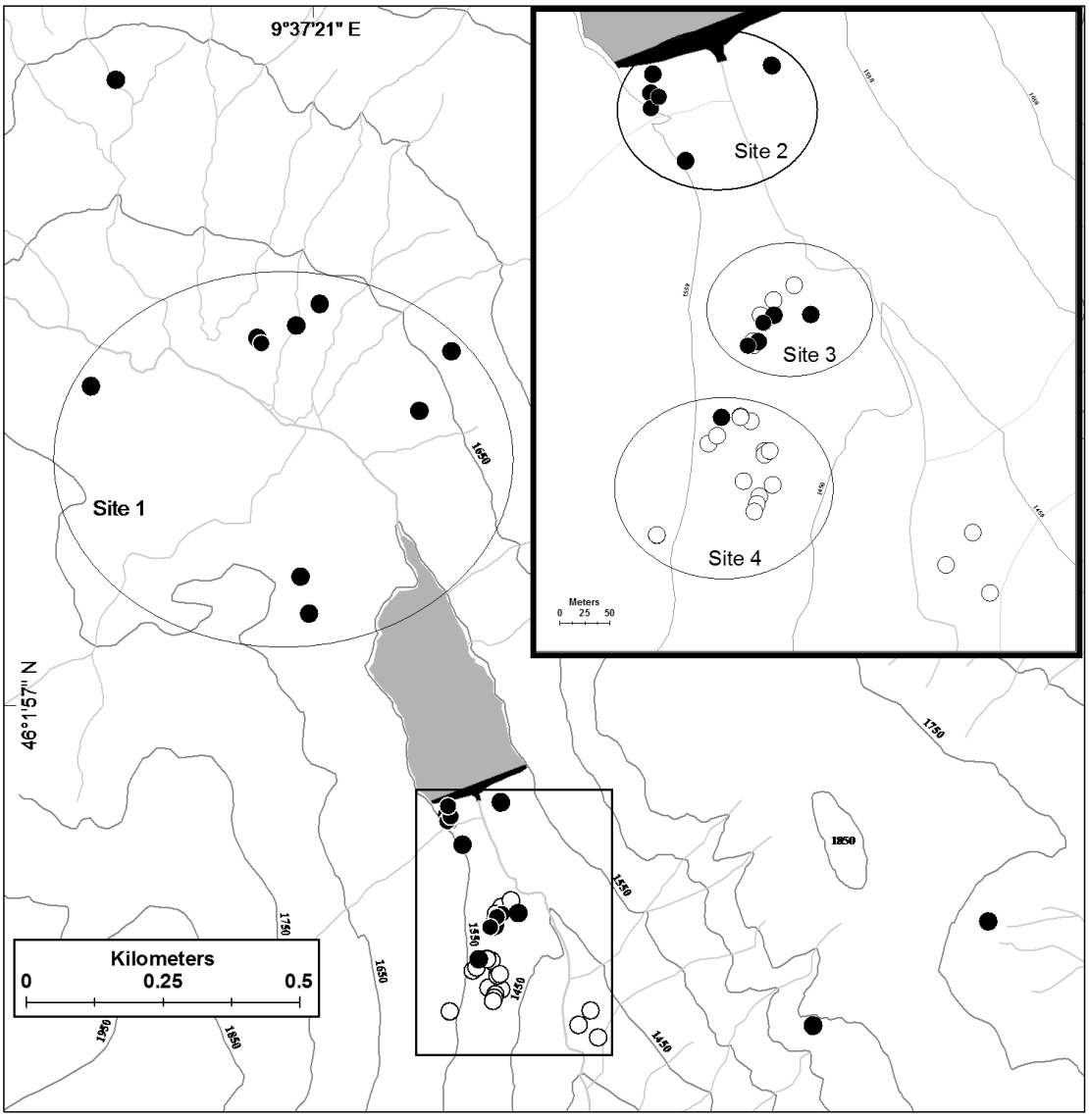

Figure 3.1. Detailed map of sampling area; closed circles represent capture site of *Z. v. vivipara* and open circles represent capture site of *Z. v. carniolica*, identified to 'subspecies' according to cytb haplotype. Ovals describe the four sites used for lizard abundance estimation.

	Abundance	
site	Mean	95% CI
Site 1	7.0	3 - 13
Site 2	13.9	8 - 22
Site 3	16.9	10 - 26
Site 4	41.3	31 - 50

Table 3.1. Empirical Bayes estimation of lizard abundance in the four sites within the study area.

Before releasing lizards, we photographed the ventral side of each individual; these photographs were used for counting the number of femoral pores on the inside of each hind limb. The mean number of femoral pores was calculated as the arithmetic mean between the number of pores on the right and left limbs (Guillaume *et al.* 2006).

Three mm tail tips were collected and stored at room temperature in 95% ethanol until DNA extraction. All sampling procedures complied with the current laws of the Italian Ministry of Environment and the Environmental Unit (DPN/2D/2003/2267). Genomic DNA was extracted using the QIAGEN DNeasy Tissue Kit and QIACUBE automated DNA extractor (QIAGEN Inc., Hilden, Germany). For each sample, a 385-base pair (bp) fragment of the mitochondrial gene cytochrome b (cytb) was amplified using the primers MVZ04 and MVZ05 (Smith & Patton 1991). Cytb is the most extensively sequenced marker for the *Zootoca* genus and, therefore, is useful for comparison of our results with previous studies, and to confirm subspecies identification, since no morphological traits unequivocally distinguish the two forms. PCR amplification was carried out according to Cornetti *et al.* (2014). PCR products were purified and sequenced in both directions on an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Thirteen microsatellite (STR) markers (Lv-4-alpha, Lv-2-145, Lv-4-X and Lv-4-115 from Boudjemadi *et al.* 1999; B114 from Remon *et al.* 2008; Lacviv04, Lacviv06, Lacviv26, Lacviv07, Lacviv27, Lacviv30, Lacviv05 and Lacviv17 from Stevens *et al.* 2012) were also amplified in seven multiplexed runs under the following conditions: initial incubation at 94°C for 10 min, followed by 30 cycles of 94°C for 1 min, annealing temperature (Ta) for 45 s, and 65 °C for 1 min, with a final extension of 65 °C for 10 min (Ta: 50°C for B114, Lv-2-145; Ta: 51°C for Lv-4-X; Ta: 52°C for Lv-4-alpha; Ta: 53°C for Lacviv07, Lacviv27, Lacviv30; Ta: 55°C for Lv-4-115, Lacviv04, Lacviv06, Lacviv26; Ta: 57°C for Lacviv05, Lacviv17). PCR amplifications were optimized in a 20µl reaction volume containing 1µl of DNA, 2 µl HotMaster™ Taq Buffer 25 mM Mg2 (Eppendorf, Westbury, NY), 100 µM dNTPs, variable proportion of labeled forward primers and reverse primers, 1 unit of HotMaster™ Taq Polymerase (Eppendorf, Westbury, NY), and double distilled water. PCR products were run with an internal lane standard (LIZ) on an ABI 3130 (Applied Biosystems, Foster City, CA, USA); alleles were scored using GeneMapper® software.

Sequence fragments were edited with FinchTV 1.4.0 (Geospiza, Inc. Seattle, WA, USA; http://www.geospiza.com), assembled using Sequencher 4.7 (Gene Codes. Corporation, USA) and aligned using Clustal X (Thompson *et al.* 1997). These and all publicly available haplotypes found across the Alpine chain were collapsed into a median-joining network using Network 4.6.1.1 (http://www.fluxus-engineering.com/sharenet_rn.htm), so that the subspecies of each of our samples could be identified.

The STR data were tested for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using Genepop 4.0 (Rousset 2008). Possible genotyping errors (presence of null alleles, large allele dropout and stuttering) were assessed with Micro-Checker 2.2.3 (Van Oosterhout *et al.* 2004). Since some markers had null alleles (see Results section), we used FreeNa (Chapuis & Estoup 2007) for calculating if such null alleles induced a positive bias in the estimates of F_{st}. Genetic variation at STRs and subspecies differentiation were investigated using the R package diveRsity (Keenan *et al.* 2013); number of alleles (N_a), allelic richness (A_r), observed and expected heterozygosity (H_o and H_e, respectively) were calculated for each subspecies, while F_{st} was calculated between subspecies. Factorial Correspondence Analysis (FCA) implemented in Genetix (Belkhir *et al.* 2004) was used for visualizing the distribution of genetic variation across individuals.

NewHybrids 1.1 beta (Anderson & Thompson 2002) and Structure 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003) were used for inferring hybridization between subspecies; that is, individual lizards were categorized as belonging to either parental subspecies (pure *vivipara*, pure *carniolica*) or one of the hybrid categories (F1, F2, or backcross) using a Bayesian algorithm and Markov chain Monte Carlo (MCMC) sampling. We ran 10 independent analyses using uniform priors, and a burn-in of $2.5x10^5$ followed by 10^6 iterations. In order to detect possible hybrids, we also ran 10 independent analyses of Structure using K=2 clusters, representing the two hybridizing subspecies (burn-in of $2.5x10^5$ followed by 10^6 iterations).

Finally, we used microsatellites data for estimating the divergence time between *Z. v. carniolica* and *Z. v. vivipara* using the Approximate Bayesian Computation (ABC) framework implemented in DIYABC 2.0 (Cornuet *et al.* 2010; for details about prior distribution of parameters see Table 3.3). We modeled a single scenario describing the divergence of the two populations, without gene flow.

Results

All 60 samples were successfully sequenced for cytb. Three haplotypes were identified corresponding to previously deposited sequences (OS3, VB1 and VL_26; accession numbers: AF444038, AF247976, KF898394). On the basis of haplotype, our sample set consists of 29 *Z. v. carniolica* (all with haplotype OS3), and 31 *Z. v. vivipara* (23 of the VL_26 and eight of the VB1 haplotype). The median-joining network shown in Figure 3.2a highlights the high level of divergence (19 mutations) between *Z. v. vivipara* and *Z. v. carniolica* populations in the contact zone studied here (see Figure 3.2a).

All thirteen STRs were successfully genotyped for all samples. MicroChecker results suggest the presence of null alleles for four markers; however, these are distributed evenly among subspecies (Lv115 and Lacviv04 in *Z. v. carniolica* and Lacviv07, Lacviv30 in *Z. v. vivipara*). In addition, three of these loci (all except Lacviv30), showed significant deviation from Hardy Weinberg equilibrium ($P<0.05$), after correction for multiple testing using False Discovery Rate (FDR; Benjamini & Hochberg 1995). Only one out of 78 locus pairs showed significant genotypic linkage ($P<0.05$; Lv-4-X and Lacviv30). However since subsequent analyses of the dataset with or without deviant loci led to very similar conclusions, we will only present here the results of analyses including all 13 STRs.

Visualization of the overall genotypic variation in STRs (Figure 3.2b) suggests a marked genetic difference between individuals belonging to the two cytb clades, corresponding to *Z. v. vivipara* and *Z. v. carniolica* subspecies, with no mitochondrial introgression. Genetic variability within the two populations was similar, and although *Z. v. carniolica* had lower estimates for all indices, these differences were not significant (t-test, $P>0.05$; Table 4.2). The mean number of private alleles was 2.6 (56%) and 4.0 (66%) in *Z. v. carniolica* e *Z. v. vivipara*, respectively; the F_{st} value between populations was high and significant (0.381) and very similar to the F_{st} calculated excluding null alleles with FreeNa (0.372).

Subspecies	N	N_a	A_r	H_o	H_e
Z. v. carniolica	29	4.69	4.34	0.47	0.51
Z. v. vivipara	31	6.08	5.35	0.51	0.57

Table 3.2. Genetic variation within the *Z. vivipara* subspecies *carniolica* and *vivipara* from one syntopic site. Number of alleles (N_a), allelic richness (A_r), observed and expected heterozygosity (H_o and H_e).

Admixture analyses using NewHybrids clearly show the lack of hybrid individuals in our sample set, and all samples were assigned to their pure parental subspecies with a probability above 99%. Similarly, Structure estimated a mean posterior probability of ranking *Z. v. carniolica* individuals to one cluster of 99.7% and *Z. v. vivipara* individuals to the other, of 99.5% (Figure 3.2c).

Finally, with ABC approach, we obtained an estimated divergence time between the two subspecies as being about 100.000 years before present (95% CI 18.000-780.000, considering a generation time of 3 years, Corti *et al.* 2010; for details see Table 3.3).

We successfully counted the number of femoral pores from all individuals except 12 juveniles, which presented underdeveloped and/or undeveloped femoral pores. The range of the mean number of femoral pores per individual slightly overlaps between subspecies (*Z. v. vivipara*: 8.5-11.5; *Z. v. carniolica*: 11.5-15.0), but their means are highly statistically different (t-test, $P<10^{-12}$). The range of number of femoral pores in females overlaps slightly (*Z. v. vivipara*: 8.5-11.5; *Z. v. carniolica*: 11.5-15.0), but that of males does not (*Z. v. vivipara*: 9.0-11.0; *Z. v. carniolica*: 12.5-14.5).

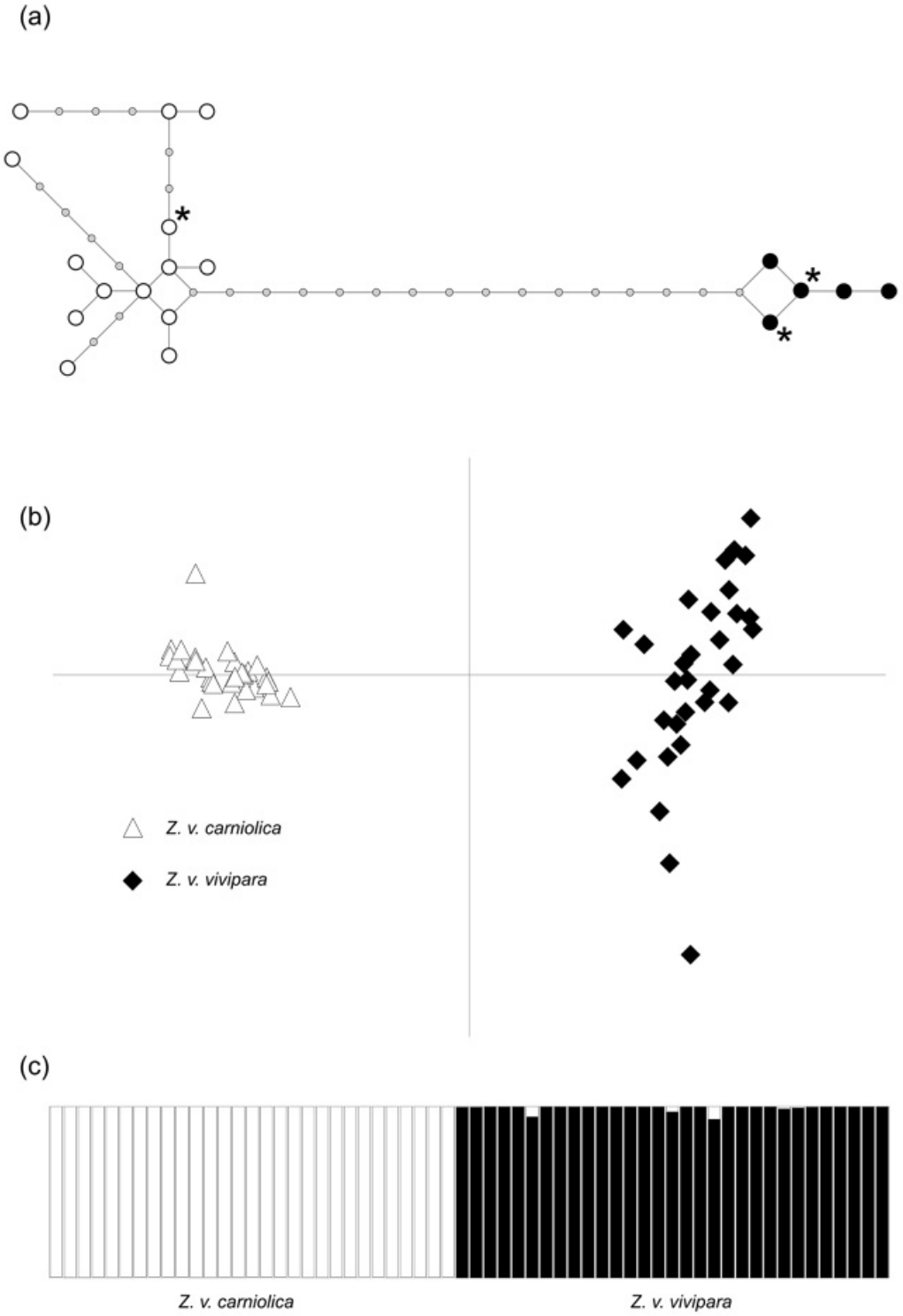

Figure 3.2. Analysis of mitochondrial and nuclear genetic variation of *Z. vivipara* in the Valmora contact zone. (a) Network analysis including deposited sequences from Alpine distributions of common lizard subspecies: closed and open circles represent mtDNA haplotypes of *Z. v. vivipara* and *Z. v. carniolica* respectively; circles indicated by asterisks correspond to haplotypes found in this study; grey dots represent diverging mutations between observed haplotypes. (b) FCA of genotypic variation between individuals divided according to cytb assignment. (c) Plot representing q-value of individuals belonging to predefined mtDNA clusters as estimated by STRUCTURE.

	Prior		Posterior			
	min	**max**	**mean**	**median**	**Quantile 2.5%**	**Quantile 97.5%**
N_carn	10	10000	4050	3810	697	8990
N_viv	10	10000	6190	6420	1660	9700
t	0	500000	56400	39500	5980	266000
Mutation rate	0.00005	0.001	0.00017	0.00013	0.00006	0.00054
p	0.1	0.5	0.41	0.42	0.26	0.5

Table 3.3. Prior and posterior distribution of demographic and mutational parameters used in the ABC framework for estimating the time of divergence between *Z. v. vivipara* and *Z. v. carniolica*. All one-sample and two-samples summary statistics available were used (for details see DIYABC 2.0 maunal). N_carn and N_viv are the effective population size *of Z. v. carniolica* and *Z. v. vivipara*, respectively; t_div is the time of divergence between subspecies, expressed in generations; mean_μ is the mean mutation rate for microsatellite markers; mean_P is the mean parameter of geometric distribution.

Discussion

Here, for the first time, we investigated the genetic pattern in a syntopic area of *Z. v. vivipara* and *Z. v. carniolica* for understanding the process of divergence between viviparous and oviparous lineages and the role of reproductive mode. Our results show that speciation is complete since our multi-locus analyses confirm two highly distinct groups and the presence of hybrid individuals can be confidently excluded. The absence of introgression suggests that the isolation between the two subspecies is not recent, and was likely complete before the last glaciation, possibly as a result of a switch in reproductive mode. In addition, some preliminarily data on morphological traits related to reproduction suggest that reinforcement may have occurred in the past in the contact zone we analysed.

In the studied area, speciation between the oviparous *Z. v. carniolica* and the viviparous *Z. v. vivipara* is complete, without ongoing evidence of gene flow. MtDNA sequences confirmed the deep haplotypic divergence between lineages (Figure 3.2a), previously suggested by a mitochondrial phylogenetic study, that estimated the divergence time between *Z. v. carniolica* and *Z. v. vivipara* at about 4.5 Mya (95 % CI 6.1-2.6; Cornetti *et al.* 2014). We also reported profound genotypic differentiation (FCA, Figure 3.2b), likely determined by lack of gene flow over time, corroborated by a high and significant F_{st} value between subspecies (0.381) and a high

percentage of private alleles (56% and 66% in *Z. v. carniolica* e *Z. v. vivipara*, respectively). In addition, with ABC analysis of microsatellites, we estimated a median divergence time between the two subspecies of about 0.12 Mya years (95% CI 0.018-0.78 Mya), or at least before the Last Glacial Maximum (LGM; Ivy-Ochs *et al.* 2008). Thus, molecular analyses clearly illustrated the lack of gene flow between oviparous and viviparous lineages in this contact zone.

Absence of hybrid genotypes of any category (F_1, F_2 and backcrosses) highlighted that the occurrence of natural hybrids is extremely unlikely (Figure 3.2c), and confirms that speciation is complete in this area. In fact, convincing evidence of natural hybridization between oviparous and viviparous *Z. vivipara* has never been reported. Only hybridization in captivity has been noted (Arrayago *et al.* 1996), with these authors suggesting that the geographically isolated *Z. v. louislantzi* and *Z .v. vivipara* can successfully hybridize, although the fitness of F_1 hybrids was lower than that of parental forms. However, *Z. v. louislantzi* and *Z. v. vivipara,* contrary to *Z. v. carniolica* and *Z. v. vivipara,* have a very similar karyotype (Odierna *et al.* 2001), and they are phylogenetically closer to each other than *Z. v. carniolica* and *Z. v. vivipara.* Since post-zygotic isolation and genetic distance are generally positively correlated (e.g., Presgraves 2002; Mendelson 2003), we expect a more reduced viability/fertility in a *carniolica* x *vivipara* F_1 hybrid than a *louislantzi* x *vivipara* one. Furthermore, hybridization in captivity may be not indicative of processes occurring in nature. Lindtke *et al.* (2010) claimed that natural hybridization occurs between wild population of *Z. v. carniolica* and *Z. v. vivipara*, but this analysis was based on phenotypic traits that taken singularly are known to lead to erroneous conclusions (Allendorf *et al.* 2001). In the only other contact zone reported for these subspecies (Carinthia, Austria), Lindtke *et al.* (2010) captured two putative hybrid females showing advanced development of embryos at oviposition, shortened incubation period of their eggs and reduced eggshell thickness; however, the authors admitted that genetic analyses were needed in order to confirm the hybrid origin of these individuals. More detailed studies in this second hybrid zone should be carried out to confirm whether hybridization has also ceased in other parts of the *Z. v. vivpara* and *Z. v. carniolica* ranges.

Our molecular results do not allow us to establish when the transition from oviparity to viviparity in *Z. vivipara* occurred, but in reptiles this switch is consistently associated with colonization of cold climates (Shine 2005; Pincheira-

Dinoso *et al.* 2013). Similarly, for *Z. vivipara*, oviparity is considered ancestral, and it has been demonstrated that the evolution and distribution of viviparous and oviparous populations have been mainly shaped by Pliocene/Pleistocene climatic oscillations (i.e. 5.3 to 0.01 Mya; Surget-Groba *et al.* 2001). A previous study based on mtDNA concluded that Quaternary glacial phases pushed the distribution of oviparous *Z. vivipara* populations to southern areas of Europe. It is hypothesized that colder climatic conditions exerted a strong selective pressure on populations between the Balkan Peninsula and southern Russia (when further southern range expansions were impeded by the sea), giving rise to viviparity. Viviparity then permitted the recolonization of northern Eurasia by these populations during interglacial periods (see also Figure 1 in Surget-Groba *et al.* 2006). Surviving oviparous populations in the Italian peninsula, currently classified as *Z. v. carniolica*, presumably remained well-adapted to the warmer climate, since their spatial and demographic re-expansion after glacial waves was limited to areas south of the Alps (Surget-Groba *et al.* 2002). The above hypothesis would indicate that the switch to viviparity in *Z. vivipara* occurred between 5.3 to 0.01 Mya, in the same range as mitochondrial phylogenetic analysis and simulation-based analyses of microsatellite data suggest that *vivipara* and *carniolica* began to differentiate (4.5-0.12 Mya), long before their secondary contact in Valmora. Interestingly, it has been previously demonstrated that shifts in reproductive mode are usually correlated with genetic divergence of lineages (Fairbairn *et al.* 1998; Schulte *et al.* 2000; Smith *et al.* 2001; Velo-Anton *et al.* 2012; Boomer *et al.* 2012).

There appears to be a discrepancy among estimated times of divergence between subspecies calculated using mtDNA (4.5 Mya, 95 % CI 6.1-2.6) and nuclear microsatellites (0.12 Mya, 95% CI 0.018-0.78). Such a discrepancy is frequently observed (e.g., Portnoy *et al.* 2010; Rodriguez *et al.* 2010; Charruau *et al.* 2011), and may be related to the calibration of the mtDNA clock, the estimated parameters of the different mutation models, the generation time assumed in the ABC analysis, and/or to the fact that the TMRCA (time to the most recent common ancestor) of the mtDNA genealogy tend to overestimate the age of the population split if the ancestral population size was very large. However, these contrasting results do not undermine the hypothesis that divergence between *Z. v. vivipara* and *Z. v. carniolica* occurred long before their secondary contact in the Valmora valley, and was almost certainly complete before the LGM.

The evolutionary transition from oviparity to viviparity requires major structural, physiological and, therefore, genetic changes (Thompson & Speake 2006; Murphy & Thompson 2011). Nonetheless, and perhaps remarkably, this switch has been reported at least 115 times in squamate reptiles, out of a total of 140 switches for vertebrates (Sites *et al.* 2011). Thus, this change in reproductive mode in *Z. v. vivipara* may have determined the genetic differentiation between these subspecies. The ecological shift that coincided with the evolution of viviparity would have resulted in an allopatric distribution of the two subspecies and in the Alps in different altitudinal distributions (mean: 1200 (range: 450-1880) m asl and 1700 (1160-2160) m asl, for *Z. v. carniolica* and *Z. v. vivipara*, respectively; Cornetti *et al.* 2014), where drift may have promoted further differentiation.

In addition to a switch in reproductive mode and drift, karyotypic divergence may also have posed significant post-zygotic barriers upon secondary contact, such as hybrid subfertility, sterility or inviability (Coyne & Orr 2004; Kitano *et al.* 2009). The karyotype of *Z. v. carniolica* and *Z. v. vivipara* differ by a fusion between the W sex chromosome and an autosome, so that males and females of *Z. v. carniolica* have the same number of chromosomes (2n=36), whereas *Z. v. vivipara* males have 2n=36 and females, 2n= 35 (Odierna *et al.* 2001). F_1 hybrids between *carniolica* males x *vivipara* females are expected to carry trivalents which may misalign and/or fail to segregate regularly during meiosis, causing germ cell death and/or resulting in inviable aneuploid gametes. Essentially, this type of chromosomal rearrangement may cause lowered hybrid fitness, potentially limiting gene flow between the two lineages (Rieseberg 2001; Faria & Navarro 2010 and references therein). Chromosome fusions are known to have played an important role in the speciation process in many vertebrates (White 1969), including lizards (Leache & Sites 2010), especially when a sex chromosome is involved (Qvarnström & Bailey 2009). During secondary contact of the two highly differentiated oviparous and viviparous subspecies after the last LGM, if hybridization between *Z. v. vivipara* and *Z. v. carniolica* occurred and did not result in completely sterile hybrids, the lowered fitness of F_1 hybrids caused by genetic and karyotypic differences may have promoted speciation by reinforcement. There is some morphological evidence for such a process within this hybrid zone. Although the two subspecies do not show clear morphological differences, *Z. v. carniolica* is said to be distinguishable from *Z. v. vivipara* for its larger body size, lower number of ventral scale rows and higher number of femoral pores. However,

the ranges of each of these measurements greatly overlap between the two lineages when individuals from the entire distribution range are analyzed (Guillaume *et al.* 2006). In contrast, in the contact zone studied here, we found that the ranges of the mean number of femoral pores slightly overlap between subspecies and, more significantly, the ranges of number of femoral pores in males do not overlap at all (9.0-11.0 and 12.5-14.5 in *Z. v. vivipara* and *Z. v. carniolica,* respectively). In lizards, femoral pores are closely related to the production of chemical compounds involved in reproduction; therefore, differences in the number of male femoral pores may influence female mating choice and could provide the basis for premating reproductive isolation (Martin & Lopez 2000; Mason & Parker 2010; Gabirot *et al.* 2012). The fact that there is very little overlap in ranges within the contact zone (but highly overlapping outside of it) suggests that this morphological trait has been selected during speciation, effectively reducing hybridization between the subspecies (speciation by reproductive character displacement; Hoskin & Higgie 2010 and references therein).

On the basis of genotypic results found here and previous studies about karyotypic and phylogenetic divergence between *Z. v. vivipara* and *Z. v. carniolica*, we hypothesize that the speciation process between the two lineages was complete or almost complete before their secondary contact in the Alpine chain as a result of a switch in reproductive mode some time before the LGM. If reinforcement happened, as suggested by the divergence in the number of femoral pores in males within the hybrid zone as compared to the whole range of the species, it was many generations ago, and all signs of gene flow have since disappeared. In this scenario, the role of reproductive mode may have made a strong contribution to genetic differentiation, although drift was almost certainly a contributing factor in allopatry.

Given the high level of genetic divergence and lack of gene flow between *Z. v. vivipara* and *Z. v. carniolica*, these two 'subspecies' should be considered as separate management units for conservation purposes. Since the most suitable habitats for *Z. v. carniolica* are considered threatened by climate change and anthropization (Moore *et al.* 2002), conservation measures should be urgently re-evaluated since *Z. vivipara* is currently considered of Least Concern (IUCN 2013).

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Fourth study: ***Zootoca vivipara*** **as a model species to analyse the evolutionary transition from oviparity to viviparity in squamate reptiles: a genomic approach**

Manuscript in preparation

Abstract

Among the 140 switches in reproductive mode from oviparity to viviparity observed in vertebrates, at least 115 occurred in squamate reptile. However, very rare examples of species showing both the reproductive modes exist. *Zootoca vivipara*, across its wide distributional range, presents egg-laying and live-bearing populations, providing an interesting model for studying the evolutionary transition from oviparous to viviparous reproductive mode. Here, I studied samples from the whole distributional range of the species using a recently developed method for reducing the complexity of a genome (RAD tag sequencing) that allows to simultaneously discover and analyse thousands of Single Nucleotide Polymorphisms (SNPs). I found about two hundreds SNPs statistically associated to the switch in reproductive mode; sequences physically linked to these polymorphisms were blasted against the *Anolis carolinensis* genome. Some of the sequences showed sequence similarity with genes potentially involved in physiological functions, such as vascularization and immune system, known to differ in groups with oviparous and viviparous reproductive mode. Although additional investigations should be performed on the newly identified genes, this study provides the first attempt to analyze transition from oviparity to viviparity at genomic level, with the consciousness that this shift is a very complex physiological change, probably mediated by hundreds of genes.

Introduction

The evolutionary transition from oviparity to viviparity requires some major structural and physiological changes (Thompson & Speake 2006), but this switch has occurred at least 115 times in squamate reptiles, out of 140 switches in vertebrates (Sites et al 2011). The genetic basis of this transition remains uncertain (Murphy & Thompson 2011). The lizard *Z. vivipara,* showing both oviparous and viviparous populations, provides an interesting natural setting for studying the genetic basis of this evolutionary shift in reproductive mode.

In reptiles, the evolution of viviparity is usually promoted by colder climatic conditions (Shine 2005; Pincheira-Dinoso *et al.* 2013); it has been also demonstrated for *Z. vivipara* that the distribution of viviparous and oviparous populations has been mainly shaped by Pliocene/Pleistocene climatic changes (Surget-Groba *et al.* 2001). *Z. vivipara* shows viviparous populations (*Z. v. vivipara*) in most of its distributional range and oviparous populations (*Z. v. carniolica* and *Z. v. louislantzi*) in the southern margin of species distribution. Oviparous populations of *Z. v. carniolica* living in northern Italy are considered the ancestral form of the species, from which viviparity likely evolved, permitting the recolonization of the entire continent during interglacial periods by *Z. v. vivipara* populations. Although the current discontinuous distributions between the western oviparous populations (*Z. v. louislantzi*) and viviparous (*Z. v. vivipara*) populations, phylogeographic analyses indicated that they are closely related to each other, suggesting a likely reversal from viviparity to oviparity in French/Spanish populations (Surget-Groba *et al.* 2006; Cornetti *et al.* 2014), as documented for other reptiles (Lee & Shine 1998; Lynch & Wagner 2010; Fenwich *et al.* 2012).

Z. vivipara offers a unique model for investigating at genome level and within a single species the transition between oviparous and viviparous reproductive mode. In this study I analyzed this transition using a Next Generation Sequencing (NGS) approach.

Methods

Forty tail tips of *Z. vivipara* were collected in order to cover the whole distribution of the species, including both viviparous (*Z. v. vivipara*, hereafter called V) and oviparous (*Z. v. carniolica* and *Z. v. louislantzi,* hereafter called O) populations (Figure 4.1 and Table 4.1).

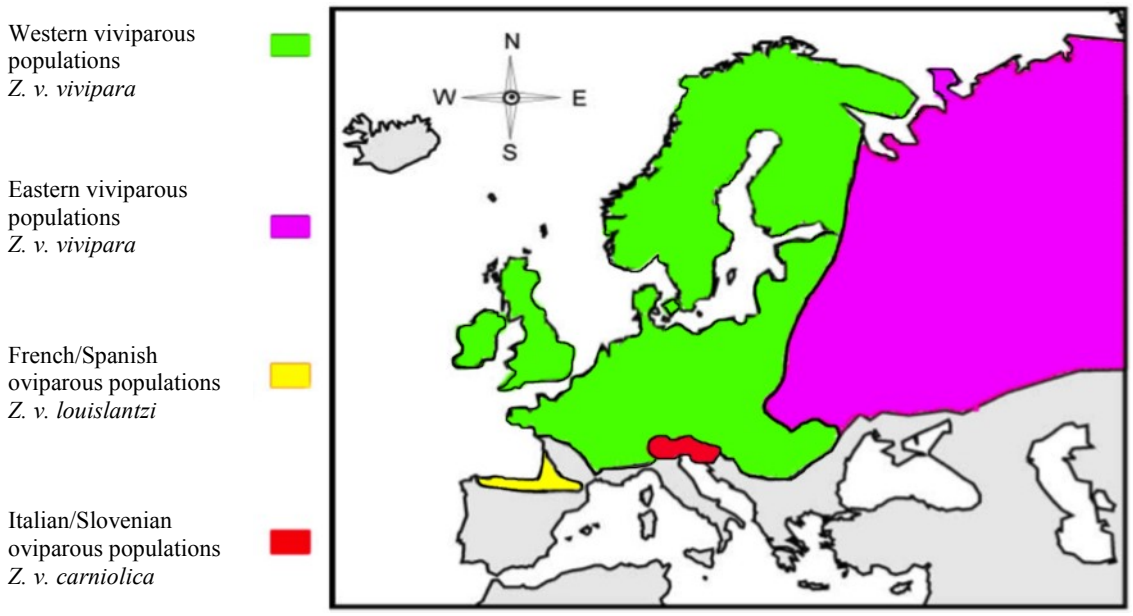

Figure 4.1 *Z. vivipara* subspecies distribution. Modified from Surget-Groba *et al.* 2001.

Genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit (QIAGEN Inc., Hilden, Germany). DNA was treated with RNaseA (QIAGEN) and successively quantified with the fluorometer Qubit 2.0 (Invitrogen). RADtag sequencing (Baird *et al.* 2008) and Illumina technology was used to study oviparous and viviparous samples of *Z. vivipara* for simultaneously discover and analyse thousands of SNPs at genomic level. RADtag is a NGS technique for genotyping by sequencing that reduces the complexity of a genome taking advantage of the usage of a restriction enzyme. I digested 1µg of genomic DNA for each individual sample, with *SbfI* restriction enzyme in a 50 µl reaction volume. P1 adapter, containing unique barcode, was ligated onto complementary compatible ends for each sample. Individually barcoded samples were pooled and then sheared to an average size of 500 bp using the ultrasonicator Covaris S220 (Covaris, Inc., Woburn MA, USA). In order to restrict the size range of tags to that which can be efficiently sequenced on an Illumina flow cell (300-500 bp), a size selection by means of agarose gel extraction

was performed. Library preparation was completed after ligating P2 adapters that allowed amplification of fragments that incorporated both P1 and P2 (see Figure 4.2). The library was run on the Illumina flow cell using Illumina HiSeq2000.

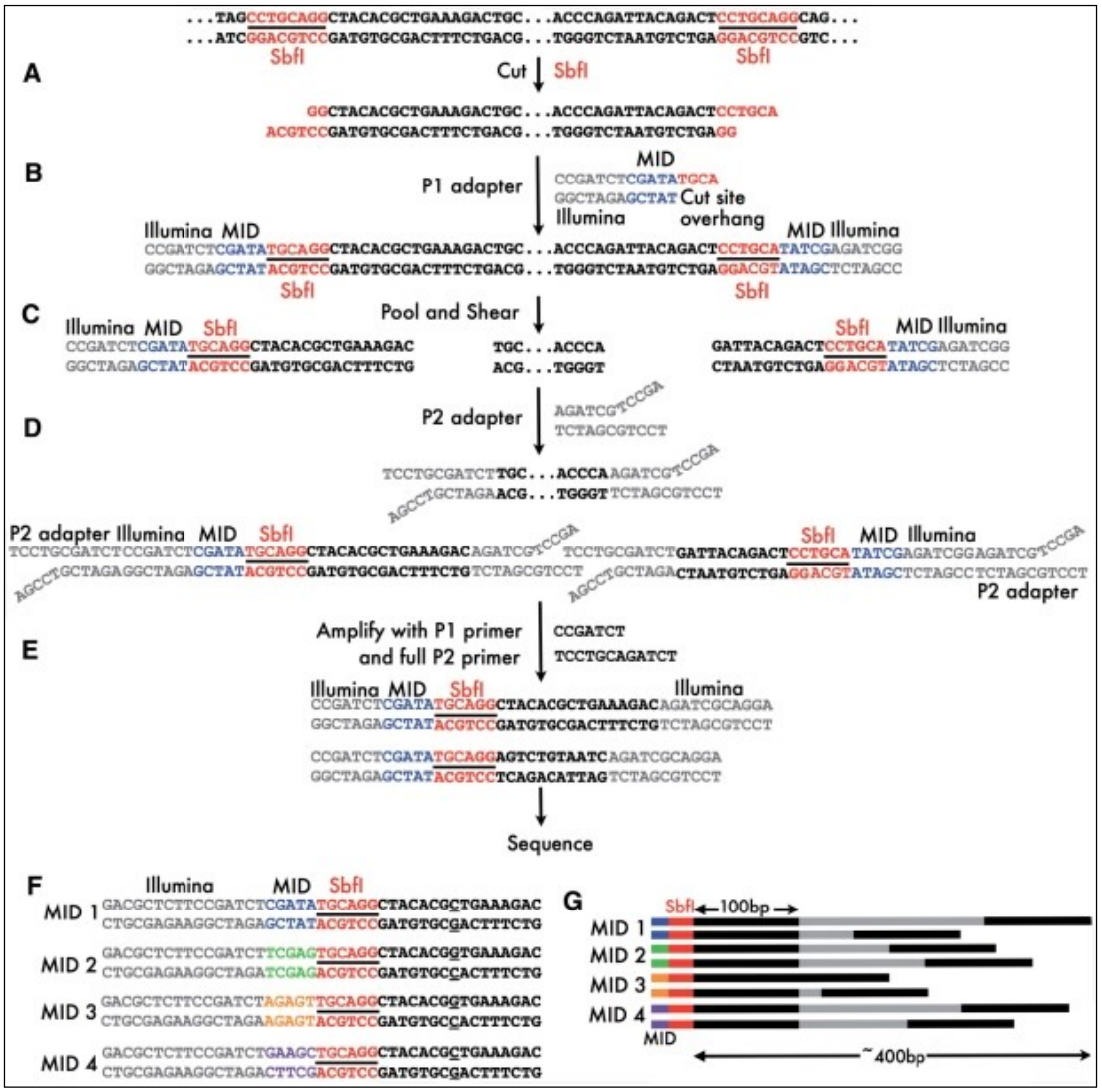

Figure 4.2 The process of RADSeq. **(A)** Genomic DNA is sheared with a restriction enzyme of choice (SbfI in this example). **(B)** P1 adapter is ligated to SbfI-cut fragments. The P1 adapter is adapted from the Illumina sequencing adapter (full sequence not shown here), with a molecular identifier (MID; CGATA in this example) and a cut site overhang at the end (TGCA in this example). **(C)** Samples from multiple individuals are pooled together and all fragments are randomly sheared. Only a subset of the resulting fragments contains restriction sites and P1 adapters. **(D)** P2 adapter is ligated to all fragments. The P2 adapter has a divergent end. **(E)** PCR amplification with P1 and P2 primers. The P2 adapter will be completed only in the fragments ligated with P1 adapter, and so only these fragments will be fully amplified. **(F)** Pooled samples with different MIDs are separated bioinformatically and SNPs called (C/G SNP underlined). **(G)** As fragments are sheared randomly, paired end sequences from each sequenced fragment will cover a 300–400 bp region downstream of the restriction site (from Davey & Blaxter 2011).

Sample	MtDNA clade	Subspecies	Origin	Reproductive mode	N of reads	Mean coverage
13_11_A	A	*Z. v. carniolica*	Italy	Oviparous	274639	9
193_09_A	A	*Z. v. carniolica*	Italy	Oviparous	629051	11
22_11_A	A	*Z. v. carniolica*	Italy	Oviparous	210776	8
26_11_A	A	*Z. v. carniolica*	Italy	Oviparous	488794	9
37_08_A	A	*Z. v. carniolica*	Italy	Oviparous	244868	8
3_08_A	A	*Z. v. carniolica*	Italy	Oviparous	344593	9
42_08_A	A	*Z. v. carniolica*	Italy	Oviparous	1579670	16
43L_A	A	*Z. v. carniolica*	Italy	Oviparous	271596	8
60L_A	A	*Z. v. carniolica*	Italy	Oviparous	207022	9
63L_A	A	*Z. v. carniolica*	Italy	Oviparous	254436	9
10H_B2	B	*Z. v. louislantzi*	France	Oviparous	891764	12
15H_B1	B	*Z. v. louislantzi*	France	Oviparous	643905	10
20H_B1	B	*Z. v. louislantzi*	France	Oviparous	1015182	13
26H_B1	B	*Z. v. louislantzi*	France	Oviparous	439002	9
35H_B2	B	*Z. v. louislantzi*	France	Oviparous	493454	10
4H_B2	B	*Z. v. louislantzi*	France	Oviparous	409953	10
7H_B2	B	*Z. v. louislantzi*	France	Oviparous	1139493	12
59H_D	D	*Z. v. vivipara*	Russia	Viviparous	622262	11
61H_D	D	*Z. v. vivipara*	Russia	Viviparous	232424	8
62H_D	D	*Z. v. vivipara*	Russia	Viviparous	763275	12
65H_D	D	*Z. v. vivipara*	Romania	Viviparous	229294	9
16_11_E	E	*Z. v. vivipara*	Italy	Viviparous	330444	9
21_11_E	E	*Z. v. vivipara*	Italy	Viviparous	190891	9
30_11_E	E	*Z. v. vivipara*	Italy	Viviparous	324216	9
31_11_E	E	*Z. v. vivipara*	Italy	Viviparous	229458	9
33L_E	E	*Z. v. vivipara*	Italy	Viviparous	611685	11
34_11_E	E	*Z. v. vivipara*	Italy	Viviparous	683032	12
35Lb_E	E	*Z. v. vivipara*	Italy	Viviparous	773848	11
36Lb_E	E	*Z. v. vivipara*	Italy	Viviparous	118707	10
40_11_E	E	*Z. v. vivipara*	Italy	Viviparous	203261	9
42L_E	E	*Z. v. vivipara*	Italy	Viviparous	332969	10
46L_E	E	*Z. v. vivipara*	Italy	Viviparous	830270	11
47L_E	E	*Z. v. vivipara*	Italy	Viviparous	590771	11
50L_E	E	*Z. v. vivipara*	Italy	Viviparous	200897	9
63_08_E	E	*Z. v. vivipara*	Italy	Viviparous	637247	9
806_09_E	E	*Z. v. vivipara*	Italy	Viviparous	239211	9
80_09_E	E	*Z. v. vivipara*	Italy	Viviparous	209499	8
811_09_E	E	*Z. v. vivipara*	Italy	Viviparous	472981	9
79H_F	F	*Z. v. vivipara*	Austria	Viviparous	559903	9
80H_F	F	*Z. v. vivipara*	Austria	Viviparous	349964	9

Table 4.1. Details of samples analysed in this study. Mitochondrial clade according to Surget-Groba *et al.* (2006), subspecies, origin and reproductive mode. In addition, number of retained reads and mean coverage per samples are reported.

I examined raw reads, corrected for sequencing errors, demultiplexed the data and isolated single nucleotide polymorphisms (SNPs) with the pipeline software Stacks 1.02 (Catchen *et al.* 2013). SNPs were then used for descriptive (R packages, 2013), population genetics (4P, Benazzo *et al.* submitted), and GWAS (Gemma, Zhou & Stephens 2012) analyses.

Polymorphisms possibly related to the reproductive mode were identified using two different approaches. First, looking for F_{st} outliers, here defined as SNPs that simultaneously satisfied the following conditions: paiwise $F_{st} \geq 0.5$ between *vivipara* (V) and *carniolica* (O) populations; pairwise $F_{st} \geq 0.5$ between *vivipara* (V) and *lousilantzi* (O) populations; $F_{st} \leq 0.05$ between different *vivipara* (V) populations. Second, using the method Gemma (Zhou & Stephens 2012), which calculates statistical genotype-phenotype association implementing the Genome-wide Efficient Mixed Model Association algorithm.

Results and discussions

The RAD sequencing experiment produced 146.439.826 paired-end raw reads. Reads with ambiguous barcodes (24.270), ambiguous restriction sites (31.474.031) and showing low quality (base call accuracy < 99.99%, 5.830.599) were discarded. The retained 109.110.926 reads were used for further analyses. In order to have a non-redundant dataset, reads identified as PCR clones (i.e. identical in both paired-ends and corresponding to 59,4% of the total) were reduced to a single copy. Genotypic calling was performed using the 22.197.925 single end reads (sequences flanking the restriction sites), by means of alignments between individuals using 5 as minimum depth coverage (most of NGS study on non-model species rely on <5x coverage per site per individual, Nielsen *et al.* 2011). I identified a total of 46.314 contigs and 82.494 SNPs, selected from the 75bp single-end reads showing no more than 5 SNPs. These polymorphisms were used to describe the overall genetic variation within and between subspecies.

The multidimensional scaling plot confirmed at genomic level the existence of one viviparous and two oviparous clades (Figure 4.3). In the viviparous group, some level of geographical substructure related to the distinct geographic origins can be clearly identified.

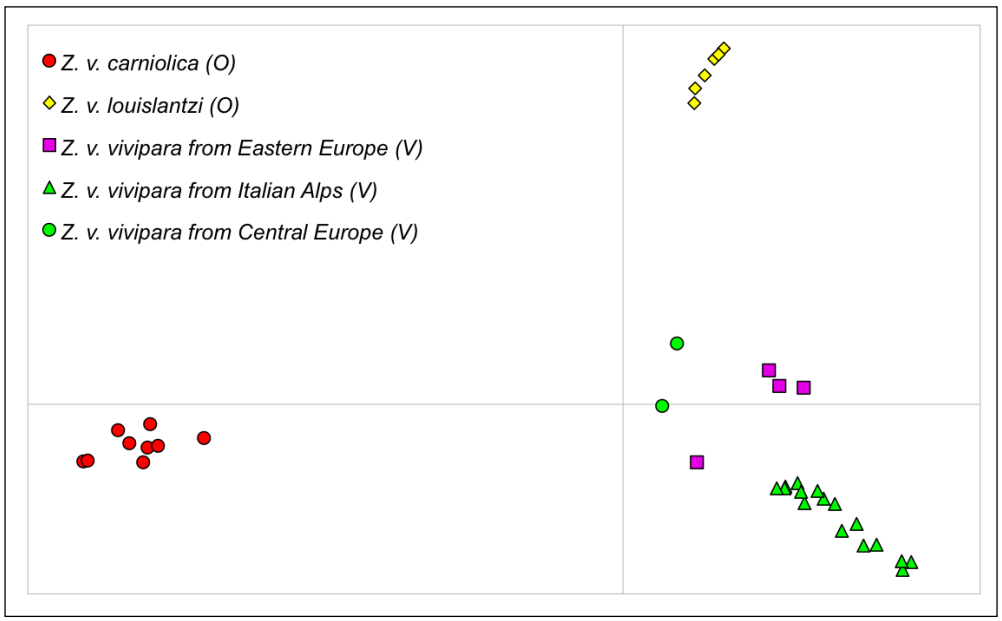

Figure 4.3. MDS resulting from about 82k SNPs about *Z. vivipara* subspecies.

I then analyzed a restricted marker-set (4908 SNP) with a reduced impact of missing data (less than 50% of missing data). 217 SNPs were identified as outliers using both the approaches I applied. Genomic sequences (200-500 bp long, achieved with Illumina Paired-end protocol) physically linked to these markers were then BLASTed against the only reptile genome available (*Anolis carolinensis*, which share the most recent common ancestor with *Z. vivipara* about 180 mya, Alfoldi *et al.* 2011). Among these genomic fragments I found sequence similarities in about 25% of them using an E-value threshold of 0.1.

A detailed analysis of the genes identified as putatively selected during the reproductive mode transition, and annotated in the *Anolis* genome, has not been performed yet. Here I note however that some of them are involved in physiological pathways known to differ in groups with oviparous and viviparous reproductive mode (Murphy & Thompson, 2011), and some others (e.g., cytokines, progesterone receptors, angiopoietin) have been already found to be potentially correlated with the switch in reproductive mode (Paulesu *et al.* 2005; Paolucci & Di Cristo 2002; Brandley *et al.* 2012). The most interesting candidate genes found in this study are listed in Table 4.2, and are reproductive hormones and genes implicated in the immune system and vascularization. This result is compatible with the idea that viviparity poses a major immunological hurdle for mother and fetus (Medawar 1953),

and that placental development substantially alters the maternal-fetal endocrine regulation and vascularization (Moffett & Loke 2006).

All the genes identified as putatively under selection and mapping into the *A. carolinensis* genome are reported in Table S4.1

Genes (predicted proteins)	Function
Suppressor of cytokine signaling V-set and immunoglobulin domain Immunoglobulin superfamily member Interleukin-8-like	Immune system
Vasopressin V1A Angiopoietin-related protein	Vascularization
Progesterone binding factor	Hormone receptor

Table 4.2. Genes identified using Illumina paired-end sequencing and Fst outlier-GWAS approaches, categorized according to their function.

So far, only candidate gene approaches have been taken for studying the evolutionary transition in reproductive mode. The advent of NGS technologies allowed moving from gene-by-gene to genome approach and using non-traditional model organisms for studying evolutionary processes. This study provided the first attempt to analyze the oviparity/viviparity transition at genomic level, with the consciousness that this shift is a very complex physiological change, probably mediated by hundreds of genes.

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Supplementary materials

Predicted protein	Score	E-value
plectin-like	410	3,00E-114
vasopressin V1a receptor	318	1,00E-86
DDB1 and CUL4 factor	316	5,00E-86
ubiquitination factor E4A	260	3,00E-69
coronin-6-like	230	6,00E-60
SWI/SNF related	217	4,00E-56
taste receptor type 1	208	2,00E-53
prolyl 3-hydroxylase	194	4,00E-49
C2 calcium-dependent domain	194	4,00E-49
transcription factor E3-like	179	9,00E-45
eukaryotic translation initiation	179	9,00E-45
glutamate [NMDA] receptor	165	2,00E-40
putative RNA-binding protein	152	1,00E-36
myogenic factor 6-like	149	2,00E-35
protein Wiz-like	149	2,00E-35
rho guanine nucleotide exchange	147	5,00E-35
semaphorin-4C-like	132	1,00E-30
pro-opiomelanocortin A-like	122	2,00E-27
protocadherin gene	98,7	2,00E-20
v-set and immunoglobulin domain	91,5	4,00E-18
solute carrier family 41	89,7	1,00E-17
vinexin-like	86	2,00E-16
cytochrome P450 2G1-like	71,6	3,00E-12
angiopoietin-related protein	66,2	1,00E-10
suppressor of cytokine signaling	64,4	5,00E-10
acetyl-coenzyme A synthetase	57,2	8,00E-08
lysozyme C	50	1,00E-05
engulfment and cell motility	48,2	4,00E-05
TBC1 domain family	48,2	4,00E-05
SWS1 opsin	46,4	1,00E-04
nucleoporin-like protein	46,4	1,00E-04
recombining binding protein	44,6	5,00E-04
lactoylglutathione lyase-like	44,6	5,00E-04
hect domain and RLD	41	6,00E-03
collagen alpha-1(XXII)	39,2	2,00E-02
neuron-specific protein	39,2	2,00E-02
immunoglobulin superfamily member	37,4	0,071
zinc finger protein 407	37,4	0,071
myotubularin-related protein	37,4	0,071
vacuolar protein	37,4	0,071
interleukin-8-like	37,4	0,071
keratin aHA1	37,4	0,071

zumo sperm-egg fusion	37,4	0,071
ryanodine receptor 3-like	37,4	0,071
progesterone binding factor	37,4	0,071
laminin	37,4	0,071
chromatin assembly factor	37,4	0,071
vomeronasal type-2 receptor	37,4	0,071
aquaporin-2-like	37,4	0,071
protein fat-free-like	37,4	0,071
myoblast determination protein	37,4	0,071
ankyrin-2-like	37,4	0,071
calmin-like	37,4	0,071

Table S4.1 Predicted proteins identified blasting contigs obtained by paired-end reads physically linked to putative polymorphisms under selection for the reproductive mode. Genes showing sequence similarity with *A. carolinensis* genome are listed ordered by increasing *E-value* (number of distinct alignments that are expected to occur in the database search by chance, with an equivalent or better score than the query sequences). The *score* is a value representing the overall quality of an alignment.

General discussion

The main aim of conservation biologists is to try to preserve global biodiversity at ecosystem, species and genetic levels. Ecosystem biodiversity is undoubtedly threatened by global change as demonstrated by Quintero and Wiens (2013). Predictions in this article are particularly alarming, since projected rates of climate change exceed usual rates of ecosystem evolution by 100000-fold among suitable ecological niches for vertebrate species. In this context, the analysis of genetic variation pattern is necessary for understanding populations' adaptive capacity in this period of climate changes.

The main effects of changing climatic conditions on wild species distribution are predicted to be changes in geographical location and in the extent of the range in line with habitat modifications. It has been already demonstrated that in order to react to climate warming, many species are shifting their geographic distribution toward higher latitudes or altitudes (Chen *et al.* 2011); however, species characterized by limited dispersal capabilities and restrictive requirements for reproduction or survival could be even more heavily affected by consequences of environmental changes. *B. variegata* and *Z. vivipara* are both characterized by reduced dispersal and specific ecological needs. Species range shifts, when possible according to suitable habitat availability, also have an impact on genetic variability. Only a restricted part of the original genetic variation moves to the newly colonized habitat causing repeated founder effect events and leading to low levels of genetic diversity (Pauls *et al.* 2013). This aspect is particularly worrisome when the original genetic variation and effective population size is already low.

The yellow-bellied toad *B. variegata* is currently considered of least concern (LC) by the IUCN (IUCN, 2013). However, population decline and fragmentation, as well as local extinctions, have been reported across its whole distributional range. In the *first* study of this thesis, I investigated the level of genetic variation and demographic pattern of some populations in Northern Italy. I found low levels of genetic variation at microsatellite markers, clear signals of population fragmentation and reduced estimates of effective population size. The genetic pattern observed in *B. variegata* was found to be mostly associated with a demographic decline occurred several thousands of generation ago, probably during the postglacial colonization of the Alps, but, at least in some areas, recent decline due to human related-processes

were inferred. These results, along with ecological requirements of the species must be taken into serious consideration considering the predicted environmental modifications and that small populations showing low genetic variation have reduced capacity to adapt to global changes (Willi *et al.* 2006). In fact, *B. variegata* generally prefers temporary habitats (e.g. puddles, stream loops) to permanent sites (e.g. ponds, tarns) for reproduction and consequently water availability is fundamental for its reproductive success; the fact that Brunetti *et al.* (2001) demonstrated that annual precipitation in north-eastern Italy decreased of about 7% in the last century, and that rainfall may experience a 30% decrease in annual precipitation in some areas by 2100 (IPCC 2007) could mean that *B. variegata* persistence is at risk. In a context of changing climatic conditions, the integration of genetic results obtained in this thesis and predicted environmental modifications suggest that conservation measures for *B. variegata* northern Italian populations should be considered with urgency.

The reduced estimates of genetic variability and effective population size I obtained for many populations of the yellow-bellied toad can be considered alarming and deserve consideration for future conservation plans. In addition, when the effective population size is low, the effects of genetic drift are amplified; both the methods I used for calculating N_e suggested very low estimates, comparable to ones obtained in similar studies on anuran species considered endangered by the IUCN. I also found strong population differentiation even in a restricted area, such as the Province of Trento, meaning that in many cases, gene flow between populations is prevented because of the combined effect of reduced dispersal capabilities of the toad and natural or man-made environmental features. Population fragmentation is enhanced when habitat discontinuity is promoted by anthropization; emblematic is the case of the populations of Nago and Loppio, which are only 2.7 Km apart but negligible traces of migration were identified between them (Fst = 0.16). Since in similar conditions (see for example Pozzolago and Pra which are separated by 3.3 Km, but *Fst* = 0.01 and not significant) genetic homogeneity was identified between populations in rural ecosystems, it was possible to hypothesize that anthropogenic barriers prevented migration, between Nago and Loppio, in the tourist area of Garda Lake.

Anthropization has also played a major role in the disappearance of *Z. v. carniolica* populations from the northern Italian lowlands. In fact, marshlands in the Po Plain, before human-mediated environmental alteration (for example, drainage and

reclamation of wetlands), were suitable habitats for the oviparous lineage. At present, the few remaining relict host populations show no mitochondrial variation (*second* study), likely because of the effects of genetic drift. The expected reduction in number and surface of wetland areas (Moore, 2002) threaten the persistence of *Z. v. carniolica* in northern Italy, both in lowlands and at mid altitude. On the contrary, the viviparous subspecies *Z. v. vivipara* showed signals of spatial and demographic expansions (*second* study). *Z. v. vivipara,* in fact, taking advantage of its reproductive mode tends to occupy higher altitudes than *Z. v. carniolica* and, consequently, less anthropized habitats. The evolution of the viviparous reproductive mode, during Pliocene/Pleistocene period, allowed *Z. v. vivipara* to colonize cold climates (both high altitude and latitude), making it the northernmost reptile species. Viviparous lacertid species, adapted to cold climates, can become threatened by the rapid projected increase of temperature worldwide and in particular in mountain regions (Brunetti *et al.* 2009). It has been demonstrated that viviparity itself increases the extinction risk among viviparous lineages belonging to the genus *Liolaemus* (Pincheira-Dinoso *et al.* 2013), because this parity mode is likely to be irreversible. Nevertheless, in this thesis, I found that, in the *Zootoca* genus, reversion from viviparity to oviparity likely occurred in currently oviparous French/Spanish populations (*Z. v. louislantzi*) according to phylogenetic analyses (*second* study), supported by a recent paper that suggests that transitions from oviparity to viviparity are less constrained than previously thought (Pyron & Burbrink 2014). This finding will not necessarily protect viviparous lineages of *Z. vivipara* from the exceptional temperature increase that is affecting the Alpine chain during the last two centuries (Brunetti *et al.* 2009). In addition, for the common lizard, whose distribution in the Mediterranean area is strictly correlated with moist habitats, changes in precipitation rates could have serious consequences for the persistence of this species. Wetlands, in fact, are one of the more impacted by environmental changes; the interaction of temperature, precipitation and atmospheric CO_2 variations in the future may change peatbog ecosystem composition with substantial consequences on the species they host (Heijmans *et al.* 2008), with obvious consequences for *Z. vivipara*.

In the *third* study, I analysed the contact area between *Z. v. vivipara* and *Z. v. carniolica*. This location in the central Alps, identified during the field work of the *first* study, provided a suitable and rare (only one other contact zone has been identified, so far) natural setting for investigating the level of gene flow between

oviparous and viviparous lineages and the role of reproductive mode in speciation. The clear absence of hybrid individuals without current evidence of reinforcement showed that the speciation process is already completed in this contact zone. These results, however, did not clarify if the switch in reproductive mode triggered the speciation process; in fact, as in other examples, it was not trivial to understand if the transition from oviparity to viviparity was driven by natural selection (environmental pressures), by genetic drift (spatial isolation) or by the combination of these two factors.

The occurrence of oviparous and viviparous populations within the same species makes *Z. vivipara* a unique model for studying the evolutionary transition from oviparity to viviparity. In fact, although other two lacertid species (the Australian scincid lizards *Lerista bougaunvilli* and *Saiphos equalis*) exhibit a reproductive bimodality, only *Z. vivipara* has overlapping and potentially hybridizing egg-bearing and live-bearing populations.

In the *fourth* study, I applied a Next Generation Sequencing technique that permitted the rapid discovery and analysis of thousands of SNPs, using viviparous and oviparous populations from the whole geographic distribution of *Z. vivipara*. This approach confirmed the unlikely occurrence of hybrid individuals as well as geographic and phenotypic genetic substructure. More interestingly, this study provided the first attempt at investigating, using a genomic approach, the genetic basis of the transition from oviparity to viviparity and highlighted some genes possibly involved in this switch. Some of those genes have been already studied, others have been identified for the first time in this thesis.

The strong genetic divergence between *Z. v. vivipara* and *Z. v. carniolica* outlined at the genome level strengthened the results obtained with traditional genetic markers. In fact, phylogenetic analyses performed with mitochondrial and nuclear sequences confirmed that *Z. v. carniolica* should be considered a separate ESU (*second* study). The species *Z. vivipara* is considered of least concern by the IUCN; nevertheless, according to the results from this thesis *Z. v. carniolica* should deserve specific conservation measures because of its genetic distinctiveness together with the vulnerability of its most suitable habitats. In addition, the lack of hybrid individuals in the contact zone between *Z. v. vivipara* and *Z. v. carniolica* (*third* study) suggested that they are two 'good species' according to the biological species concept (Mayr, 1942). This substantial distinction, if confirmed in other contact zones, could call for a

taxonomic revision and for specific conservation actions for the subspecies *Z. v. carniolica*.

Conclusions and future perspectives

Genetic investigations demonstrated that signals of low genetic variability, patterns of reduced effective population size and fragmentation are affecting both the toad *B. variegata* and the lizard *Z. vivipara* (in particular the subspecies *Z. v. carniolica*). These two vertebrate species have in common low dispersal capability and restrictive ecological requirements. In particular they both live in wetland habitats, which are among the environments most likely to be threatened by climate warming and anthropization. Considering that the adaptive potential of a population depends on genetic diversity, specific action tailored to increase or at least preserve current genetic variation should be evaluated.

Papers published, submitted or in preparation

- Salvidio S, **Cornetti L,** *et alii.* (2011) Assessing the status of amphibian breeding sites in Italy: a national survey. *Acta Herpetologica,* **6**, 119-126.
- **Cornetti L,** Menegon M, Giovine G, Heulin B, Vernesi C (2014) Mitochondrial and nuclear DNA survey of *Zootoca vivipara* across the eastern Italian Alps: evolutionary relationships, historical demography and conservation implications. *PLoS ONE,* **9**, e85912.
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- **Cornetti L,** Benazzo A, Vernesi C, Bertorelle G (2014) Small effective population size and fragmentation in Alpine populations of *Bombina variegata*: the combined effects of recent bottlenecks and postglacial recolonization. *To be submitted to Molecular Ecology.*
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- **Cornetti L,** *et al. Zootoca vivipara* as model for studying evolutionary transition form oviparity to viviparity in squamate reptiles: a genomic approach. *In preparation.*

- **Cornetti L,** *et al.* Contrasting genetic structure, historical demography and ecological requirements of oviparous and viviparous populations of *Zootoca vivipara* across the Alpine chain. *In preparation.*

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