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RELATIONSHIPS AMONG THE THREE LEVELS OF BIODIVERSITY - GENES, SPECIES, AND ECOSYSTEMS: AN EMPIRICAL STUDY WITH ALPINE AMPHIBIANS FROM TRENTINO

Direttore della Scuola: Ch.mo Prof. Gianni Barcaccia
Supervisore: Ch.mo Prof. Andrea Battisti
Co-supervisore: Cristiano Vernesi

Dottorando: Alexis Marchesini

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Summary

In the ongoing global biodiversity crisis, amphibians are the most endangered group of vertebrate, with increasing reports of population declines and extinctions worldwide. Alpine ecosystems are heavily affected by climate change and habitat alteration: range shifts and contractions are predicted, particularly for cold-adapted species, leading to an increased fragmentation of populations. A growing body of evidence is showing that the different levels in which biological diversity may be divided (i.e. genes, species, and ecosystems) are broadly linked, and ecological processes result from the complex interactions between these levels. As a result, modern conservation biology is increasingly recognizing the need for an integrative approach. In this project, we adopted such approach, investigating the evolutionary and ecological processes affecting the amphibian populations and communities, within a systemic perspective.

We focused on a south-eastern Alpine region, Trentino, choosing a model organism, the common frog (*Rana temporaria*), as target species for investigating patterns of diversity at the genetic level.

In Chapter 2, we investigated the past evolutionary history of *Rana temporaria* in the Trentino region, by means of a phylogeographic study based on mtDNA data (540 individuals from 54 sites). We highlighted a complex scenario, with three different Pleistocene glacial refugia located in the southern slopes of the Alps, routes of post-glacial recolonization following irregular patterns (reflecting the complex orography of the region), and a contact zone among different evolutionary lineages in the eastern part of the region. Notably, different lineages exhibited different levels of genetic diversity at mtDNA.

In Chapter 3, we conducted a population and landscape genetics study, using microsatellite markers (1522 individuals from 79 sites), for evaluating patterns of current genetic variability and genetic structure in *Rana temporaria* populations. We detected a main barrier to gene flow, the Adige river valley, separating the region in two genetically differentiated clusters. Comparing our findings with the pattern emerged from Chapter 2, we concluded that current levels of intra-population genetic variability seem to be shaped by a combination of past (e.g. recolonization processes) and present (e.g. isolation) factors. The two sub-regions, West and East Trentino, showed different spatial patterns for both genetic variability and fine-scale population structure.

In Chapter 4, we studied the relationship between species diversity of amphibian

communities and genetic diversity of the model species, *Rana temporaria*. We found a strong negative correlation, and we demonstrated that the recorded pattern was due to the opposite influence of environmental factors on the two levels of biological diversity.

Genetic diversity is fundamental for the persistence of populations in the face of environmental change: in the context of the ongoing biodiversity declines, it is therefore of crucial importance monitoring its levels and understanding the underlying processes. Our approach, based on two different types of genetic markers (mtDNA and microsatellites), provided evidence that the legacy of past evolutionary history is still largely evident in the genetic patterns of small, low-vagility vertebrates such as amphibians, even at fine spatial scale. Identifying different evolutionary lineages, "hotspots" of genetic diversity, as well as evaluating current connectivity patterns should be considered an essential preliminary step for developing effective conservation strategies. In addition, the detected negative correlation between species and genetic diversity, perhaps the most important finding of this study, suggests that species diversity cannot be universally used as proxy for genetic diversity in conservation planning.

Choosing a common, widespread species allowed us to capture a detailed (although not exhaustive) picture of amphibian biodiversity in the Trentino region, and this study may be used as a term of comparison with more endangered species, or for testing specific hypothesis in future research investigations.

Riassunto

Nell'attuale crisi globale della biodiversità, gli anfibi sono considerati il gruppo di vertebrati più a rischio, con declini ed estinzioni di popolazioni riportati in misura sempre più frequente da tutto il mondo. Gli ecosistemi alpini risentono in modo particolare del cambiamento climatico e dell'alterazione degli habitat: sono previsti cambiamenti e riduzioni nell'areale, in modo particolare per le specie adatte al freddo, con conseguente aumento della frammentazione delle popolazioni. Evidenze sempre più numerose stanno mostrando come i diversi livelli in cui la biodiversità può essere suddivisa (i.e. geni, specie, ed ecosistemi) siano in realtà strettamente connessi, e come i processi ecologici derivino dalle complesse interazioni tra le singole componenti. Di conseguenza, la moderna biologia della conservazione sta progressivamente riconoscendo in misura maggiore la necessità di un approccio integrato. In questo progetto si è adottato un tale approccio, analizzando i processi evoluzionistici ed ecologici nelle comunità e popolazioni di anfibi, in una prospettiva sistemica.

Focalizzando la nostra attenzione su una regione delle Alpi Sud-Orientali, il Trentino, abbiamo scelto un organismo modello, la rana di montagna (*Rana temporaria*), come specie target per lo studio dei pattern di diversità a livello genetico.

Nel Capitolo 2, abbiamo analizzato la storia evolutiva passata di *Rana temporaria* nell'area di studio, mediante uno studio di filogeografia basato su dati di DNA mitocondriale (540 individui da 54 siti). Ne è emerso uno scenario complesso, con tre diversi rifugi glaciali localizzati nelle aree più meridionali delle Alpi, rotte di ricolonizzazione irregolari (che riflettono la complessa orografia della regione), e una zona di contatto tra diverse linee evoluzionistiche nella parte orientale della regione. Inoltre, le diverse linee evoluzionistiche hanno evidenziato diversi livelli di diversità genetica.

Nel Capitolo 3, abbiamo realizzato uno studio di genetica di popolazione e genetica del paesaggio (*landscape genetics*), mediante l'utilizzo di marcatori microsatellite (su 1522 individui da 79 siti), al fine di valutare i livelli attuali di diversità genetica e la struttura genetica nelle popolazioni di *Rana temporaria*. È stata identificata un'importante barriera al flusso genico, la Valle dell'Adige, che si è rivelata dividere la regione in due gruppi geneticamente differenziati. Confrontando i nostri risultati con quanto emerso nel Capitolo 2, abbiamo potuto concludere che gli attuali livelli di variabilità genetica intra-popolazione

sembrano essere influenzati da una combinazione di fattori storici (processi di ricolonizzazione) e attuali (isolamento).
Nel Capitolo 4, abbiamo studiato la relazione tra la diversità specifica delle comunità di anfibi, e la diversità genetica della specie modello, *Rana temporaria*. È stata riscontrata una forte correlazione negativa, che abbiamo dimostrato essere dovuta ad un'influenza opposta dei fattori ambientali sui due livelli di diversità biologica.

La diversità genetica è fondamentale per il persistere delle popolazioni al mutare delle condizioni ambientali: nel contesto dell'attuale crisi globale della biodiversità, è pertanto di cruciale importanza il monitorarne i livelli e comprendere i processi che ne stanno alla base. Il nostro approccio, basato su due tipi diversi di marcatori molecolari (DNA mitocondriale e microsatelliti), ha permesso di evidenziare come la storia evolutiva passata abbia lasciato un'impronta ancora fortemente evidente nei pattern genetici di vertebrati di piccola taglia e ridotta mobilità, come gli anfibi, persino a scala spaziale ridotta. L'identificazione di diverse linee evolutive, degli "hotspot" di diversità genetica, così come la stima dei pattern attuali di connettività dovrebbe essere considerata un passo preliminare essenziale per lo sviluppo di strategie conservazionistiche efficaci. Inoltre, la correlazione negativa riscontrata tra diversità specifica e genetica, che costituisce forse il risultato più importante di questo studio, indica che la diversità specifica non può essere utilizzata come proxy universale della diversità genetica nella pianificazione conservazionistica.
La scelta di una specie comune ed ampiamente diffusa ha permesso di ottenere un quadro dettagliato (sebbene non esaustivo) della biodiversità anfibia del Trentino, e questo studio può fungere da termine di paragone con altre specie, maggiormente a rischio, o per testare specifiche ipotesi nell'ambito di progetti di ricerca futuri.

Chapter 1. Introduction

1.1 Amphibians biodiversity conservation within a systemic approach: a global perspective

1.1.1 Global biodiversity decline

In its most general meaning, biological diversity or biodiversity is the "variety of life" at all levels of biological organization (Gaston & Spicer 2004).

The term "biodiversity" was first used in a publication by the sociobiologist and entomologist E.O. Wilson in 1988. Since this period, the term has been widely used by scientists, environmental managers and policy-makers, leading to a large number of formal definitions. At the same time, awareness has grown regarding the value of biodiversity, as well as the concern about its loss due to human activities.

The global importance of biodiversity was one of the key points of the 1992 Rio de Janeiro Earth Summit, which resulted in the Convention on Biological Diversity (CBD), ultimately ratified by 193 countries. The goals of the CBD are "*the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits from the use of genetic resources*". CBD provided the following definition of biodiversity, which has become one of the most widely accepted: "*Biodiversity is the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are a part; this includes diversity within species, between species and of ecosystems*" (UNEP, 1992). Therefore, CBD recognized three main components of biodiversity. The biological meaning of this subdivision and its rationale will be further discussed later in the following sections.

Worldwide biodiversity is threatened by an ongoing crisis, directly or indirectly determined by human activities. Global change continuously stresses populations, ecological communities and natural environments, exposing them to new adaptive challenges. The major threats to biodiversity include: overexploitation (e.g. overfishing and overhunting), habitat destruction, degradation and fragmentation, pollution, introduction of alloctonous species, spread of emerging infectious diseases and, perhaps the most challenging one, human driven climate change. All these factors interact in complex ways, giving rise to self-reinforcing processes (amplifying feedbacks and cascading effects; Brook *et al.* 2008) and the extinction of a species is often driven by synergistic causes. As such, conservation actions which only target single species or single threats might be inadequate in the long term.

As a result of this anthropic pressure, current rates of biodiversity loss are now faster than ever before in human history. The phenomenon is so huge that scientists from different fields argue we are entering or in the midst of the "sixth mass extinction", being the entity of species loss comparable to that of the five great mass extinctions occurred during the history of life on our planet (Leakey & Lewin 1995). Unlike past mass extinction events, the "sixth extinction" seems to be almost entirely driven by a single species, *Homo sapiens* (Ehrlich & Ehrlich 1981), since all the major causes are due to human impact and ultimately linked to humanity's population growth. The increase of species extinction rate is a key measure for assessing the severity of this decline and initial studies estimated current rates to be 100–10000 times higher than pre-human or background extinction rates (Pimm *et al.* 1995; Lawton *et al.* 1995; Myers 1993). Nevertheless, these initial estimates have been criticized by some authors, arguing they were based on assumptions that overestimated the crisis (He & Hubbell 2011; Stork 2009). However, recent data are now confirming and reinforcing the hypothesis stating that we are on the brick of a mass extinction event. In 2014, Pimm and colleagues published a global analysis in *Science* journal, showing that current extinction rate are at least 1000 times faster than in the past, and are poised to increase in the future. In the same year, an independent study concluded that current rates are 1,000 times higher than natural background rates and future rates are likely to be 10,000 times higher (De Vos *et al.* 2014). Another study, focusing on vertebrates, found species loss over the last century to be up to 114 times higher than the background extinction rate, emphasizing however that these estimates were highly conservative (Ceballos *et al.* 2015). Despite the disagreement in numbers (derived from different models and assumptions), substantial evidence suggests that a mass extinction event is underway. As an example, numbers illustrating the decline of worldwide vertebrate species are astonishing. According to IUCN Red List of Threatened Species, recognized as the world's most comprehensive inventory of the conservation status of plant and animal species, the percentage of species threatened among vertebrates ranges from 13 percent of birds to 41 percent of amphibians (IUCN 2016). However, the scenario could even be worse, since taxonomic catalogues are still far to be complete, with many species going extinct before they have even been discovered (Lees & Pimm 2015). WWF Living Planet Report predicts for vertebrate populations a global decline by 67% by 2020 (WWF 2016). It is worth noting that once a population is reduced below a certain threshold its eventual extinction is virtually assured (Shaffer 1981). Indeed, small populations generally display decreased fitness and are extremely vulnerable to diseases and

environmental change, due to demographic (Flather et al. 2011) and genetic processes (Frankham 2005).

The importance of conserving biodiversity is widely recognized because of its immeasurable and multiple value. Beyond its intrinsic value, biodiversity is the source of goods that are crucial for human survival, including food (crops, livestock, game and fish, wild fruits and vegetables, etc...), medicines (both current and potential) and industrial products (e.g. wood, rubber, fibre, coal, fuels and other extractable resources). In addition, wild species and natural environments provide a vast array of direct and indirect essential services to mankind (the so called "ecosystem services"), such as regulation of global processes (e.g. atmospheric composition, climate, soil formation and fertility, nutrient and water cycling, etc.), pollution breakdown and absorption, pollination and seed dispersal, control of agricultural pests and invasive species, tourism, recreation and countless other services we often take for granted. Moreover, each living system is a masterpiece of natural engineering, evolution and optimization, offering a vast source of information for scientific research and technology. Biodiversity is also a key component of human culture, playing a central role in our language, tradition, spirituality and arts. Translating these "ecosystem services" into economic value, scientists estimated for the entire biosphere a current value in the range of US $16–54 trillion per year (Costanza *et al.* 1997).

Finally, a special mention needs to be made for the role of biodiversity in maintaining ecosystem functioning and stability. The diversity and complexity of biological communities (in terms of species, compositional structure, trophic interactions, functional traits, etc.) are thought to be crucial to their own integrity and stability through time. Indeed, there is a growing body of evidence that more diverse ecosystems generally have higher productivity (van der Heijden *et al.* 1998; Tilman *et al.* 1996) and ability to withstand perturbations and environmental stress (Mc Cann 2000; Folke *et al.* 2005). This aspect of biodiversity is clearly relevant for both planet Earth and humankind, since our survival ultimately depends on ecosystem functioning and persistence.

For all these considerations, biodiversity erosion has various and tremendous ecological and societal consequences: we therefore urgently need to improve our efforts for monitoring this global phenomenon, correctly understanding its different causes and quickly implementing appropriate mitigation measures. Despite the dramatic ongoing decline, according to scientists it is still possible to avoid a complete sixth extinction through intensified conservation efforts, but rapid action is needed (Ceballo *et al.* 2015). Several

global attempts in this direction have been made in recent past, so far producing, however, few concrete positive results. In 2002, at the sixth Conference of the Parties of the Convention on Biological Diversity, world leaders committed “to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national level”. This statement has become known as the 2010 Biodiversity Target. Unfortunately, this goal was not met: biodiversity loss does not appear to be slowing, while pressures on nature are increasing (Butchart *et al.* 2010; Gordon *et al.* 2010). In response to this failure, in 2010, the tenth meeting of the Conference of the Parties, held in Nagoya (Japan), adopted a revised and updated Strategic Plan for Biodiversity, fixing new targets for the 2011-2020 period, known as "Aichi Biodiversity Targets" (SCBD 2010).

Aichi Biodiversity Targets are summarized as follows:

(1) Strategic Goal A: Address the underlying causes of biodiversity loss by mainstreaming biodiversity across government and society;
(2) Strategic Goal B: Reduce the direct pressures on biodiversity and promote sustainable use;
(3) Strategic Goal C: To improve the status of biodiversity by safeguarding ecosystems, species and genetic diversity;
(4) Strategic Goal D: Enhance the benefits to all from biodiversity and ecosystem services;
(5) Strategic Goal E: Enhance implementation through participatory planning, knowledge management and capacity building.

As in CBD, genes, species and ecosystems diversity are recognized as main components of biodiversity in “Aichi targets” and the preservation of all three levels is advocated (see Strategic Goal C).

1.1.2 Global amphibian decline

In the context of global biodiversity decline, amphibians deserve special attention. Since the 80’s, herpetologists increasingly reported amphibian population declines and extinctions worldwide and in 1989, at the First World Herpetology Conference, scientists officially recognized that amphibians were suffering from an unprecedented and dramatic widespread decline (Barinaga 1990). In order to fulfil the lack of comprehensive data on amphibians distribution, conservation status and threats, IUCN (International Union for Conservation of Nature), in partnership with Conservation International, undertook the first Global Amphibian Assessment (GAA), which was completed in 2004. The GAA found that at least

43.2% of amphibian species were experiencing some form of population decrease, 32.5% were globally threatened and 22.5% were too poorly known to assess their conservation status (Stuart *et al.* 2004). It is now an undeniable evidence that amphibians are facing a dramatic declining at a global scale (Houlahan *et al.* 2000; Mendelson *et al.* 2006; Stuart *et al.* 2008): they are considered the most endangered group of vertebrates (Stuart *et al.* 2004; IUCN 2016) and for this reason they have recently received a great deal of scientific attention.

In the general scenario of biodiversity crisis, the global amphibian decline is characterized by three distinctive features: (1) the reported increase in amphibian population declines and extinctions is more recent than in other groups of animals (starting in the 1980s); (2) simultaneous declines are occurring in vast regions of the planet, spanning from different habitats and climates; and (3) even natural and protected areas are often affected by this phenomenon (Collins & Storfer 2003; Drost & Fellers 1996; Knapp & Matthews 2000). The last point is perhaps the most challenging, indicating that habitat protection alone could be in some cases an insufficient measure to ensure protection to amphibian populations.

No single cause has been universally recognized for global amphibian decline, with numerous different factors being invoked as potential explanations on a case-by-case basis. Collins and Storfer, in their global review (2003) identified six leading hypotheses (i.e. stressing factors), divided into two major groups according to the following classification:

Class I hypotheses: (1) introduction of alien species; (2) overexploitation; (3) land use change;

Class II hypotheses: (4) global change (including increased UV radiation and climate warming); (5) environmental contaminants; (6) emerging infectious diseases.

According to the authors, Class I includes factors that have negatively affected amphibian populations for more than a century and we have reached a good understanding of the underlying processes. On the contrary, the impact of Class II factors is relatively more recent, involving more complex and subtle dynamics and often indirect effects. In the following, we will provide a brief discussion of the six factors.

1. Introduction of alien species

Introduced species can often cause declines or even local extinctions of native amphibians, affecting their populations in different ways, among which the most frequent are: predation (mainly of eggs and larval stages), competition (between one or more life stages), introduction of pathogens and hybridization (Collins & Storfer 2003).

These biotic interactions can cause direct and indirect effects on amphibians, such as reduced growth or survivorship, alterations in behaviour or habitat use and possibly, at the landscape scale, disruption of metapopulation structure (Pilliod & Peterson 2001). The majority of studies reporting negative effects of alien species introduction on local amphibian populations are from temperate regions and in North America they are considered a primary cause of decline (Fisher & Schaffer 1996). However, introductions play an important role also in tropical areas: as an example, in South America about 30% of the amphibian species are classified by the IUCN as threatened by alien invaders (Rodriguez 2001). Among the most dangerous amphibian predators we may include introduced fish, other amphibians such as bullfrogs (*Rana catesbeiana*) and cane toads (*Bufo marinus*), and crayfish (Kats & Ferrer 2003). Well documented examples of severe amphibians declined caused by introduced fish come from North America, e.g. the yellow-legged frogs (*Rana muscosa*) (Knapp & Matthews 2000), the long-toed salamander (*Ambystoma macrodactylum*) and the Columbia spotted frog (*Rana luteiventris*) (Pilliod & Peterson 2001). In Italy, several examples of amphibian decline due to predation by alloctonous salmonids and cyprinids are reported, mainly from alpine and apennine lakes (Lapini, 2005).
Concerning the effects of alien amphibians on native species, population losses due to the predation by introduced bullfrogs (*Rana catesbeiana*) are reported from North America (Blaustein & Kiesecker 2002) and Europe, e.g. from Po Plain (Italy) where this large frog has become established (Lanza *et al.* 2009).

Beyond direct effects like predation, the introduction of alien species may have negative effects via other more complex mechanisms. Indeed, alien species (both fish and amphibians) may act as vectors for emerging pathogens (Daszak *et al.* 2004; Kiesecker *et al.* 2001), or disrupt local adaptation and fitness of local populations due to hybridization. A particular example of this last phenomenon is represented by the introduction of marsh frogs (*Pelophylax ridibundus)* in several areas of western and central Europe and their impact on the autochthonous water frog *P. lessonae*. Due to a complex process of hybridogenesis prior to meiosis, hybridizations among the two species favour the replacement of the *lessonae* genome by *P. ridibundus* alloctonous genes, and therefore to the disappearing of *P. lessonae* in these areas (Vorburger & Reyer 2003). Although the role and different effects of exotic species in amphibian declines is relatively well understood, managing ongoing biological invasions is generally not straightforward: many alien species are difficult to eradicate and, in addition, conflicts may arise among conservationists and other social partners, since the

introduction of alien fish in many areas reflect primarily economic and recreational goals (e.g. sporting fishery).

2. Overexploitation

Many amphibian species have long been exploited as a food resource in different parts of the world. However, large-scale effect of amphibian harvesting by humans is poorly known, although evidence is growing that it can be significant in areas of intense harvesting (Lannoo *et al.* 1994; Jennings & Hayes 1985). Other forms of amphibian exploitation for other purposes include collection for pet trade, education and medical research (Jensen & Camp 2003). In Europe, amphibian harvesting may be considered only a secondary cause of decline, with dangerous consequences only for already declining populations (Lapini 2005).

3. Habitat destruction, degradation and fragmentation

Habitat destruction and alteration are major causes of reduced biodiversity globally and amphibians are not an exception. Massive deforestation, wetland drainage for agriculture, urbanization, road construction and other forms of land use change lead to terrestrial and aquatic habitat loss, degradation or fragmentation, often preventing migrations to breeding sites and gene flow among populations (Beebee & Griffiths 2005; Hels & Buchwald 2001). Even apparently less severe habitat modifications, such as ecologically unsustainable forest practices, can result in changes in microclimate, soil moisture and habitat complexity, with detrimental effects for the most sensitive amphibian species (deMaynadier & Hunter 1998; Gardner 2001; Felix *el al.* 2010; Semlitsch *et al.* 2009). Land use change has been identified as an important driver of local and regional amphibian population declines and extinctions from temperate to tropical zones (Alford & Richards 1999, Collins & Storfer 2003). However, while in Europe and North America temporal patterns of amphibian declines seem to identify historic habitat loss as the primary driver, in other areas of the planet habitat loss is a more recent threat for amphibian populations (Houlahan *et al.* 2000). Despite the obvious negative impacts of habitat modifications, measuring and fully understanding their consequences is often not so straightforward, because of common time-lag responses (often with a delay of up to several decades, e.g. Findlay & Bourdages 2000) and because of long-range consequences, which may be difficult to detect or to relate to the source of disturbance (Houlahan & Findlay 2003). Amphibians are small vertebrates characterized by low desiccation tolerance, different life stages with different habitat requirements and low vagility: for all these reasons, they are highly susceptible to environmental changes occurring from broad to very small spatial scale (Rowe *et al* 2003, Hopkins 2007). In addition, they

often have a patchy distribution leading to metapopulation structure (although this is not a universal rule; see Smith & Green 2005): connectivity plays an important role in amphibian population dynamics and genetic diversity patterns. Therefore, the effects of fragmentation seem to be particularly severe for this group of animals (Marsh & Trenham 2001; Cushman 2006). Fragmentation can lead to a decrease in within-population genetic diversity and inbreeding depression, and there is little doubt that these genetic processes ultimately increase the extinction risk of natural populations (Frankham *et al.* 2006).

4. Global change

Ongoing climate change can potentially affect amphibians at individual, population and community level. Latitudinal and altitudinal range shifts are generally hypothesized in response to global warming, together with negative effects on amphibian survival, growing rates, reproduction and dispersal ability (Blaustein *et al.* 2010). Moreover, changes in climatic regime may lead to habitat alterations, particularly in terms of vegetation, soil moisture and hydrology. These alterations may in turn affect food availability and predator-prey interactions, as well as intra- and interspecific competition (Alford 1989; Alvarez *et al.* 2002; Cunningham *et al.* 2009).

In addition, climate change can modify host-pathogen dynamics and favour the spread of emerging infectious diseases (Pounds *et al.* 2006). Finally, an increase in UV radiation caused by the reduction of stratospheric ozone may, according to some studies, contribute to the decline of some populations and species, although observations from empirical studies are discordant (Collins & Storfer 2003). There is growing empirical evidence that amphibians are already experiencing the negative effects of climate change in Europe. Declines in some species have been related to changes in climatic conditions, particularly in areas where wetlands are already scarce and aridity is predicted to increase, like Mediterranean regions (Henle *et al.* 2008).

Long-term studies on European amphibians show many evidences of phenological changes, with a general tendency to earlier breeding in response to increased temperatures (Blaustein *et al.* 2003). At this regard, herpetologists think that amphibians in Central and Northern Europe may suffer from an increased risk due to late frosts, with the risk of freezing for spawn or adults (Hänninen 1991; Walther *et al*. 2002). This is particularly the case of early breeding amphibian, such as brown frogs (*Rana arvalis, R. dalmatina, R. temporaria*) and *Bufo bufo.* Moreover, less snow cover and warmer winters may disrupt normal rhythms of hibernation, leading to energy depletion in adults, which in turn negatively affects survival

and/or reproduction, as suggested by Reading (2007). Finally, an increased likeliness of extreme events, particularly of extreme droughts, may cause dramatic fluctuations in population size due to complete reproductive failures (Pechman *et al.* 1989, Semlitsch 2003). Complete failures in larval development or metamorphosis have already been reported in Portugal for a multispecies assemblage breeding in temporary water bodies (Malkmus 2006), and for *Rana temporaria* in Poland (Jedrzejewska *et al.* 2003) and Finland (Piha *et al.* 2007).

5. Environmental contaminants and acidification

Contamination of both terrestrial and freshwater habitats from agricultural and industrial pollutants can have deleterious consequences on amphibians (Linder *et al.* 2003; Egea-Serrano *et al.* 2012). Negative effects of agricultural pollutants (such as pesticides, herbicides and fertilisers) on amphibians have been confirmed by both observational and experimental evidence, with embryonic and larval stages being particularly vulnerable (Carey & Bryant 1995). Well documented direct effects include: larval mortality, developmental delay and physical deformations, alteration of feeding behaviour (e.g. Boone *et al.* 2003; Bridges 1999; Ortiz *et al.* 2004; Griffis-Kyle 2007; Shinn *et al*., 2008; Marco & Blaustein 1999). The case of atrazine deserves a special mention: this herbicide, widely used worldwide but banned in the European Union since 2004, has be proven to cause feminisation of frogs even at extremely low concentrations, with negative consequences at the population level (Hayes *et al*., 2002). Beside direct effects, the importance of indirect influences on food resources, predation, and competition cannot be neglected (Boone *et al.* 2007; Relyea 2009, Bridges & Semlitsch 2000). Another potential cause of observed amphibian declines is acid precipitation. Low pH of ground and pond water may be responsible for enhanced embryo and larval mortality, reduced egg and larval growth, and, again, a number of dangerous indirect effects (Freda & Dunson 1986; Waldman & Tocher 1998; Alford & Richards 1999). Negative effects of acidification at the individual level have been confirmed for some species by laboratory studies (e.g. the wood frog *Rana sylvatica*; Freda & Dunson 1986) while some field surveys reported vast detrimental consequences at population level (e.g. reduction in range size of natterjack toad *Bufo calamita*; Beebee *et al.* 1990). Because of atmospheric transport of contaminants, acid precipitation and other forms of atmospheric pollution may affect even pristine environments far from the primary source of contamination, and amphibians have provided important evidence for these large-scale effects (Davidson 2004; Davidson *et al.* 2002; Fellers *et al.* 2004; Sparling *et al.* 2001). However, it must be remembered that amphibians exhibit marked interspecific variation in

their susceptibility to both agricultural chemicals and acidification (Bridges & Semlitsch 2000; Pierce 1985; Snodgrass *et al.* 2008).

6. Infectious diseases

Amphibians are afflicted with a wide variety of diseases, such as viral, bacterial, fungal and trematode infections (Carey *et al.* 2003; Wright & Whitaker 2001).

Dramatic mass mortalities caused by outbreaks of emerging pathogens have been reported for many different species and geographical regions, suggesting that diseases may play a significant role in global amphibian declines (e.g., Cunningham *et al.*, 1996; Berger *et al.* 1998; Lips 1999; Daszak *et al.* 2003). In particular, concern is growing on two major amphibian emerging infectious diseases: chytrid fungus (*Batrachochytrium dendrobatidis*) and ranaviruses (Iridoviridae) (Carey *et al.* 2003; Collins & Storfer 2003).

The chytrid fungus *Batrachochytrium dendrobatidis* is probably the most worrying amphibian pathogen so far discovered and in the last 20 years. It has been associated with population or even species extinctions in South and North America, Africa, Europe, Australia and New Zealand (e.g. Berger *et al.* 1998; Bosch *et al.* 2001; Bradley *et al.* 2002; Garner *et al.* 2005; Lips *et al.* 2006; Weldon *et al.* 2004). The fungus attacks keratinized tissues of juvenile and adult amphibians causing the disease known as chytridiomycosis, consisting in disorders of the adult epidermis and mouthparts of larvae, which in turn lead to a vast array of symptoms, from disrupted respiration and osmoregulation to anorexia, behavioural changes and eventually death (Berger *et al.* 1998; Kilpatrick *et al.* 2010). The first recognized case of chytridiomycosis in Europe dates back to 1997-1999, when it was identified as the cause of mass mortalities of the common midwife toad (*Alytes obstetricans*) in central Spain (Bosch *et al.* 2001). In Italy, the fungus was first detected during a survey on populations of the Apennine yellow-bellied toad (*Bombina pachypus*), and later its widespread occurrence was demonstrated for this species both in contemporary and historical samples (Lanza *et al.* 2009; Canestrelli *et al.* 2013).

The disease is now broadly distributed across Europe, affecting many amphibian species belonging to different families, with records from Spain, Portugal, Italy, Switzerland, Luxembourg, France, Hungary, Germany, Denmark and Great Britain (reviewed in Olson *et al.* 2013). Catastrophic declines in many distant areas of the world, together with molecular studies seem to indicate that the pathogen is expanding its geographic distribution and/or increase in virulence (Morehouse *et al.* 2003). However, amphibians exhibit great inter- and intraspecific variation in susceptibility to this pathogen and some species may even carry the

fungus without showing clinical signs, potentially acting as pathogen reservoirs (Daszak et *al.* 2004).

A second species of chytrid fungus (*Batrachochytrium salamandrivorans*) is recently emerging in Europe, severely threatening European urodele amphibians (newts and salamanders) (Martel *et al.* 2014). It was first described in 2013 in the Netherlands, where this new pathogen caused dramatic mass mortality events in fire salamanders (*Salamandra salamandra*), bringing the species to the edge of local extinction (Spitzen-van der Sluijs *et al.* 2013; Martel *et al.* 2013). Recent surveys are now showing evidence for a rapid spread to Belgium and Germany, with infections extending to alpine newts (*Ichthyosaura alpestris*) and smooth newts (*Lissotriton vulgaris*) (Spitzen - van der Sluijs *et al.* 2016).

Another important group of emerging amphibian pathogens is represented by *Ranavirus,* a genus of viruses that infects fish, reptiles and amphibians and can be transmitted between these taxonomic classes of vertebrates (Beebee & Griffiths 2005; Duffus *et al.* 2015). Ranaviruses are known to have caused amphibian die-offs on five continents (Gray *et al.* 2009). In North America, they have been associated with high levels of mortality in tiger salamanders (*Ambystoma tigrinum; J*ancovich *et al.* 1997). In Europe, Ranaviruses outbreaks have been documented at least since the 1980s, when large-scale mortalities in common frog (*Rana temporaria*) populations, unambiguously related to this group of pathogens, were reported in southeast of England.

(Cunningham *et al.* 1996). Afterwards, infections have been reported from Croatia, Denmark, Netherlands, Switzerland, Spain and Portugal, mainly on European green frogs (*Pelophylax* spp.), *Alytes obstetricans* and several European newt species (reviewed in Gray *et al.* 2009). As for chytridiomycosis, the susceptibility, long-term impacts and resilience to *Ranavirus* infections vary widely among amphibian species and populations (Hoverman *et al.* 2010; Teacher *et al.* 2010).

For both *Ranavirus* and chytrid fungus infections, an important role is supposed to be played by anthropogenic spread, e.g. via importation of fish and amphibians for pet trade, tourism or even herpetological surveys (Collins & Storfer 2003; Jancovich *et al.* 2003; Bletz et al. 2015; Halliday 1998). However, the scenario could be more complex and disease outbreaks seem to often originate from complex dynamics involving different ecological drivers, e.g. climate change (Pounds *et al.* 2006), increased UVB radiation (Kiesecker *et al.* 2001), pollution and water eutrophication (Johnson & Chase 2004).

The complex interactions characterizing emergent amphibian diseases seem to be the rule rather than the exception in explaining global amphibian declines. Indeed, many scientists now argue that amphibian declines are complex phenomena: although some simplifications can be made in specific situations, they seem to be generally driven by multiple abiotic and biotic stressors acting simultaneously. Moreover, the relative contribution of various stressors to population declines may differ from region to region, among species, among populations of the same species, and even among life stages within a population, giving rise to a vast array of complicated local interaction (Blaustein & Kiesecker 2002; Blaustein & Bancroft 2007; Blaustein *et al.* 2011). Due to this ''context dependency', general conservation strategies are difficult to develop and often inadequate: there is a strong need for local studies in order to find effective local solutions (Grant *et al.* 2016).

Lastly, beyond the external factors potentially threatening amphibian populations, in the context of declines we must not forget the evolutionary role of genetic diversity: it is universally recognized that a decrease in genetic variation can lead to a reduction in fitness and adaptability to environmental changes (Frankham *et al.* 2006). Amphibians, due to their particular breeding strategy (often characterized by small effective population sizes and whole clutch mortality), their low dispersal and high philopatry (limiting connectivity), seem to be especially prone to such genetic processes. Indeed, a growing number of studies are showing a reduction in genetic variation for many amphibian species or populations. The potential negative effects of external drivers (e.g. habitat fragmentation) on genetic diversity have been already discussed above, now it must be remembered that low genetic diversity can in turn magnify the effects of other stressing factors and eventually leading to extinction vortex (Höglund 2009). Genetic monitoring should therefore be considered a fundamental aspect in the study of amphibian declines, together with a better understanding of the underlying evolutionary processes (Allentoft & O'Brien 2010; Blaustein & Bancroft 2007).

Amphibians as bioindicators and model organisms in ecological and evolutionary studies.
Amphibians have been widely recognized as "biological indicators" of environmental stress and ecosystem health (Vitt *et al.* 1990; Mendelson *et al.* 2006). Indeed, their peculiar physiology (e.g. thin and permeable skin, unshelled eggs) make them particularly sensitive to changes in microclimatic conditions, environmental pollution and skin diseases (Rowe *et al.* 2003). Moreover, because of their complex life cycle, generally including both terrestrial and aquatic phase, they are exposed to the effects of alterations and loss of both habitat types (Dunson *et al.* 1992).

In addition, the importance of amphibians in environmental monitoring is linked to their high trophic importance. Indeed, several amphibian species may represent the highest fraction of vertebrate biomass in many aquatic and terrestrial ecosystems (Blaustein *et al.* 1994), being important components of food webs. They occupy different habitats during different life stages, they are able to exploit energy- poor micro-environments, with larvae characterized by high conversion efficiency: therefore, they play a crucial role in energy and nutrients flows, connecting different trophic levels and habitats. As a consequence, any factor negatively affecting amphibian populations may in turn have a great impact on entire ecosystems, with local extinctions having large-scale and ecosystem-level effects (Hopkins 2007; Whiles *et al.* 2006).

For all these reasons, they have been widely used as model organisms in ecological studies, and concern about their global decline is also due to their value as "sentinels" of environmental perturbations. Scientists believe that understanding amphibian declines may shed light on the causes and mechanisms involved in the decline of other animal species (Collins & Storfer 2003). Furthermore, given the complexity of the underlying dynamics, research in this field may foster the development of models which implicitly include context dependency, a property that seems to be prevalent in ecological systems, promoting advances in general ecological theory and conservation biology (Blaustein & Kiesecker 2002).

Beside their primary role in ecology and conservation, the peculiarities of their biology make amphibians perfect candidates for genetics and evolutionary biology studies, too. Because of their patchy distribution, low mobility and high sensitivity to environmental change, they are indeed particularly suitable for investigating micro- and macro-evolutionary processes at the population and species level. Moreover, many amphibians (and particularly pond breeding anurans) are often philopatric and gregarious during the breeding seasons, making them easy to sample (at adult or larval stage) with a relatively minimal effort. As a result, they have become popular subjects in population genetics, molecular ecology (i.e. the application of molecular tools for addressing fundamental ecological questions; see McCartney-Melstad & Shaffer 2015, for a review on amphibians) and phylogeography (i.e. the study of the principles and processes governing the geographic distribution of genetic lineages within and among related species; Avise 2000). For example, amphibian species are commonly chosen in studies aimed at: (1) investigating the genetic effects of current population dynamics (e.g. population structure, isolation due to habitat fragmentation, reduction of population size, local adaptation, etc.; see Beebee 2005, for a review) and (2)

understanding the consequences of past evolutionary processes (e.g. range reductions/expansions due to climatic oscillations; see Zeisset & Beebee 2008, for a review). Ecological and evolutionary scientists, thus, have had a long-standing interest in amphibians, and this group of animals contributed largely to the body of knowledge of these scientific fields. Academic researchers and environmental managers both benefit from collaborations which consist in the application of state-of-the-art scientific methods and theory to important conservation questions, specifically focusing on amphibians or using amphibians as models for drawing more general conclusions (Blaustein & Kiesecker 2002; McCartney-Melstad & Shaffer 2015).

1.1.3 The three levels of biodiversity: genes, species and ecosystems

As already introduced, according to the definition included in the text of the Convention of Biological Diversity (see section 1.1.1 for the complete citation), biodiversity encompasses three fundamental levels, or key components: (1) genetic diversity; (2) species diversity; (3) ecosystem diversity. This definition has become the "increasingly conventional" definition of biodiversity (Gaston, 1996). However, scientists are still debating on the validity of this perhaps too simplistic definition. For example, recently a fourth aspect of biodiversity has been highlighted: functional diversity, namely the extent of functional differences among the species in a community (Tilman 2001). Functional diversity has been recognized as a crucial determinant of ecosystem functioning (Loreau 2000), however, since it is difficult to develop simple standardized measures (but see Petchey & Gaston 2002), this component of biodiversity has received minor attention so far in conservation practice. Because of its limited operational use, functional diversity will not be discussed hereafter, although its incorporation in future conservation strategies is strongly advocated. Following, for each of the three conventionally recognized elements of biodiversity a brief description is provided.

Genetic diversity

Genetic diversity represents the most fundamental level of biodiversity and refers to the variation at the level of genes. Genes are sequences of nucleotides in a particular region (locus) of DNA molecules which contain the information for protein production. Therefore, genetic diversity ensure variation in functional, biochemical, morphological or behavioural traits, which in turn is responsible for differences in reproductive rate, survival or behaviour of individuals (Frankham *et al.* 2004). For these reasons, it is also clear that genetic diversity has a crucial evolutionary importance. Indeed, it provides the raw material for evolution by

natural selection (Fisher, 1930), being therefore required for populations and species to adapt to a changing environment (Booy *et al.* 2000; Reusch *et al.* 2005; Höglund 2009). In other words, the genetic variation in a population can be viewed as the basis for its "evolutionary potential" (Conner & Hartl 2004). Moreover, a loss in genetic diversity is often associated with inbreeding depression and reduction in fitness, with detrimental consequences for the persistence of viable populations (Frankham *et al.*, 2002; Hansson & Westerberg 2002; Reed & Frankham 2003). Genetic diversity can be divided into two different hierarchical levels: (1) genetic variability among individuals within local populations; (2) genetic differentiation among populations within the same species (Allendorf *et al.* 2007).

Species diversity

Species diversity is perhaps the most commonly known level of biodiversity. Species are distinct units of biological organization and they are generally easy to identify and count in the field, without the need of expensive laboratories techniques (such as for genetic diversity). For this reason, biodiversity is most commonly described in terms of species diversity (Gaston & Spicer 2004; Baillie *et al.* 2004) and the terms are often improperly used as synonyms. As an example, ongoing biodiversity decline is commonly identified merely in terms of species loss (extinctions).

Two basic complementary measures of species diversity are: (1) species richness, which is the total number of different species in a community and (2) species evenness, which takes into account the abundance in individuals per species (Hill 1973). However, it must be specified that both species richness and evenness are local measures of diversity (alpha diversity), and general ecological theory identifies other two dimensions of diversity: beta diversity and gamma diversity. Beta diversity describes the variation in species composition between sites (a measure of species turnover), while gamma diversity is the total species diversity of a landscape (a measure of regional diversity) (Whittaker 1960, Tuomisto 2010; Jost *et al.* 2011).

Closely linked to species richness is the concept of "biodiversity hotspots". Biodiversity hotspots are by definitions regions with unusually high concentrations of endemic species that also have suffered severe habitat destruction. More specifically, this approach takes the number of endemic plant species as a proxy for biodiversity and the percentage of primary vegetation that has been destroyed as an index of the level of threat (with a threshold of 70 percent for qualifying a region as a hotspot). Examples of biodiversity hotspots are tropical rainforests, but also oceanic islands and Mediterranean ecosystems, all of them showing

exceptionally high rates of plant endemism (Myers 1988; Myers *et al.* 2000). The biodiversity hotspots concept was initially intended largely as a rapid measure to face the impelling priority of large-scale extinctions and did not neglect the importance of implementing also other conservation strategies focused on different aspects of biodiversity (Myers *et al.* 2000). However, due to the simplicity of its idea and to the provision of measurable criteria, this approach has become the dominant conceptual framework for conservation strategies and the major determinant of global conservation funding. Nevertheless, many researchers now believe that directing conservation efforts exclusively on hotspot-based strategies is a controversial and dangerous oversimplification. In particular, Kareiva & Marvier (2003) stressed the need to consider other important aspects of biodiversity and not to neglect the importance of vast species poor ecosystems, such as temperate ecosystems, tundra and glacial environments, which encompass the last major wilderness landscapes, providing habitat for wide-ranging animal species, and playing a crucial role for global and local ecosystem processes (the so-called "biodiversity coldspots").

Ecosystem diversity

Ecosystem diversity encompasses the variety of ecosystems at the level of biological communities, their particular habitats and the physical condition under which they live (Wilson 1992). Ecosystem diversity is perhaps the more complex dimension of biodiversity: the above mentioned “sub-levels” are more theoretical conceptualizations rather than directly measurable elements. For example, different habitat types are generally defined arbitrarily, setting boundaries that does not correspond to the continuous nature of ecological systems. Moreover, the concept of habitat itself doesn't have a unique definition and it is species and scale dependent (Mitchell 2005). In addition, ecosystem diversity includes also abiotic components that, together with biological systems, give rise to complex ecological processes (Sodhi & Ehrlich 2010). A comprehensive measure of ecosystem diversity covering all its complexity is therefore a utopian goal, and often, for its assessment, scientists need to use some type of proxy (see Magurran 1988; e.g. Kerr *et al.* 2001; Rocchini & Neteler 2012).

1.1.4 New approaches to the conservation of biodiversity: conservation genetics and its implementation in conservation policy

The evolutionary consequences of a loss of genetic diversity have already been discussed. The growing awareness of this problem in the scientific community led to the origin of a

new interdisciplinary discipline, conservation genetics, consisting in the study of genetic diversity in natural populations and its related evolutionary processes. More generally, conservation genetics could be defined as the application of genetic theory and methods to the conservation and restoration of biodiversity (Frankham *et al.* 2004; Allendorf *et al.* 2007). The origin of conservation genetics as a research field could possibly date back to the 80's (Frankel & Soulé 1981). However, it started to reach a full development only from year 2000, when the journal *Conservation Genetics* was first published (Frankham *et al.* 2002). Researchers involved in conservation genetics come from a variety of fields, including population genetics, molecular ecology, evolutionary biology, systematics, etc., but the common underlying approach relies in the use of standard (e.g. Polymerase Chain Reaction, Sanger sequencing, microsatellite genotyping) or newly developed (e.g. Next Generation Sequencing technologies) molecular biology techniques for the study of genetic polymorphism in individuals and populations. With modern laboratory protocols, DNA can now be obtained even from a few milligrams of biological samples, leading to the possibility of implementing semi- or non-invasive sampling techniques (e.g. the collection of hair, faeces, feathers, buccal swabs, etc.), which are especially useful for rare or endangered species.

Conservation genetics is now a multifaceted scientific field and its major aims, reflecting different evolutionary processes and genetic issues, may be synthesized in the following points (Frankham *et al.* 2003; Frankham 2003; DeSalle & Amato 2004):

(1) studying the deleterious effects of inbreeding on the fitness of populations (inbreeding depression);
(2) understanding the consequences of a loss of genetic diversity for evolutionary potential and adaptability;
(3) resolving population structure, understanding population connectivity and assessing the effects of fragmentation and reduced gene flow;
(4) investigating the processes of natural selection and local adaptation;
(5) resolving taxonomic uncertainties;
(6) identifying "Evolutionary Significant Units" (ESUs), namely evolutionary distinct groups of populations within a species (Moritz 1994; Avise 1995), which should correspond to different management units (MUs);
(7) identifying populations of major concern and defining prioritization strategies;

(8) using molecular genetic analyses to understand aspects of species biology, ecology and ethology which are important for conservation and management;
(9) detecting hybridization processes (genetic pollution) and studying the deleterious effects on fitness that may occur as a result of outcrossing (outbreeding depression);
(10) managing reintroductions and captive populations;
(11) detecting and defining invasive species;
(12) using molecular genetic analyses in forensics (e.g. for contrasting poaching and illegal trade of animals);
(13) genetically identifying species and monitoring the species richness of communities (e.g. via DNA barcoding and metabarcoding).

Due to the need to assess the influence of habitat modifications, fragmentation and landscape heterogeneity on gene flow and genetic diversity, a new separate subfield of conservation genetics has recently emerged: "landscape genetics". Landscape genetics was formally recognized as a specific research area by Manel *et al.* (2003) in their seminal paper, where it was defined as an "amalgamation of landscape ecology and population genetics". Later, Storfer *et al.* (2007) more precisely defined landscape genetics "research that explicitly quantifies the effects of landscape composition, configuration and matrix quality on gene flow and spatial genetic variation". Landscape genetics analyses consist in combining approaches and statistical methods of different disciplines (e.g. landscape ecology, population and evolutionary genetics, GIS and spatial analysis, etc.) in order to integrate population genetic data, adaptive or neutral, with data of landscape structure (Holderegger & Wagner 2008). Therefore, the basic steps of landscape genetics are the detection of spatial genetic patterns (at the individual or population level), and the analysis of correlation among these recorded genetic patterns with landscape and ecological features, with the aim of studying contemporary microevolutionary processes generally at local or regional scale (Holderegger & Wagner 2008; Anderson *et al.* 2010; Wang e*t al.* 2010). The number of published landscape genetics studies is growing quickly and this young discipline is a rapidly evolving, both from a methodological and a theoretical point of view (see Balkenhol *et al.* 2016 for a review on methods and applications).

The importance of preserving genetic diversity and natural genetic processes for the management and conservation of natural populations is recognized not only scientifically but also politically (Laikre *et al.* 2009a; Hoban *et al.* 2013). For example, in the new strategic plan of the Convention on Biological Diversity (the already mentioned "Aichi Biodiversity

Targets"; see Section 1.1.1), Target 13 explicitly addresses the conservation of genetic diversity (SCBD 2010). Nevertheless, despite the powerful methodological and theoretical means of conservation genetics and landscape genetics, the distance between theoretical research and applied conservation is still considerable. Indeed, the implementation of genetic monitoring as well as direct actions to protect genetic diversity are still largely lacking (Laikre *et al*. 2009b). Moreover, genetic diversity is rarely considered in the identification of hotspots for conservation prioritization (Vandergast *et al.* 2008) and its evaluation is not directly implemented in IUCN conservation rankings (Willoughby e*t al.* 2015). Consequently, a much closer partnership between (a) conservation geneticists and (b) conservation managers and policy makers is urgently needed, with the aim of a direct incorporation of genetic data and analysis in conservation decision-making (Hoban *et al.* 2013).

1.1.5 New approaches to the conservation of biodiversity: a systemic perspective considering the three levels of biological diversity and their local interactions

It is now well established that the three main levels in which biological organization is commonly divided are indeed intimately linked and ecological processes results from the complex interactions between them (Gaston & Spicer 2004). The above presented subdivision must be therefore viewed as a mere operational classification: from a practical point of view, each level requires different monitoring techniques, different analytical methods and different approaches to management. For these reasons, the different aspects of biological diversity have so far been confined to separate lines of research, corresponding to the different fields of evolutionary biology and ecology. Nonetheless, this historical division led to severe limitations. We still have a very poor knowledge about the fundamental relationships among the three levels of biological diversity and we are far from the formulation of general theoretical framework which could help us in a better understanding of current crisis. Therefore, modern conservation biology is now increasingly recognizing the importance of investigating the intrinsic connections among the different components of biological diversity within a systemic perspective (Magurran 2005).

In this context, of particular interest are a growing number of studies addressing how genetic (within-species) and species diversity can be correlated in space (the so-called species-gene diversity correlation, SGDC), and how ecological factors can affect these two organizational levels. In 2005, Vellend proposed a first tentative theoretical model in order to explain this relationship, inspired by a review on previous empirical study suggesting a

general positive correlation (Vellend, 2003). In this seminal theoretical work, based on the universally accepted theory of island biogeography (MacArthur & Wilson 1967) and on the island model of population genetics (Wright 1931), the author hypothesized that species diversity and genetic diversity should be positively correlated in space "as the result of processes that influence the two levels of diversity in parallel ways". According to this hypothesis, a parallel action of evolutionary processes such as drift, migration, and spatially varying selection may have the same effects on species diversity and genetic diversity, giving rise to a positive correlation (Vellend 2005; Vellen & Geber 2005).

The relevance of this potential correlation was immediately clear. Indeed, genetic diversity, despite its recognized evolutionary importance, is often neglected in conservation strategies since it is difficult and costly to measure at large scale (CIT). On the other side, species richness is the most widely applied measure in biodiversity assessment and conservation. A positive correlation among these two levels of biodiversity could imply the use of species richness as surrogate of species diversity. Indeed, under a positive SGDC, a conservation prioritization based on species diversity should ensure the preservation of genetic diversity, too. However, caution is needed. Indeed, the few empirical studies available are still contradictory, showing variegate patterns including examples of negative correlation (e.g. Taberlet *et al.* 2012). Moreover, recent theoretical models including previously overlooked parameters such as mutation and speciation, are now showing a more complex and heterogeneous scenario. The predicted SGDCs assume different values, from positive to negative, depending on the strength of different processes such as mutation, competition or non-neutral evolutionary forces (Laroche *et al.* 2015). Therefore, the generality of a positive correlation between species richness and genetic diversity remains controversial and the extent to which one level of biodiversity can act as a surrogate of the other remains uncertain. In conservation practice, the three main levels of biodiversity should be therefore adequately considered, and/or their relationships should be evaluated in order to avoid the negative consequences of implementing wrong assumptions.

Finally, a further criticism to traditional biodiversity assessment and conservation prioritization came from the consideration that they generally focus on global biodiversity, while a major imminent threat is the loss of local biodiversity. Indeed, ecosystems functioning is profoundly local, depending on local populations of plants and animals and their local interactions. Therefore, while much concern focuses on the global extinction of species, the effects of regional and local extinction should not be underestimated and conservation efforts should also be directed in order to prevent the local loss of biodiversity

(Stork 2009). In addition, while most species are still globally distributed, their regional contribution to ecosystems has often been reduced because of widespread population-level extinctions, threatening "ecosystem services" taking place at a local level and being the prelude to species-level extinction (Ceballos *et al.* 2009).

To conclude, from the vastness of theoretical and empirical studies focusing on biodiversity and its ongoing decline, a common pattern strongly emerges: the complexity of biological and ecological systems. Biodiversity may still be a valuable concept for bridging the gap between scientists and non-experts, but could not be oversimplified and merely reduced to the number of species in a region. Conservation strategies need to be reconsidered, fostering interdisciplinary approaches aiming at the long-term conservation of species, ecosystems and evolutionary processes. Moreover, an enhanced dialogue among scientists, conservationists, local administrations and other stakeholders should be promoted.

1.2 Amphibians biodiversity conservation within a systemic approach: a local perspective

1.2.1 Study area

Our study area is Trentino (Autonomous Province of Trento, Italy), a mountainous region of 6212 km^2 located central-eastern Italian Alps, including part of the Dolomites and Prealps. Elevation ranges from 65 to 3764 m above sea level; more than 70% of the region lies above 1000 m a.s.l and about 20 % above 2000 m. The region is characterized by a complex terrain, with numerous valleys, among which the Adige river valley (130–270 a.s.l.) represents the major discontinuity, dividing the area into western and eastern halves, with a north-south orientation. The geology of the region is variegated, including sedimentary rocks ranging from the middle of the region to the Venetian Prealps in the south-eastern part, a vast porphyric area (Athesian porphyric platform) flanking the Adige river valley, a crystalline complex in the west (Adamello-Presanella) and dolomitic rocks mainly located in the north-eastern part of the region. Due to the complex relief, the climate is also heterogeneous, varying from the typical Alpine climate in the most elevated areas, to the sub-continental moderate climate of the minor valleys, and the sub-Mediterranean conditions approaching the southernmost part of the region, characterized by the mitigating effect of Lake Garda.
The landscape is characterized by forests, covering more than 56% of the area, mainly composed of spruce (*Picea abies*; 59.2%), followed by European larch (*Larix decidua*; about 17.3%), other conifers, and broad-leaved trees (only 5.4%). The rest of the region includes

grassland and pastures, high-elevation heaths (overall 30%), with agricultural crops (mainly intensive vineyards and orchards) and urban areas limited to medium-low elevations.
The region is sparsely populated (population density: 77 people /km²; below the average for Italy); about 50 % of the population is concentrated in the valley floors, below 250 m a.s.l. (APPA 2012).

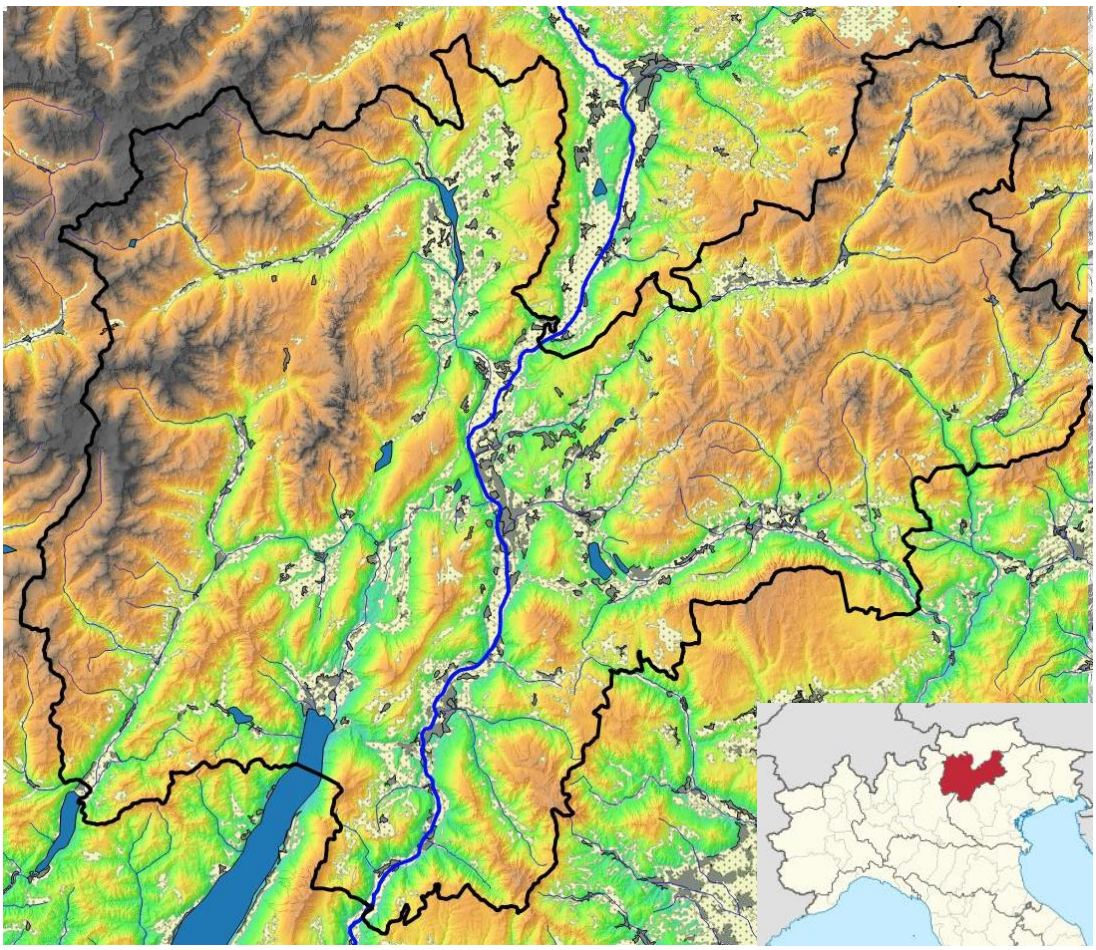


Fig. 1 Geographical map of the Trentino region (Autonomous Province of Trento). Yellow-spotted areas correspond to agricultural areas; grey areas to urban areas. The Adige river is depicted in blue in the middle of the region. The inset shows the location of the study area in the Italian peninsula.

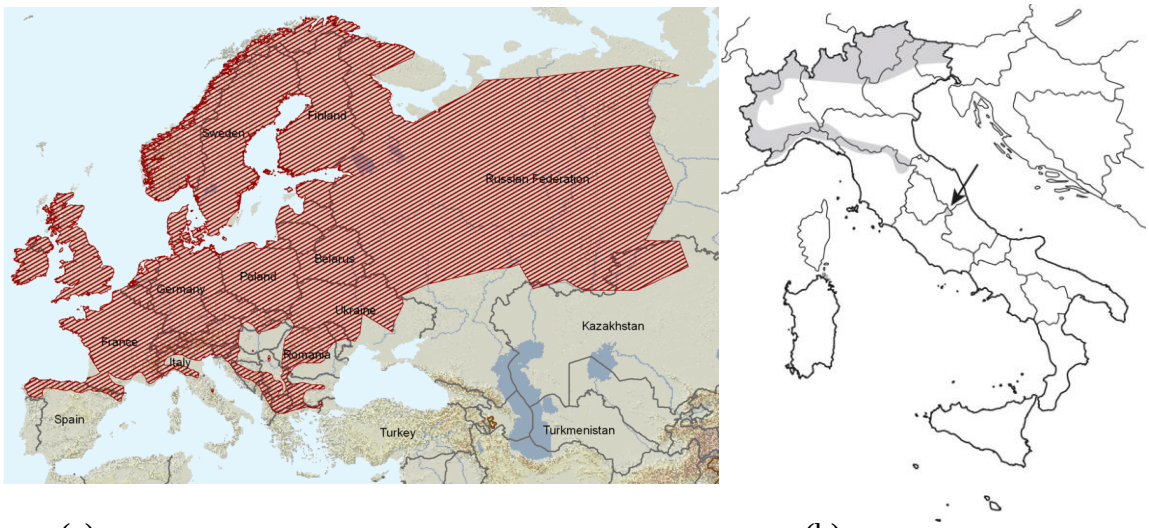
1.2.2 Focal species for the evaluation of diversity patterns at the genetic level: *Rana temporaria*

Our focal species for the assessment of genetic diversity patterns is the common frog (*Rana temporaria* Linnaeus, 1758). Here we provide a brief description of the species, together with a justification for its choice.

Geographical and altitudinal distribution

The common frog (*Rana temporaria* Linnaeus, 1758) is one of the most widespread amphibian in Europe, inhabiting a wide range of environments from northern Spain to the Scandinavian Peninsula in the north, and to the Urals, western Siberia and northwestern Kazakhstan in the east (Gasc 1997; see Fig. 2a).

In the Italian peninsula, the species is common in the Alps and the Ligurian Apennines, while being more scarce in the Tuscan-Emilian Apennines (Lanza *et al.* 2009). A relict population of the species survives in the Laga Mountains (NE Latium; see Fig. 2b). In Italy, its altitudinal range spans from about 20 m a.s.l. (Savona; Liguria) to at least 2760 m a.s.l. (Gran Paradiso National Park; Bernini & Razzetti 2006).



(a) (b)

Figure 2 a) Global (adapted from Kuzmin *et al.* 2008) and b) Italian (adapted from Lanza *et al.* 2009) distribution range of the common frog (*Rana temporaria).* The arrow indicates the relict population in the Laga Mountains (NE Latium)

Description and biology

The common frog, with a length of about 6-10 cm is the largest of the Italian "brown frogs" (genus *Rana*), and is characterized by a robust body with relatively short hind legs. Other distinctive features include the large dark temporal spot (Fig. 3a), a prominent tympanum and horizontal pupils. The colour is variable from olive-green to grey-brown, brown, grey, yellowish and reddish (Fig. 3b). Males can be distinguished from females due to hard excrescences, called nuptial pads, on the first finger. Moreover, they tend to turn greyish-blue during the mating season, particularly in the throat area.

(a)

(b)

Figure 3 *Rana temporaria*: a) close-up showing the characteristic large dark temporal spot; b) variation in colour pattern among different individuals

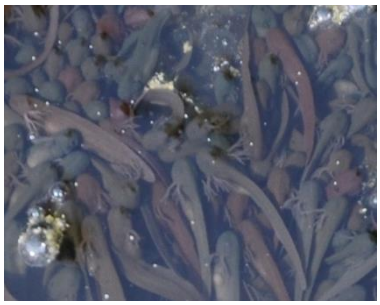
Outside of the breeding season, *Rana temporaria* is mainly a terrestrial frog, although in some sites individuals may remain by the water for most of the season. Across its distribution range, the species can be found in a great variety of habitats, but in Italy seems to prefer forested areas (particularly coniferous forests, but also mixed woods), alpine grasslands and pastures, bogs and mires, brooks, etc. (Lanza *et al.* 2009).

The species is an "explosive breeder": the breading season usually lasts only 1-2 weeks, generally from early spring to early summer, although it may vary widely according to latitude and altitude (exceptionally also in early autumn at low elevations). Males reach the breeding sites before the females, starting to call for attracting them. The call is of medium intensity, usually emitted at dusk and at night, but sometimes also in the daytime, often in water.

Eggs are laid in the shallow (5-50 cm) waters of small lakes, ponds, swamps, peat bogs, ditches, stream- and brook pools, temporary water bodies, etc. Each spawn consists of about 700-3000 eggs, combined in 1 or 2 globular clutches, often forming aggregates from a few up to several hundred of clutches (Fog *et al.* 1997; Fig. 4a).

Larvae hatch after 2-3 weeks and appears as 6-9 mm long, blackish-brown tadpoles with well-developed external gills (Fig. 4b). Metamorphosis is completed in 2-4 months, with a high variability according to different environments (Merilä *et al.* 2000).

(a)



(b)

Figure 4 *Rana temporaria*: a) typical aggregate consisting of several egg clutches; b) larvae at hatching

Common frogs hibernate through the winter, either in water (e.g. at the bottom of ponds) or on land (especially juveniles). Frogs usually reach sexual maturity at second or third year, but in high-altitude elevation even at 4-6 years (Miaud *et al.* 1999); mean life-span in the wild is about 5 years, although cases of longevity up to 15 years are documented (Lanza *et al.* 2009).

Among Italian anurans, *R. temporaria* is the most resistant to cold, sometimes being active at temperatures near or even below 0 °C. On the other side, the species is highly sensitive to high temperatures, particularly when associated with low humidity (Lanza *et al.* 2009).

Intraspecific variability

Across its wide distribution, the species shows high morphological variability, which has led to the description of numerous subspecies (Grossenbacher 1997; Veith *et al.* 2003; Lanza *et al.* 2009):

1. *R. t. parvipalmata* (Cantabrian Mountains, NE Spain);
2. *R. t. canigoensis* (high elevation areas of the Pyrenees);
3. *R. t. gasseri* (lowland areas adjacent to the Pyrenees);
4. *R. t. aragonensis* (Aragon; NE Spain);
5. *R. t. honnorati* (Basses-Alpes, SE France);
6. *R. t. temporaria* (occurring in most of the distribution range).

However, the different morphotypes do not always correspond to genetically divergent lineages. For example, phylogenetic analyses (Veith *et al.* 2002; Veith *et al.* 2012) showed that low-altitude Pyrenean populations (including "*R. t. gasseri*") and high-altitude Pyrenean frogs (including "*R. t. aragonensis*" and ‘"*R. t. canigonensis*") do not constitute different evolutionary lineages, and also the subspecific status of *R. t. honnorati* is questionable based on genetic data (Stefani *et al.* 2012). By contrast, *R. t. parvipalmata* is genetically well differentiated and is supposed to have diverged during the Pleistocene (approximately 1.1 Ma; Veith *et al.* 2003). Among the different supposed subspecies, only the nominal form *R. t. temporaria* is present in Italy (Stefani *et al.* 2012).

Status and conservation

Overall, *Rana temporaria* is widespread and abundant in Europe (IUCN 2016), as well as in the Italian Alps (Lanza *et al.* 2009), and there are no global threats to this species (IUCN 2016). Nevertheless, significant local declines have been reported in different areas.

Specifically, disease-driven mass mortalities have occurred in England since the 1980s (see Section 1.1.2). In Italy, at the southern limit of its range the species is more fragmented and there is a tendency towards local declines and extinctions, particularly in the Prealps and northern Apennines (Lanza *et al.* 2009). Local threats to the persistence of common frog populations include the destruction and alteration of breeding sites and surrounding terrestrial habitats, the introduction of fishes and exotic species in alpine lakes and ponds and road mortality occurring during migrations to and from the breeding sites. The situation is particularly critical for the relict population in the Laga Mountains, which is completely isolated and threatened by severe habitat alterations (Lanza *et al.* 2009).
The common frog is included in Appendix III of the «Berne Convention» and in Appendix V of the «Habitat Directive» 92/43/EEC. In the IUCN Red List (2016), the species is classified as LC (least concern).

Rana temporaria: a model organism in ecology, evolutionary biology and conservation

Being widespread and often abundant, *Rana temporaria* is an important component of many ecological communities (Luiselli *et al.* 1995; Lodé 1996). This anuran is characterized by high adaptability to different ecological conditions, showing local adaptations even at short geographic distance (Muir *et al.* 2014; Richter-Boix *et al.* 2010) and high phenotypic plasticity (Laurila *et al.* 2002; Johansson *et al.* 2013). Moreover, the species has the greatest genetic variability of all western Palearctic brown frogs (Veith *et al.* 2003). For all these reasons, *Rana temporaria* is a model organism for ecological (e.g. Loman 2004; Vos 2007; Decout *et al.* 2012), evolutionary (e.g. Miaud 1999; Laugen *et al.* 2003), phylogeography (e.g. Palo *et al.* 2004; Teacher *et al.* 2009; Stefani *et al.* 2012) and population genetics studies (e.g. Hitchings & Beebee 1997; Johansson *et al.* 2006).

Finally, a special mention must be made to the role of common species in conservation biology. Indeed, although conservation practice has focused almost entirely on rare species (e.g. those in the red lists of threatened species) so far, recent studies are emphasizing the pivotal importance of naturally common species -those that are abundant and widespread- to both terrestrial and marine ecosystems. Being often keystone species and dominant in terms of numbers or biomass, even relatively small and local declines in their populations can result in disruption of ecosystem structure and functioning, posing at risk the ecosystem services that they provide (Gaston & Fuller 2008; Gaston 2011). Thus, conservation biologists are now recognizing the abundance of a species as a "conservation value" (Gaston 2010; Redforf *et al.* 2013) and are stressing the importance of protecting not

only threatened species, but also common taxa, also considering that there are already several known examples of declines in once naturally common species (Ellison *et al.* 2005; Gaston & Fuller 2008; Jiguet *et al.* 2010).

The common frog is a key component of many ecological communities, particularly in low-productivity Alpine habitats, where both larval and adult stages play a crucial role in trophic and nutrient networks (Luiselli *et al.* 1995; Lodé 1996; Sztatecsny *et al.* 2013). Therefore, we stress the conservation relevance of monitoring the status and trend of common frog populations, since even local declines may have ecosystem-level impacts in fragile Alpine ecosystems. This consideration is particularly relevant in the face of ongoing climate change, which is predicted to heavily affect both the distribution of the species (e.g. Bartolini *et al.* 2014) and Alpine ecosystems in general (e.g. Bragazza 2008; Maiorano *et al.* 2013; Tafani *et al.* 2013).

1.2.3 Focal animal group for the evaluation of diversity patterns at the species level: alpine amphibians

Alpine amphibians are species-poor communities, and a total of 12 autochthonous species are known for the Trentino region (Caldonazzi *et al.* 2002). Among these, 7 species were recorded in the study sites: common frog (*Rana temporaria*; focal species for the study of genetic diversity), common toad (*Bufo bufo*), Alpine newt (*Ichthyosaura alpestris*), fire salamander (*Salamandra salamandra*), green frog (*Pelophylax* synkl. *esculentus*), yellow-bellied toad (*Bombina variegata*) and agile frog (*Rana dalmatina*). Here is a synthetic outline of the considered amphibian species (for *Rana temporaria*, see previous section).

Although numerous cases of decline are reported, all species are globally classified as LC (least concern) in the IUCN Red List (2016), therefore we report only their status in Habitat Directive (Appendix II = species that must be protected under the Natura 2000 Network, through the creation of Sites of Community Importance; Appendix IV = species of community interest, which should be strictly protected across their entire natural range within the EU; Appendix V= species whose exploitation and taking in the wild can be restricted by European law).

Information on local distribution and trend is retrieved from Caldonazzi *et. al* (2002).

Yellow-bellied toad (*Bombina variegata*)

Key features: small anuran with a compact body, between 3.5-5.5 cm in length; top side is grey-brown, under side is black-blue with yellow or orange spots

Reproduction: 1-4 matings usually from May to September; eggs are laid isolated or in small groups (up to 160 eggs); pristine breeding habitat consisting in temporary water bodies along brooks and rivers; adapted to anthropic habitats such as puddles, small ditches, drinking troughs, artificial basins, agricultural cisterns, etc.
Habitat in the study region: riparian wetlands, broad-leaved and mixed forests with ponds, pastures, agricultural areas and other anthropic habitats (e.g. pits)
Distribution in the study region: mainly localized in the central part of the region (Adige river valley), some adjacent valleys (Valle dei Laghi, Valle di Cembra) and Monte Baldo; altitudinal range: 180-1700 m a.s.l, but usually at low elevation (below 500 m)
Status in Habitat Directive: Appendix II, Appendix IV
Local population trend: rare and declining

Common toad (*Bufo bufo*)
Key features: large sized anuran with robust body, up to 15 cm in length
Reproduction: explosive breeder; breeds in spring-early summer; eggs are laid in double strings; spawning takes place in lakes, ponds, ditches, etc.
Habitat in the study region: generalist: wide variety of habitats, including urban areas
Distribution in the study region: widespread from 100 to 2000 m a.s.l, but more frequent below 1000 m
Status in Habitat Directive: /
Local population trend: common, generally stable, locally impacted by high road mortality

Agile frog (*Rana dalmatina*)
Key features: medium size anuran, smaller and thinner than the common frog; pointed snout; white underside, without spots; the hind legs are unusually long
Reproduction: explosive breeder; breeds in early spring; egg clutches are deposited on underwater vegetation, in permanent or temporary ponds
Habitat in the study region: mainly riparian wetlands and swamps, generally associated with broad-leaved or mixed forests, rarely bogs, pastures in the Monte Baldo massif
Distribution in the study region: mainly localized in the central part of the region (Adige valley and Valle dei Laghi), some adjacent valleys (e.g. Valle di Cembra) and Monte Baldo; altitudinal range: 200-1500 m a.s.l, but usually at medium-low elevation (below 1000 m)
Status in Habitat Directive: Appendix IV
Local population trend: not common; data deficient

Fire salamander (*Salamandra salamandra*)

Key features: medium-large sized urodele, generally 12-20 cm in length; black with vivid yellow or orange-yellow spots or stripes

Reproduction: eggs are fertilized internally; females deposit the larvae in slow-flowing sections of brooks and streams, generally in spring

Habitat in the study region: usually broad-leaved or mixed forests

Distribution in the study region: generally common, but rarer in the internal valleys and absent from the highest mountain massifs; altitudinal range: 100-1500 m a.s.l, but usually found at low elevation (below 1000)

Status in Habitat Directive: /

Local population trend: generally stable; a potential threat is represented by the anthropic alteration of stream beds and margins

***Alpine newt** (Ichthyosaura alpestris)*

Key features: medium sized urodele, up to 12-20 cm in length; top side is grey-brown, under side is orange with black spots on a white strip on the flank; during the mating season, males exhibit dark-blue color on their backs and a black and yellow crest along entire dorsum

Reproduction: breeding season in spring-summer; eggs (60-300) are laid singly or in very small groups (2-5), attached to aquatic vegetation, generally in ponds, alpine lakes or oligotrophic water bodies

Habitat in the study region: bogs, margin of alpine lakes, forest and shrubby areas in the proximity of water habitats

Distribution in the study region: irregular, almost completely lacking from the western mountain massifs (Adamello-Brenta); altitudinal range: 200-2400 m a.s.l, but more frequent above 1500 m a.s.l

Status in Habitat Directive: /

Local population trend: relatively common in the area of presence; generally stable, but local declines/extinctions are reported, due to introduction of fishes or destruction of the water habitat (particularly in low elevation areas; Omizzolo *et al.* 2002)

Green frog (also known as edible frog; *Pelophylax* synkl. *esculentus*)

Key features: medium size anuran (10-15 cm), prevalently aquatic, inhabiting various types of wetlands and water bodies (usually also used as breeding sites)

Reproduction: breeding season in spring-summer; each female lays 1000-4000 eggs, divided into a few roundish masses (6-7 cm in diameter), usually anchored to aquatic vegetation
Note: The species is indeed a hybridogenetic complex composed by the pool frog (*P. lessonae*), the marsh frog, (*P. ridibundus* or the balcan species *P. kurtmuelleri*) and their hybrid form *P.* kl. *esculentus*. *P. lessonae* and the hybrid form *P.* kl. *esculentus* are sympatric and very difficult to distinguish on a morphological basis (Lanza et al. 2009; Bovero et al. 2013). For the purpose of this study, *P. lessonae* and the hybrid *P.* kl. *esculentus* are considered as a single species, according to the classification used in Caldonazzi et al. (2002) and in the regional monitoring program (PAT)
Habitat in the study region: strictly linked to water habitats, mainly riparian wetlands, natural or artificial ponds, channels; frequent in agricultural areas
Distribution in the study region: mainly localized in the central part of the region (Adige river valley and Valle dei Laghi), a few low elevation valleys (e.g. Valsugana) and medium elevation hills (e.g. Altopiano del Calisio, Laghestel); altitudinal range: 100-1200 m a.s.l, but usually at low elevations
Status in Habitat Directive: Appendix IV, Appendix V
Local population trend: declining, many habitats are threatened by intensive agriculture and strong anthropization; locally threatened by the alloctonous amphibian *P. kurtmuelleri* in a few sites

1.3 Aims of the thesis

In this thesis, I combined different methods for the study of amphibian biodiversity in an alpine region (Trentino, Italy), with the aim of both shedding light on patterns of diversity at the different levels of biological organization, and gaining a better understanding of the underlying -past and present- evolutionary processes.

The key points of our approach are: (1) the use of molecular methods for assessing genetic diversity levels in a model species, the common frog (*Rana temporaria*) and investigating their main drivers; and (2) the integration of information on different levels of biodiversity in amphibian communities, in order to evaluate their relationships under a theoretical and applied perspective, with the ultimate goal of developing effective conservation strategies.

The research project can be divided into three separate studies, with the following major objectives:

Study 1. Phylogeography of the common frog (*Rana temporaria*) in the Trentino region: past evolutionary processes and their genetic legacy.

Objectives:

a) Investigating the phylogeographic history of the species in the study region, identifying potential Pleistocene glacial refugia and post-glacial recolonization routes (using mtDNA);
b) Identifying "historical hotspots" of genetic diversity and "Evolutionary Significant Units" (ESUs)

Study 2. Population and landscape genetics of the common frog (*Rana temporaria*) in the Trentino region: assessing current levels of intrapopulation genetic variability and present connectivity.

Objectives:

a) Assessing the current levels of genetic variability for the species in the study region (using microsatellite markers), differentiation and population structure;
b) Identifying "hotspots" and situations of criticality;
c) Investigating patterns of present connectivity

Study 3. Species-genetic diversity correlation: the case study of the common frog (*Rana temporaria*) and amphibian communities in an alpine region.

Objectives:

a) empirically testing the correlation between genetic and species diversity using alpine amphibian communities as model system, and the common frog (*Rana temporaria*) as focal species for genetic diversity assessment;
b) shedding light on the evolutionary and ecological processes underlying the observed patterns, considering the influence of environmental variables on both levels of biodiversity, as well as potential causal relationships (e.g. the effect of species diversity and community composition on genetic diversity, via interspecific competition);
c) outlining general indications for amphibian conservation planning.

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Chapter 2.

STUDY 1:

Phylogeography of the common frog (*Rana temporaria*) in the Trentino region: past evolutionary processes and their genetic legacy.

Phylogeography of the common frog (*Rana temporaria*) in the Trentino region: past evolutionary processes and their genetic legacy.

Alexis Marchesini, Andrea Battisti, Cristiano Vernesi

Abstract

The common frog (*Rana temporaria*) has been focus of several broad scale phylogeographic studies, revealing a deep split between eastern and western European populations, induced by the onset of the Pleistocene glaciations. Anyway, the identification of glacial refugia, as well as the understanding of recolonization processes and their genetic legacy remain far from complete. A recent survey on Italian populations revealed a previously unrecognized Pleistocene refugial area in the Italian peninsula and suggested the hypothesis of multiple separated microrefugia. However, fine-scale studies in these areas of great interest are still lacking. We examined the phylogeographic structure of 54 common frog populations (540 individuals) by means of COI mitochondrial gene, focusing on a south eastern alpine region (Trentino, Italy) with an intensive sampling design. Phylogenetic reconstruction indicated the presence of three different COI lineages, exhibiting different levels of genetic diversity, and a contact zone in the eastern part of the region. Our data supported the scenario of multiple sub-refugia, probably located in the southern slopes of the Alpine chain, where the species survived the ice ages in fragmented populations. Recolonization routes in the study region followed irregular paths, most likely due to the complicated orography, and led to complex phylogeographic patterns, generally unexpected at this low spatial scale. A correct understanding of the consequences of major past evolutionary processes on local populations is of great interest under an evolutionary and conservation perspective, particularly in the face of ongoing climate change. This study, focused on a widespread species, stands as a starting point for comparisons with other organisms and for testing more general hypotheses in biodiversity conservation.

Keywords: amphibians, *Rana temporaria*, phylogeography, Italian Alps, Trentino, postglacial colonization, mitochondrial DNA

Introduction

Phylogeography, the integration of phylogenetics and population genetics theory for analyzing the relationship between genetic structure and biogeography (Avise *et al.* 1987), since its origin 30 years ago, has rapidly become a powerful tool in the study of historical evolutionary processes and their legacy on animal and plant species (Avise 2000, 2009). Pleistocene climatic oscillations had a great impact on the distribution and demographic trends of plant and animal species. During the Ice Ages, ice sheets expanded restricting the distribution of many species to suitable areas south of the glaciated regions, the so-called "glacial refugia", while during the interglacials the species were able to recolonize previously glaciated areas. These repeated contraction-expansion had important genetic consequences, leading to (1) the genetic differentiation of populations isolated in separate glacial refugia, (2) the erosion of genetic diversity along the recolonization front, due to repeated founder events, and (3) the potential arising of contact zones, characterized by admixture of divergent lineages (Hewitt 2000, 2004; Petit *et al.* 2003). Mitochondrial DNA (mtDNA) is often a marker of choice in traditional phylogeographic studies. Indeed, due to its peculiar biological properties, (e.g. lack of recombination, putative neutrality, and smaller effective population size due to maternal inheritance) it is considered an appropriate marker for detecting the effects of past processes (Avise *et al.* 1987; Hickerson *et al.* 2010).

In general, amphibians are known to be poor dispersal and, particularly pond breeding anurans, often exhibit high philopatry to breeding sites (Beebee 1996). As a consequence, populations tend to be highly structured genetically and retain strong signals of past evolutionary processes. Consequently, they have become popular subjects in many phylogeographic studies (see Zeisset & Beebee 2008, for a review). The common frog is one of the most widespread and abundant amphibian species in Europe (Gasc 1997); moreover, the species has the greatest genetic variability of all western Palearctic brown frogs (Veith *et al.* 2003; Vences *et al.* 2013): it is therefore a perfect model organism for examining phylogeographical processes.

Large-scale phylogeographic studies (Palo *et al.* 2004; Teacher *et al.* 2009), based on cytochrome b gene (cyt b), identified two main lineages for *Rana temporaria* in the Palearctic region, with the eastern lineage mainly distributed in eastern Europe and Scandinavia (but documented also for the northern Alpine border, including an Italian population), and the western lineage widespread in France, Germany, Iberian Peninsula, and the British Isles). Teacher et al. (2009) indicated the Iberian Peninsula as main refugium for

the western lineage, with a potential secondary refugium located in Ireland, while the eastern lineage (with low genetic diversity) was supposed to originate from a single refugium situated in Italy or the Balkans. Nevertheless, Stefani *et al*. (2012), based on a genetic survey covering the whole Italian distribution of the species, proposed an alternative phylogeographic scenario. Specifically, these authors detected only the western cyt b lineages in Italy. In addition, using cytochrome oxidase I (COI) gene, which provides a better resolution, they found high genetic diversity in the Italian populations, with 5 different COI lineages: 4 located in the Alps and the remaining one in the Apennines. Therefore, the authors strongly proposed the Italian Peninsula as important refugium for the western lineage. Moreover, the recorded patterns of diversity and differentiation of Italian populations were interpreted as evidence for a "refugia-within-refugia" model, stating that the species survived the glacial ages in different isolated peripheral refugia on the southern slopes of the Alps (Stefani *et al.* 2012). Therefore, the phylogeographic history of the species appears to be more complicated than previously assumed, and important hints for a better understanding of the recolonization processes might come from the investigation of local patterns of genetic diversity, as stressed by different authors (Teacher *et al.* 2009; Stefani *et al.* 2012).

With this study, we provided a fine-scale reconstruction of the phylogeographic history of the common frog in an alpine region, by means of COI mitochondrial gene and an intensive sampling design. We focused on the Trentino region (Italy), a mountainous area characterized by complex orography and biogeography, and potentially located in the proximity of putative refugia for the species.

Specifically, we addressed the following question: (1) do our data support the hypothesis of a "refugia-within-refugia" model for the species, with different sub-refugia located in the Southern Alps? (2) if so, do the lineages originated in different sub-refugia harbor different levels of genetic diversity at mtDNA? and (3) what are the routes and modes of postglacial recolonization in the study region?

Understanding how Pleistocene refugia and recolonization processes affected biodiversity is of crucial importance in the face of ongoing climate change, in order to develop effective long-term conservation strategies (Sgro *et al.* 2011; Morelli *et al.* 2016).

Materials and Methods

Ethics Statement

All conducted experiments complied with the current laws of Italy. Sampling and monitoring procedures were approved by the Italian Ministry of Environment and the Environmental Unit of the Autonomous Province of Trento (DPN/2D/2003/2267 and 4940- 57/B-09-U265-LS-fd). Samples from Veneto were collected thanks to a collaboration with University of Padova (Dept. of Biology).

Sampling

Our study area is Trentino (Autonomous Province of Trento, Italy), a mountainous region of 6212 km^2 belonging to the eastern Italian Alps. The region is characterized by a complex terrain (elevation range: 65-3764 m above sea level; more than 70% of it lies above 1000 m a.s.l), including part of the Dolomites and Prealps as well as low elevation valleys. The Adige river valley (130–270 a.s.l.) represents the major discontinuity, dividing the area into western and eastern halves, with a north-south orientation.

We selected sampling areas in order to cover the whole geographic and altitudinal distribution of the species in the study region, as well as different ecological environments. In years 2009-2012, the selected areas were screened for common frog spawn during the breeding season. We collected eggs and larvae: one fertilized egg from each distinct clutch, and tadpoles coming from separate ponds, therefore minimizing the probability of gathering full-sibs. This sampling procedure has been widely used in earlier studies with the common frog and other pond breeding amphibians (e.g. Rowe *et al.* 1998; Brede & Beebee 2004, 2006; Stefani *et al.* 2012; Van Buskirk 2012). Tadpoles were stored in 95% ethanol until DNA extraction, while eggs were brought to the laboratory, were allowed to hatch and larvae were harvested at Gosner stage 23 (active swimming, Gosner 1960), following indications in previous studies (e.g. Brede & Beebee 2004; Stevens *et al.* 2006; Johansson *et al.* 2013). GPS coordinates of each sample were recorded, and samples coming from different ponds within the same 1 km^2 area were considered belonging to the same sampling site (Johansson 2005, 2007). Three additional areas (LPo, MP2, Pos), located outside of the political borders of the Autonomous Province of Trento were included in the study, because of their particular geographic position (at the southern margin of *Rana temporaria* distribution range in the considered part of the Alps). Specimens from these additional sites were collected during field surveys focused on other amphibian species.

A total of 1522 specimens of *R. temporaria* were collected from 90 different sites. For the purpose of this study, a subset of 54 sites were chosen and 10 samples for each site have been used in the following analysis (Fig. 1 and Table S1 in Supporting Information).

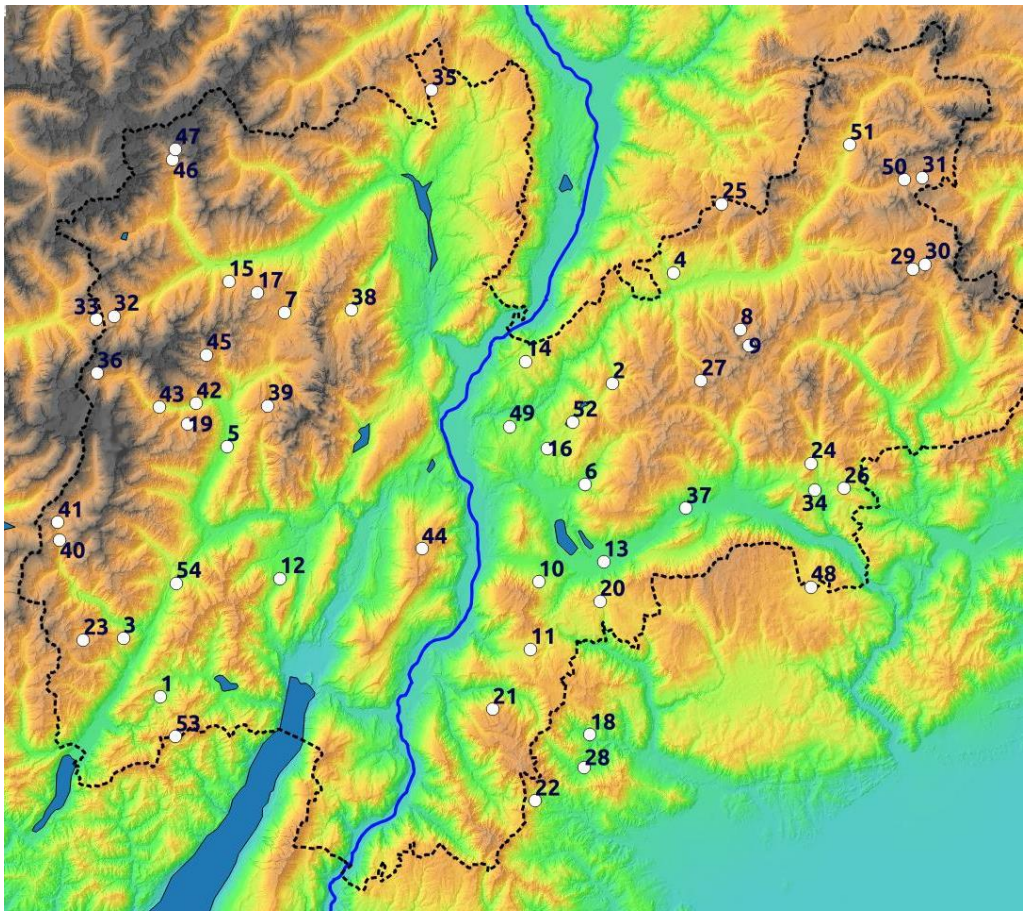

Figure 1 Sampling sites of *Rana temporaria* in the Trentino region. Labels, site names and coordinates are listed in Table S1 (Supporting Information). The blue line in the middle of the region represents the Adige river.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted using the Qiagen Dneasy 96 Well Plate Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's protocol.

For all the 540 selected samples, a fragment of 569 base pairs (bp) of mtDNA cytochrome oxidase subunit I (Cox I) region was amplified via Polymerase Chain Reaction (PCR), using the universal primer LCO1490 (Folmer *et al.* 1994) and the specific primer COItemp (Stefani *et al.* 2012). The PCR amplification was carried out in a 20 µl reaction mix containing: 1 µl template DNA, HotMaster TM Taq Reaction Buffer, 20 mM dNTPs, 5 mM of each primer and 1 unit of HotMaster TM Taq. The thermocycling regime consisted of incubation at 94 °C for 2 min, followed by 35 cycles of 94°C for 15 s, 50 °C for 1 min, and

72 °C for 1 min, with a final extension of 72 °C for 5 min. For all DNA extractions and PCR amplifications, contamination was rigorously checked by means of blank samples and PCR-negative controls. Before sequencing, the excess primers and dNTPs were removed using ExoSAP-IT (USB Corporation, Cleveland, OH). DNA sequencing was performed following the ABI Prism Big-Dye Terminator Kit v.1.1 (Applied Biosystems) standard protocol and the sequencing reaction products were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The resulting sequences were edited using Finch TV 1.4.0 (Geospiza, http://www.geospiza.com/Products/finchtv.shtml), visually checked and aligned using BioEdit 7.2.5 (Hall 1999).

Genetic and phylogeographic analysis

Sequences obtained were collapsed into haplotypes by using DnaSP v5 (Librado & Rozas 2009). DnaSP v5 was also used to calculate total number of polymorphic and parsimony-informative sites, and standard genetic diversity measures for each population (number of different haplotypes, n; haplotype diversity, h; nucleotide diversity, π; mean number of pairwise nucleotide differences, k; number of polymorphic sites, s). In order to investigate geographic patterns of intrapopulation genetic diversity, we tested the correlation between latitude, longitude and standard measures of genetic diversity using Pearson coefficient in R statistical environment (R Core Team 2016).

We performed correlation tests for the whole datasets, and for two separate subsets including only populations of the western and eastern part of the region, respectively.

Phylogenetic networks were generated using the statistical parsimony procedure (Templeton *et al.* 1992), implemented in the software TCS 1.21 (Clement *et al.* 2000), using the 95% limit for a parsimonious connection. First, we constructed a COI haplotype network, combining our sequences with all public available haplotypes found for the *Rana temporaria* in the Italian peninsula, (Stefani *et al.* 2012; EMBL codes FN813783-FN813810), in order to infer phylogenetic relationships among haplotypes. Then we built a second network considering only our sequencing, for a graphical representation of haplotype frequencies in the study region.

Pairwise PhiST values for all the populations were calculated using ARLEQUIN 3.5 (Excoffier & Lischer 2010); their significance was tested with 10,000 permutations and associated P values were adjusted for multiple comparisons using false discovery rate method (FDR; Benjamini & Hochberg 1995), as implemented in "p.adjust" R function (R Development Core Team 2006).

Population genetic structure was assessed by performing a spatial analysis of molecular variance using the program SAMOVA 2.0 (Dupanloup *et al.* 2002). SAMOVA uses a simulated annealing procedure to define groups of geographically adjacent populations, by maximizing the amount of variance among groups (FCT) and evaluating their significance by means of conventional F statistics. This approach, in contrast to conventional AMOVA does not require that the groups are defined *a priori*, allowing instead the best-fit grouping to emerge from the data. We run 100 number of independent simulated annealing processes using 10000 number of steps, for K (numbers of hypothetical groups) from 2 to 10. Afterwards, an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was carried out with the software ARLEQUIN 3.5 (Excoffier & Lischer 2010), using the best-fit grouping pattern suggested by SAMOVA and Tamura and Nei model of sequence evolution. The statistical significance of the variance components was computed by 10000 permutations.

Finally, the location of major genetic discontinuities was also assessed using the software BARRIER 2.2 (Manni *et al.* 2004). This analysis was based on the geographical coordinates for each site and the matrix of pairwise PhiST values. This approach starts with the creation a Delaunay triangulation network connecting adjacent populations, upon which a Voronoï tessellation is superimposed. Genetic barriers are then identified using Monmonier's maximum difference algorithm, by determining which of the borders between adjacent populations exhibits the highest genetic differentiation. As a result, genetic breaks are detected in areas characterized by high divergence despite geographic proximity. With BARRIER, the number of genetic barriers to be computed is determined a priori by the user. If iterated, the procedure results in the generation of a series of barriers from higher to lower ''rank". We continued adding barriers until the last one starting from a statistically significant PhiST value was included (Manni *et al.* 2004).

Results

We found a total of 12 COI gene haplotypes (569 bp long), that differed at 19 polymorphic sites (19 parsimony-informative sites). Six of these were previously unreported (Table 1). Phylogenetic reconstruction including haplotypes available from public repositories (Figure S2 in Supporting Information) led to the assignment of the detected haplotypes to three of the four COI lineages (= haplogroups) known for the Alps (Stefani *et al.* 2012).
Specifically, 9 haplotypes were found for Alpine lineage I (hereafter Alp1), 2 haplotypes for Alpine lineage IV (hereafter Alp4) and 1 haplotype for Alpine lineage II (hereafter Alp2). A phylogenetic network for the haplotypes found in the study region, with node size proportional their frequencies, is reported in Fig. 2.

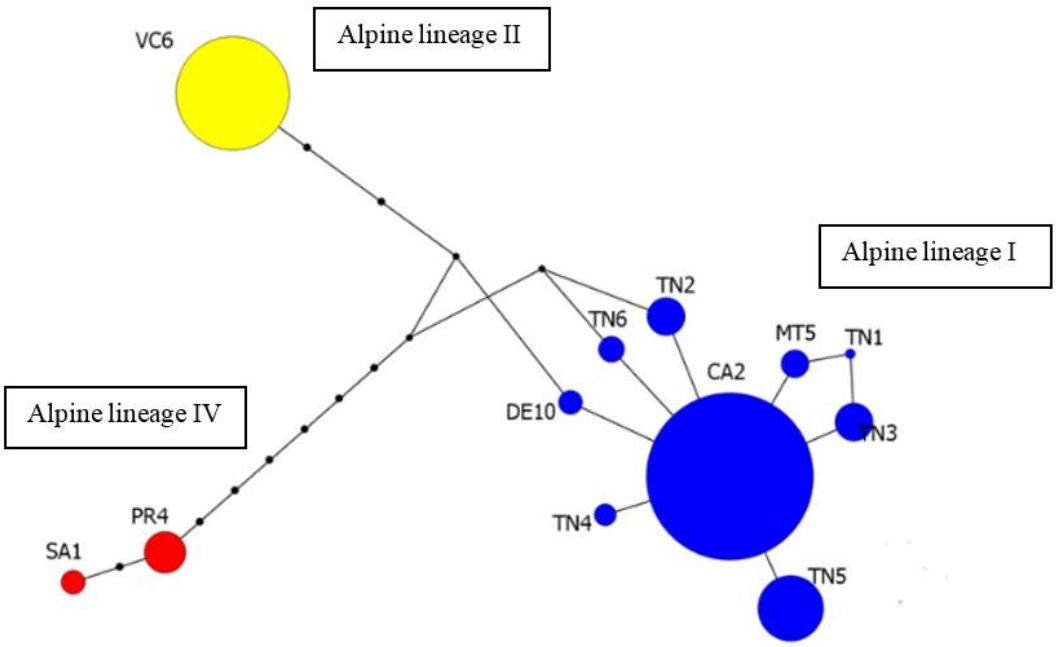

Figure 2 Phylogenetic network of the 12 COI haplotypes found among *Rana temporaria* populations in the Trentino region, based on the statistical parsimony procedure implemented in TCS. Circle sizes are proportional to haplotype frequency; missing intermediate haplotypes are shown as open dots. The different colors identify different COI lineages.

Lineage Alp1 was ubiquitous in the study region, while lineages Alp2 and Alp4 were detected only in the eastern part of the region. In particular, Alp2 seem to prevail in south-eastern populations (Venetian Prealps), while lineage 4 is present only in 10 sites, located in the north-eastern corner of the region, always in admixture with other lineages. Overall, complex spatial patterns of admixture among the three lineages were detected in the eastern part of the region (hereafter East Trentino), while the western part (hereafter West Trentino), is characterized by the presence of a single COI lineage, Alp1 (Fig. 3).

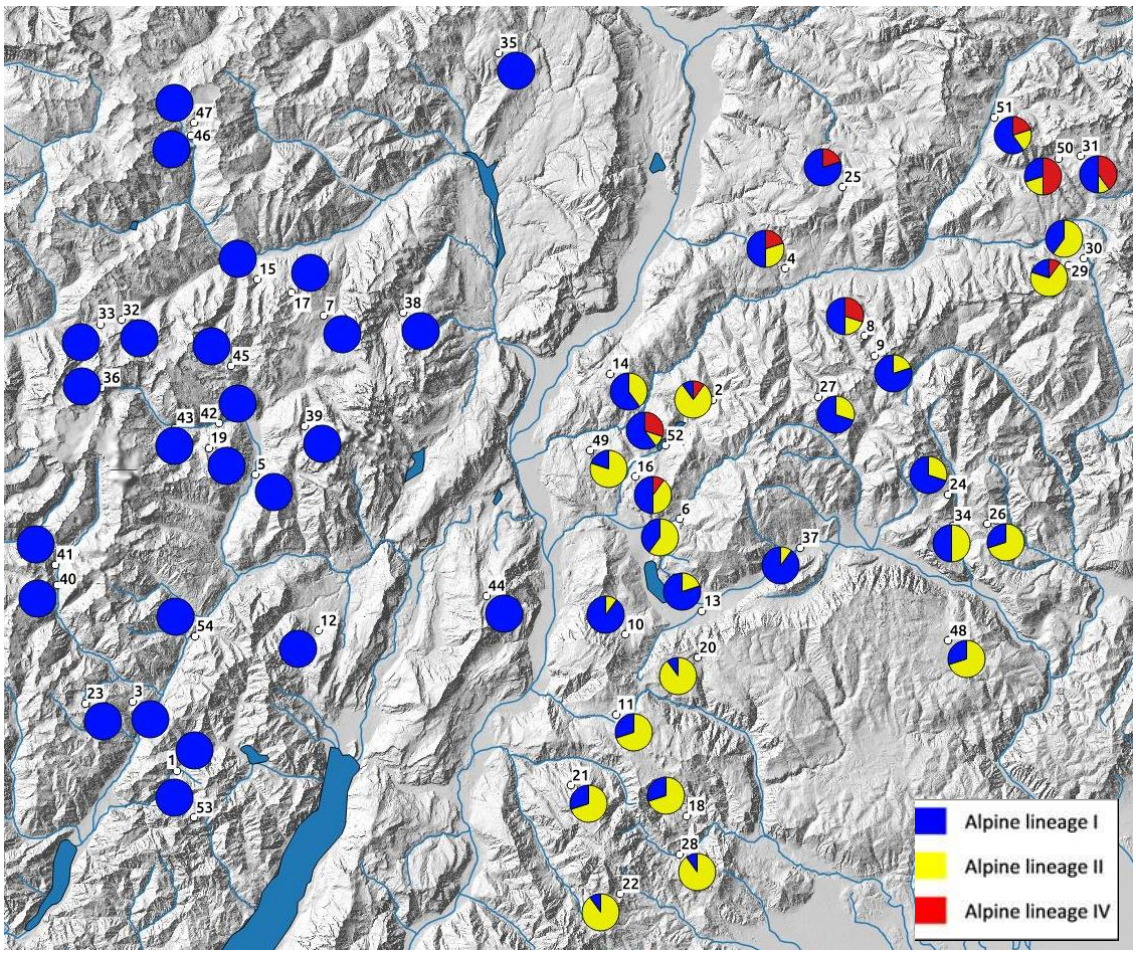

Figure 3 Spatial distribution of COI lineages in the different populations. Different colors correspond to the different lineages. Sites are numbered according to Table S1 (Supporting Information).

Overall, the haplotype with the highest frequency is CA2, belonging to lineage Alp1 (overall frequency = 0.526; see Table 1). CA2 is distributed across the whole study region and it is present in all sites, except for one (RM1). The second most frequent haplotype is VC6 (lineage Alp1; overall frequency =0.246), although being present only in East Trentino. Within this sub-region, haplotype VC6 is present in all sites except for one (PLa), and it is present with frequency ≥ 0.5 in 14/28 sites. 9 of them (64%) are located in the southern half of the region. All other haplotypes are present with global frequency < 0.1. Haplotype TN1 is present only in one site (Tre).

Table 1 Overall haplotype frequencies and distributions among sites.
Newly discovered haplotypes are highlighted in bold.

COI lineage	Haplotype	N sequences	Frequency	N sites	% sites
Alp1	CA2	284	0.526	53	0.981
Alp1	DE10	6	0.011	4	0.074
Alp1	MT5	8	0.015	5	0.093
Alp1	**TN1**	1	0.002	1	0.019
Alp1	**TN2**	15	0.028	6	0.111
Alp1	**TN3**	15	0.028	7	0.130
Alp1	**TN4**	5	0.009	2	0.037
Alp1	**TN5**	42	0.078	13	0.241
Alp1	**TN6**	7	0.013	3	0.056
Alp2	VC6	133	0.246	28	0.519
Alp4	PR4	18	0.033	7	0.130
Alp4	SA1	6	0.011	3	0.056

West Trentino harbors more haplotypes than East Trentino, despite of the presence of only one COI lineage. Considering the haplotypes belonging to this lineage, Alp1, 7 of them are exclusive of West Trentino. The spatial distribution of the different haplotypes show general patterns of geographical clustering, although with frequent irregularities, particularly at local scale (Fig. S1 in Supporting Information). Haplotype frequencies for all populations are reported in Table S1 (Supporting Information).

Different populations exhibited different levels of intra-population genetic diversity (Table S2, Supporting Information), sometimes even at short geographic distance. No correlation was found between latitude and standard genetic diversity measures. However, when considering the two separate subsets, West and East Trentino, a moderate significant correlation was detected in both cases, but with opposite sign. Specifically, number of alleles

decreased from south to north in West Trentino ($r = -0.42$, $p < 0.05$), while in East Trentino an opposite trend was highlighted ($r = 0.59$, $p < 0.05$), with genetic diversity increasing with latitude. Other measures of genetic diversity (e.g. haplotype diversity, h; nucleotide diversity, π) yielded very similar correlation values (data not shown). A correlation between longitude and h was detected only in East Trentino ($r = 0.42$, $p < 0.05$). This result seems to be driven by the presence of 3 COI lineages in the north-eastern part of this sub-area.

Pairwise PhiST values (Table S4, Supporting Information) highlighted an overall high level of genetic differentiation among populations, with 686/1431 comparisons (47.9 %) yielding significant values ($p < 0.05$, after adjustment for multiple comparisons using false discovery rate method; Benjamini & Hochberg 1995). This result is remarkable, considering the fine spatial scale of our study, and the fact that we employed a single mtDNA gene (569 bp long). Significant PhiST values were frequently found even for populations separate by less than 10 km, particularly in East Trentino (e.g. PR1-PS1, PR1-PS2, PR2-PS1, PR2-PS2, Ech-DDB, Ech-Ing, Mon-DDB, Mon-Ing, Ste-MBa, Ste-Bed). The high levels of differentiation detected in this area are most likely due to the presence of 3 different lineages, which are admixed following non-linear spatial patterns.

Spatial analysis of molecular variance (SAMOVA) indicated K=3 (3 groups) as the most likely population structure, when FCT was maximized (FCT= 0.494) and the increment of FCT was the largest ($\Delta FCT = 0.0018$) (Table S3 and Fig. S4 in Supporting Information). Group 1 include all populations (39) with a prevalence of the lineage Alp 1 (Frequency of Alp 1 > 0.5); group 2 include all populations (13) with a prevalence of the lineage Alp 2 (Frequency of Alp 2 > 0.5); group 3 include only 2 populations, characterized by admixture of 3 lineages and lineage Alp 4 present at high frequencies (≥ 0.4). Anyway, it must be noted that: (1) all the other tested grouping schemes (K) yielded similar proportions of explained variance (FCT values are relatively constant among the different K; (2) in all the tested K, the proportion of genetic variability found among populations (FST) is higher than the proportion of genetic variability found among groups (FCT).

The AMOVA analysis applied to the 3 groups inferred by SAMOVA showed a significant partitioning of genetic variation ($P<0.001$), with the largest proportion of variation explained by differences among groups (49.44 %). However, high levels of genetic variation were also found within populations (44.79 %) (Table 2). This is not surprising, since all the populations in the eastern part of the area are characterized by admixture of different COI lineages, therefore showing high inter-individual variation.

The variation explained among populations within groups was relatively low (5.77 %), indicating that the different groups inferred by SAMOVA are relatively homogeneous and therefore providing further support for the inferred broad scale spatial structure (but see point 1 in the previous paragraph).

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)	F statistics	P value
Among groups	2	242,942	1,054	49,44	FCT: 0,494	0.0001
Among poputions within groups	51	111,429	0,123	5,77	FSC: 0,114	0.0001
Within populations	486	464,161	0,955	44,79	FST: 0,552	0.0001
Total	539	818,532	2,132			

Table 2 Analysis of molecular variance (AMOVA) computed for the most likely subdivisions inferred by SAMOVA (K=3).

For the detection of major genetic discontinuities (software BARRIER), scenarios imposing from 1 to 8 barriers were investigated, until the identified discontinuities were corresponding to statistically significant PhiST values (Fig. 4). The analysis firstly indicated the isolation of single populations fixed for single haplotypes (e.g. barrier a, isolating RMa) or small groups of populations (e.g. barrier b). Then, the imposition of the third barrier (barrier c) resulted in the separation of populations from the Venetian Prealps. Adding more barriers, more general patterns started to appear. Major separations resulted from the addition of different adjacent barriers, e.g. barrier h + b, separating the whole north-eastern part of Trentino from the rest of the region. As a final output, 8 barriers lead to the almost complete separation of the western and eastern side of the Adige valley (Fig. 4). The two sides remained connected by a single corridor with east-west orientation, located in the central part of the region. Another composed barrier (barrier f + a) resulted in the separation of the populations in the north-western corner of the area.

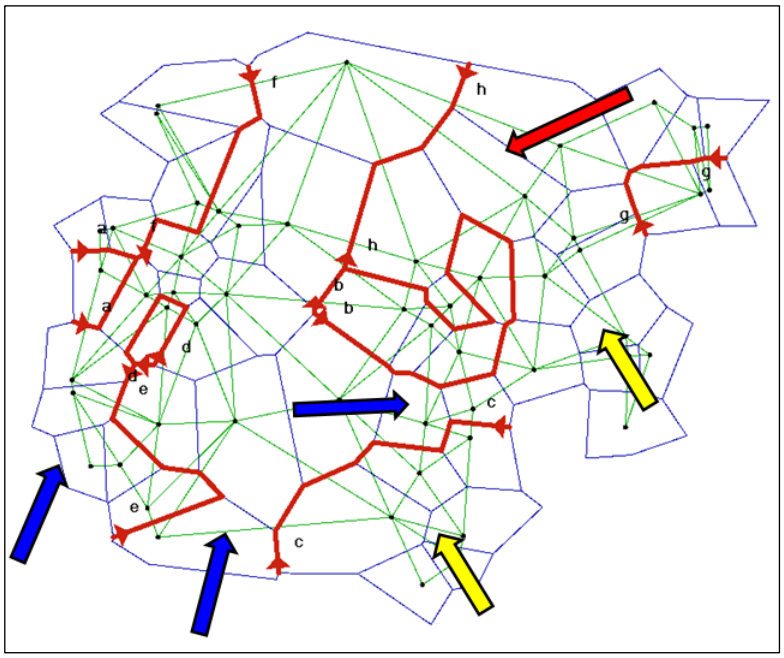

Figure 4 Output of BARRIER analysis, showing the spatial location of major genetic discontinuities. Sample points (populations) are represented by black dots, blue lines correspond to Voronoï tessellation and green lines to Delaunay triangulation. The inferred barriers are depicted with red lines and designated with letters according to their rank (a-g). Colored arrows represent hypothesized recolonization routes from different glacial refugia (see Discussion).

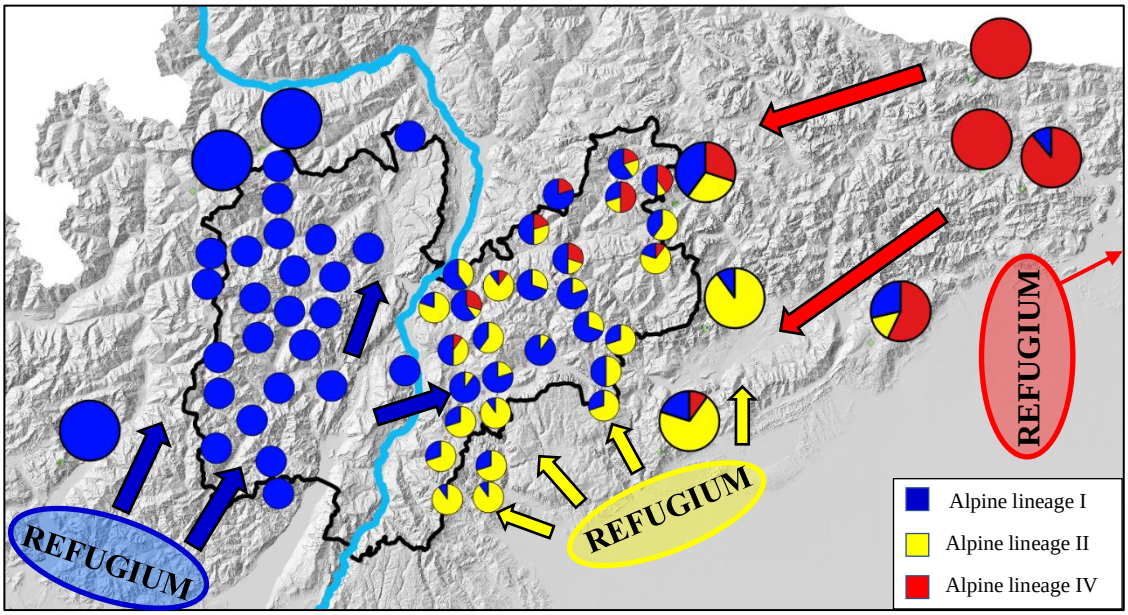

Figure 5. Tentative phylogeographic reconstruction for *Rana temporaria* in the Trentino region. The map shows the different sampling sites, colored according to the frequency of detected COI lineages, together with the approximate location of corresponding glacial refugia and the proposed recolonization routes (arrows; see Discussion). The light blue line in the middle of the region depicts the Adige river. The black line depicts the border of the study region. Big circles outside the study region mark sites for which data were retrieved from Stefani *et. al* (2012).

Discussion

Our mtDNA survey, performed using an intensive sampling design, allowed us to reveal the complex phylogeographic scenario for *Rana temporaria* in the considered southern alpine region (Fig. 5). Overall, our study provides strong support for the hypothesis of a "refugia-within-refugia" model for the species in the Italian Alps, which assume the survival of populations during the Pleistocene glaciations in different isolated peripheral refugia on the southern slopes of the Alps (Stefani *et al.* 2012).

The global levels of genetic diversity observed for the species in the Trentino region are considerably high. We found 12 different haplotypes: this number is striking considering the small spatial scale of our study. As a basis for comparison, a total of 18 haplotypes were found by Stefani *et. al* (2012), in their survey across the whole Italian Alps. Moreover, the detection of 3 different COI lineages highlighted the Trentino region as a contact zone among different postglacial recolonization routes. SAMOVA analysis, providing support for 3 main groups but identifying other grouping schemes with a similar proportion of explained variance, suggested that the inferred population structure may indeed be better interpreted as spatial clines of admixture among the 3 lineages. The spatial distribution of lineages and haplotypes clearly indicate that Alpine lineage 1 (Alp1) colonized East Trentino from the west. Indeed, Alp1 is the only lineage found in the western part of the region, where it exhibits high levels of diversity, while in the east it is present with only 2 haplotypes (including the most abundant one, CA2), and always admixed with other lineages. In addition, the negative correlation between genetic diversity and latitude, detected in West Trentino, provides evidence for a north-south orientation of the postglacial recolonization process for Alp1. Such a reduction in genetic diversity in the north may be explained by repeated founder events occurred during the recolonization, which can lead to the common pattern of "southern richness and northern purity" (Gugerli *et al.* 2001; Hewitt 2004; Canestrelli *et al.* 2014). We can therefore assume that the lineage Alp1 originated in a peripheral Pleistocene refugium probably located in the south-western mountains of the region, or in the immediate southern Lombardian Prealps, from where it spread toward the north and toward the east under favorable climatic conditions. Lineage Alp2 is instead the dominant haplogroups in the Venetian Prealps, at the southern margin of the region, suggesting this area as potential refugium. Further support for the proposed locations of the refugia for Alp1 and Alp2 lineages come from fossil records: fossils remains of *Rana temporaria* were found in Pleistocene paleontological localities in the North-Western

Lombardian Prealps (Bona *et al.* 2002) and in Lessinia, in the Venetian Prealps (Delfino 2002), providing evidence that the species survived the Pleistocene glacial cycles in these areas lying outside the current latitudinal and altitudinal distribution of the species (Bartolini *et al.* 2014; see Fig. S3b in Supporting Information). The frequency of the lineage Alp4 depicts a penetration line from north-east to the middle of the region. Its presence is marginal in the study region and does not allow speculations on its geographic origin, however data from Stefani et al. (2012) seem to indicate the far eastern margin of the Alps as its approximate potential refugial area (see Fig. S3a in Supporting Information). The increase in genetic diversity with latitude, detected in East Trentino, and opposite to the western trend, may be most likely explained by the admixture of different lineages occurring in the northern part of this sub-region. Such an addictive effect is indeed a common feature of contact zones (Petit *et al.* 2003). Interestingly, both Alp2 and Alp4, does not penetrate in West Trentino. The location of major genetic discontinuities, detected with Monmonier algorithm (BARRIER), provided further details on the potential colonization routes. Specifically, West and East Trentino appeared to be completely separated except for a strict corridor in the central part of the region. This area perfectly matches with the Valsugana valley, a west-east oriented valley that could have been used as a corridor by Alp1 in its expansion toward the east. The hypothesis of a single penetration corridor may be reinforced by the rapid loss of genetic diversity that this lineage seems to have experienced moving from west to east. Colonization occurring through narrow corridors can indeed lead to a faster decline in genetic diversity, as a result of the ‘embolism' effect (the growth of genetically uniform populations ahead of the main colonization front, Bialozyt *et al.* 2006).

On a more detailed scale, the observed high levels of genetic differentiation, with high fragmentation in small groups and populations fixed for single haplotypes, lead to the conclusion that recolonization routes followed irregular patterns, and this seems to be particularly true in East Trentino, where different lineages met. This could be due to the complex orography of the study region, characterized by different mountain massifs and deep valleys. Nevertheless, an alternative explanation for the recorded high local differentiation may be "allele surfing", a process in which a small number of individuals at the expansion front multiplies into unoccupied environments, causing some particular alleles to spread at high frequencies, and eventually increasing population structuring (Excoffier & Ray 2008). Klopfstein *et al.* (2006) found that “allele surfing” occurs more often in small, rapidly growing populations under limited dispersal, and this may be the case of our study species. The two proposed explanations are not mutually exclusive.

Another major outcome of this study is the remarkable difference in overall genetic diversity levels between Alp1 and Alp2 lineages. Indeed, Alp1 exhibited high diversity (9 haplotypes), while only 1 haplotype was found for Alp2, despite the latter being abundant in East Trentino. Stefani *et al.* (2012), in their survey covering the whole Alpine chain, found only 2 haplotypes for this lineage. Therefore, their broad-scale study, together with our intensive survey in the proximity of putative refugium, suggests that the strong detected difference cannot be due to sampling bias. This marked difference in genetic diversity might reflect different conditions experienced by the two lineages in their respective glacial refugia. Particularly, Alp1 might have persisted in a large refugium with widely connected populations and an overall high effective population size, while Alp2 might have been restricted to a smaller, less favorable area, therefore experiencing a loss of genetic diversity due to drift. The different current spatial distributions of the two lineages, with Alp1 being widespread in a large sector of central and eastern Alps, and Alp2 limited to a small portion of eastern Alps and Prealps (Stefani *et al.* 2012; see Fig. S3a), seem to corroborate this hypothesis. Furthermore, Alp1 haplotype network display a "star-like" shaped topology, with a numerical dominating central haplotype surrounded by several less abundant haplotypes, a pattern that is generally interpreted as an evidence of past population-wide demographic expansion (Rogers & Harpending 1992; Bandelt *et al.* 1995). Again, this could be interpreted assuming favorable conditions in the refugial area; however, it could also be due to the sudden spatial expansion occurred during the recolonization process. Without more specific analyses, either scenario might be possible, since difference models of population growth and different processes may lead to similar gene tree patterns (Slatkin & Hudson 1991).

The main genetic discontinuity detected in our study region, corresponding to the low elevation Adige river valley, has a strong paleoclimatic foundation. A major genetic barrier in correspondence to a broad valley was also found for the species in the Western Alps by Stefani *et al.* (2012), specifically in the valley of the Dora Baltea river. According to the authors, an explanation for this barrier effect may be found considering that, during the interglacials, broad Alpine valleys were occupied for longer by slowly retreating glaciers. During the last Alpine Last Glacial Maximum (ALGM), about 25.000 years ago, the whole Trentino region was indeed covered by the Adige glacier, approximately 1600-2000 m thick (Caldonazzi & Avanzini 2011). In contrast, Prealpine areas were only partially covered by glaciers (Bassetti & Borsato 2005). The ice sheet started to retreat between 17,000- 11,500 years ago, and in the final stage of the retreating, complete ice melt led to massive flooding in the central part of the region (Angelucci 2013). Meanwhile, forests started to cover both

sides of the region. It is likely that, in a certain phase of glacial retreating, both surrounding forests and the swampy central valley, still characterized by a fresh climate, provided a very suitable habitat for the common frog. Then, further increasing of temperature, together with complete flooding, may have contributing in generating a barrier for the species. Combining mtDNA and microsatellites, Vernesi *et al.* (2016) found strong genetic differentiation among populations from the eastern side of the Adige valley for several animal species (roe deer, red deer, mountain hare and, only for mtDNA, chamois). A similar east-west genetic differentiation along this line was also detected for the mid-altitude butterfly *Erebia euryale* (Haubrich & Schmitt 2007) and for different alpine plant species (e.g. Schonswetter *et al.* 2002; Albach *et al.* 2006). At the species diversity level, the so-called "Brenner-line", which include the Adige valley up to the Brenner pass, was formerly stressed as a barrier for plant species distributions back in the 19th century (Kerner 1870), and an analogous separation was recognized for cave-dwelling species (Ruffo 1950, 1958). Therefore, our results confirmed the biogeographic peculiarity of the Trentino region, already highlighted by past studies from different fields, but so far not recognized in an organic theoretical framework, nor in conservation planning.

However, the Pleistocene history of the common frog revealed a more complicated scenario than a simple east-west separation: lineages Alp2 and Alp4 remained confined to the eastern part of the region, but we found strong evidence that Alp1 crossed the valley, colonizing East Trentino. This asymmetric barrier effect, caused by the Adige river valley, remains an open question. As a mere speculation, we introduce here three different hypotheses: (1) recolonization by the different COI lineages occurred in different times; (2) the three different putative refugia were located at different distances from the central valley: under this scenario, glacial refugium for Alp1 should have been located closer to the central part of the region, so that this lineage reached the valley when it was not a barrier, while the other lineages arrived too late; (3) recolonization from different refugia took place at different recolonization rates.

Testing these hypotheses would require more genetic data (e.g. sequencing different genes) and more complex phylogeographic analysis, or a simulation approach. However, as a further suggestion, we propose that: (a) the supposed locations of different refugia support hypothesis 2 for explaining the failure of Alp4 to penetrate West Trentino: indeed, we detected this lineage in East Trentino only in few sites, with frequency rapidly decreasing toward the center and resembling the ending tail of a penetration line; (b) evidence supporting hypothesis 3 for explaining the failure of Alp2 may come from its low genetic

diversity. Indeed, supposing low effective population size and/or density in its corresponding refugium for explaining the low levels of genetic diversity, we could assume that the same factor negatively affected dispersal rates and connectivity, and, ultimately, recolonization potential. Considering its limited geographic distribution across the Alps, it must be noted that this lineage doesn't seem to have spread toward the east, neither (Stefani *et* al. 2012; Fig. S3a).

We provided evidence that the use of a common, widespread, species may be an effective choice for detecting historical hotspots of genetic diversity, for unravelling the fine-scale legacy of past climatic oscillations and identifying different management units of relevant evolutionary significance. For example, the finding of a genetically homogeneous gene pool in the western part of the region, opposite to the admixture patterns found in the east, clearly indicate the need for different management and conservation strategies for the species in the two sub-regions. Under a conservation perspective, past evolutionary processes related to glacial cycles are rarely considered. Nevertheless, a recent meta-analysis on European amphibians showed that the conservation status of species is negatively correlated with distance from refugia. The authors therefore proposed that the phylogeographic status of populations (i.e., refugial vs. post-glacial colonization) should be considered in conservation assessments for red lists (Dufresnes & Perrin 2015). This study may serve as a term of comparison with other (more threatened) species, in order to identify common patterns or to highlight relevant evolutionary differences among organisms.

Finally, our results may provide a basis for the study of micro-evolutionary processes affecting biological species in the face of ongoing climate change, in particular adaptation to changing ecological conditions. Indeed, recent studies are showing that different evolutionary lineages may potentially carry different ecological adaptations (Teske *et al.* 2008; Moritz *et al.* 2012). In particular, lineages that have persisted in isolated peripheral areas might have genotypes that will confer greater resistance to future climate warming (Moritz *et al.* 2012), being therefore of great conservation relevance (Hampe *et al.* 2005). The rapid spread of later-generation molecular technologies and the consequent "genomics revolution" has dramatically improved our ability to identify adaptive genes, opening the door for integrating biogeography and genomic science (Avise 2010; Stapley *et al.* 2010), and the common frog stands as a good candidate for future research in this direction (Bonin *et al.* 2006).

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Supporting Information

Supplementary Tables

Table S1 List of sampling sites with geographic coordinates (UTM 32N), average elevation and COI haplotype frequencies. Sites are numbered according to map in Fig.1.

code	site	site name	long	lat	elev	CA2	TN1	TN2	TN3	TN4	TN5	TN6	DE10	MT5	VC6	PR4	SA1
1	Amp	Lago d'Ampola	628457	5081277	795	0.4	0	0.6	0	0	0	0	0	0	0	0	0
2	Bed	Bedollo	679355	5116467	1183	0.1	0	0	0	0	0	0	0	0	0.8	0.1	0
3	Bon	Palù di Boniprati	624263	5087863	1206	0.5	0	0.3	0.1	0	0	0	0.1	0	0	0	0
4	Bro	Brozin	686249	5128930	999	0.4	0	0	0	0.1	0	0	0	0	0.3	0.2	0
5	Cad	Caderzone	635940	5109415	741	0.6	0	0	0	0	0.3	0	0	0.1	0	0	0
6	Can	Canezza	676219	5105171	653	0.4	0	0	0	0	0	0	0	0	0.6	0	0
7	CCC	Campo Carlo Magno	642438	5124460	1649	0.7	0	0	0	0	0.1	0	0	0.2	0	0	0
8	Ce1	Alpe Cermis 1	693763	5122533	2204	0.5	0	0	0	0	0	0	0	0	0.2	0.3	0
9	Ce2	Alpe Cermis 2	694710	5120705	2329	0.8	0	0	0	0	0	0	0	0	0.2	0	0
10	DDB	Dos del Bue	671037	5094193	1014	0.9	0	0	0	0	0	0	0	0	0.1	0	0
11	Ech	Torbiera Echen	670137	5086617	1273	0.3	0	0	0	0	0	0	0	0	0.7	0	0
12	Fia	Fiavé	641894	5094567	665	0.3	0	0.1	0.3	0	0.3	0	0	0	0	0	0
13	Ing	Inghiaie	678339	5096436	444	0.8	0	0	0	0	0	0	0	0	0.2	0	0
14	Lag	Lagabrun	669610	5118968	1115	0.6	0	0	0	0	0	0	0	0	0.4	0	0
15	LCa	Lago dei Caprioli	636112	5127947	1385	1	0	0	0	0	0	0	0	0	0	0	0
16	Lel	Laghestel	671966	5109197	876	0.5	0	0	0	0	0	0	0	0	0.4	0	0.1
17	LMe	Laghetti di Mezzana	639388	5126637	2061	0.6	0	0	0	0	0.3	0	0	0.1	0	0	0
18	LPo	Lago di Posina	676835	5076986	578	0.3	0	0	0	0	0	0	0	0	0.7	0	0
19	LSG	Laghi di S.Giuliano	631477	5111923	1974	0.3	0	0	0	0	0.7	0	0	0	0	0	0
20	Mon	Monterovere	677907	5092004	1240	0.1	0	0	0	0	0	0	0	0	0.9	0	0
21	MP1	Monte Pasubio 1	665846	5079866	1622	0.3	0	0	0	0	0	0	0	0	0.7	0	0
22	MP2	Monte Pasubio 2	670596	5069564	1004	0.2	0	0	0	0	0	0	0	0	0.8	0	0
23	MRe	Monte Remà	619764	5087651	1846	0.8	0	0	0.1	0	0	0	0.1	0	0	0	0
24	Mug	I Mughi	701676	5107512	1269	0.7	0	0	0	0	0	0	0	0	0.3	0	0
25	PLa	Passo Lavazé	691672	5136691	1802	0.8	0	0	0	0	0	0	0	0	0	0	0.2
26	PLC	Parco La Cascatella	705431	5104694	965	0.3	0	0	0	0	0	0	0	0	0.7	0	0
27	PMa	Passo Manghen	689343	5116779	2083	0.7	0	0	0	0	0	0	0	0	0.3	0	0
28	Pos	Posina	676186	5073349	563	0.1	0	0	0	0	0	0	0	0	0.9	0	0
29	PR1	Passo Rolle 1	713150	5129299	1947	0.2	0	0	0	0	0	0	0	0	0.7	0.1	0
30	PR2	Passo Rolle 2	714547	5129907	2039	0.4	0	0	0	0	0	0	0	0	0.6	0	0
31	PS2	Passo S. Pellegrino 2	714246	5139634	1940	0.5	0	0	0	0	0	0	0	0	0.1	0.4	0
32	PT1	Passo Tonale 1	623192	5124059	1856	0.9	0	0	0	0	0.1	0	0	0	0	0	0
33	PT2	Passo Tonale 2	621203	5123660	1873	0.9	0	0	0	0	0	0.1	0	0	0	0	0
34	PTe	Pieve Tesino	702128	5104542	833	0.5	0	0	0	0	0	0	0	0	0.5	0	0
35	PTr	Palù Tremole	658996	5149432	1738	0.7	0	0	0	0	0.1	0.2	0	0	0	0	0
36	RM1	Rifugio Mandrone 1	621304	5117641	2405	0	0	0	0	0	1	0	0	0	0	0	0
37	Ron	Palude di Roncegno	687655	5102437	401	0.9	0	0	0	0	0	0	0	0	0.1	0	0
38	Tov	Lago di Tovel	649907	5124734	1210	0.3	0	0	0	0	0.3	0.4	0	0	0	0	0
39	Va1	Valagola	640550	5113989	1689	0.4	0	0.1	0.2	0	0	0	0	0.3	0	0	0
40	VD1	Val Daone 1	617095	5098860	1651	0.7	0	0	0	0	0.1	0	0.2	0	0	0	0
41	VD2	Val Daone 2	616911	5100854	1847	0.6	0	0.1	0	0	0	0	0.2	0.1	0	0	0
42	VG1	Val di Genova 1	632491	5114234	1053	0.7	0	0	0	0	0.3	0	0	0	0	0	0
43	VG3	Val di Genova 3	628297	5113817	1233	0.5	0	0	0.3	0	0.2	0	0	0	0	0	0
44	ViT	Torbiera delle Viote	657860	5097877	1570	1	0	0	0	0	0	0	0	0	0	0	0
45	VN2	Val Nambrone 2	633610	5119702	2170	0.6	0	0	0	0	0.4	0	0	0	0	0	0
46	VP1	Val di Peio 1	629846	5141587	2155	1	0	0	0	0	0	0	0	0	0	0	0
47	VP2	Val di Peio 2	630097	5142784	2577	1	0	0	0	0	0	0	0	0	0	0	0
48	Mar	Marcesina	701694	5093616	1362	0.3	0	0	0	0	0	0	0	0	0.7	0	0
49	MBa	Monte Barco	667741	5111662	869	0.2	0	0	0	0	0	0	0	0	0.8	0	0
50	PS1	Passo S. Pellegrino 1	712169	5139382	1838	0.3	0	0	0	0	0	0	0	0	0.2	0.5	0
51	So2	Soraga 2	706085	5143303	1346	0.2	0	0	0	0.4	0	0	0	0	0.2	0.2	0
52	Ste	Palude di Sternigo	674831	5112145	1010	0.6	0	0	0	0	0	0	0	0	0.1	0	0.3
53	Tre	Monte Tremalzo	630127	5076810	1668	0.5	0.1	0.3	0.1	0	0	0	0	0	0	0	0
54	LRo	Lago di Roncone	630189	5094052	861	0.6	0	0	0.4	0	0	0	0	0	0	0	0

Table S2 Frequency of COI lineages, number of lineages (nl) and standard measures of intrapopulation genetic diversity (n= n° of haplotypes; h= haplotype diversity; π= nucleotide diversity; k= mean n° of pairwise nucleotide differences; s= n° of polymorphic sites). Sites are numbered according to map in Fig.1

N°	site	Alp1	Alp2	Alp4	nl	n	h	π	k	s
1	Amp	1	0	0	1	2	0.533	0.00094	0.533	1
2	Bed	0.1	0.8	0.1	3	3	0.378	0.00539	3.067	13
3	Bon	1	0	0	1	4	0.711	0.00152	0.867	3
4	Bro	0.5	0.3	0.2	3	4	0.778	0.00976	5.556	14
5	Cad	1	0	0	1	3	0.600	0.00117	0.667	2
6	Can	0.4	0.6	0	2	2	0.533	0.00469	2.667	5
7	CCC	1	0	0	1	3	0.511	0.00098	0.556	2
8	Ce1	0.5	0.2	0.3	3	3	0.689	0.01039	5.911	13
9	Ce2	0.8	0.2	0	2	2	0.356	0.00312	1.778	5
10	DDB	0.9	0.1	0	2	2	0.200	0.00176	1	5
11	Ech	0.3	0.7	0	2	2	0.467	0.0041	2.333	5
12	Fia	1	0	0	1	4	0.800	0.00199	1.133	3
13	Ing	0.8	0.2	0	2	2	0.356	0.00312	1.778	5
14	Lag	0.6	0.4	0	2	2	0.533	0.00469	2.667	5
15	LCa	1	0	0	1	1	0.000	0	0	0
16	Lel	0.5	0.4	0.1	3	3	0.644	0.00828	4.711	15
17	LMe	1	0	0	1	3	0.600	0.00117	0.667	2
18	LPo	0.3	0.7	0	2	2	0.467	0.0041	2.333	5
19	LSG	1	0	0	1	2	0.467	0.00082	0.467	1
20	Mon	0.1	0.9	0	2	2	0.200	0.00176	1	5
21	MP1	0.3	0.7	0	2	2	0.467	0.0041	2.333	5
22	MP2	0.1	0.9	0	2	2	0.200	0.00176	1	5
23	MRe	1	0	0	1	3	0.378	0.0007	0.4	2
24	Mug	0.7	0.3	0	2	2	0.467	0.0041	2.333	5
25	PLa	0.8	0	0.2	2	2	0.356	0.0075	4.267	12
26	PLC	0.3	0.7	0	2	2	0.467	0.0041	2.333	5
27	PMa	0.7	0.3	0	2	2	0.467	0.0041	2.333	5
28	Pos	0.1	0.9	0	2	2	0.200	0.00176	1	5
29	PR1	0.2	0.7	0.1	3	3	0.511	0.00652	3.711	13
30	PR2	0.4	0.6	0	2	2	0.533	0.00469	2.667	5
31	PS2	0.5	0.1	0.4	3	3	0.644	0.01051	5.978	13
32	PT1	1	0	0	1	2	0.200	0.00035	0.2	1
33	PT2	1	0	0	1	2	0.200	0.00035	0.2	1
34	PTe	0.5	0.5	0	2	2	0.556	0.00488	2.778	5
35	PTr	1	0	0	1	3	0.511	0.00098	0.556	2
36	RM1	1	0	0	1	1	0.000	0	0	0
37	Ron	0.9	0.1	0	2	2	0.200	0.00176	1	5
38	Tov	1	0	0	1	3	0.733	0.00176	1	2
39	Va1	1	0	0	1	4	0.778	0.0018	1.022	3
40	VD1	1	0	0	1	3	0.511	0.00098	0.556	2
41	VD2	1	0	0	1	4	0.644	0.00133	0.756	3
42	VG1	1	0	0	1	2	0.467	0.00082	0.467	1
43	VG3	1	0	0	1	3	0.689	0.00145	0.822	2
44	ViT	1	0	0	1	1	0.000	0	0	0
45	VN2	1	0	0	1	2	0.533	0.00094	0.533	1
46	VP1	1	0	0	1	1	0.000	0	0	0
47	VP2	1	0	0	1	1	0.000	0	0	0
48	Mar	0.3	0.7	0	2	2	0.467	0.0041	2.333	5
49	MBa	0.2	0.8	0	2	2	0.356	0.00312	1.778	5
50	PS1	0.3	0.2	0.5	3	3	0.689	0.01133	6.444	13
51	So2	0.6	0.2	0.2	3	4	0.800	0.00969	5.511	14
52	Ste	0.6	0.1	0.3	3	3	0.600	0.01113	6.333	15
53	Tre	1	0	0	1	4	0.711	0.0018	1.022	3
54	LRo	1	0	0	1	2	0.533	0.00094	0.533	1

Table S3 Spatial analysis of molecular variance (SAMOVA).

K	FSC	FST	FCT	Δ FCT
2	0.16341	0.5521	0.47619	0
3	0.11408	0.5521	0.49442	**0.01823**
4	0.11609	0.55104	0.49208	-0.00234
5	0.07886	0.52919	0.48888	-0.0032
6	0.06618	0.52125	0.48732	-0.00156
7	0.06576	0.51394	0.47973	-0.00759
8	0.06413	0.51402	0.4807	0.00097
9	0.01571	0.49327	0.48519	0.00449
10	0.0235	0.49362	0.48143	-0.00376

For each K (hypothesized number of groups), values of FST (proportion of genetic variability found among populations), FSC (proportion of genetic variability found among populations within groups), FCT (proportion of genetic variability found among groups) are reported.

The defined groups for K=3 (maximum Δ FCT; most likely subdivision) were:

Group 1: Amp, Bon, Bro, CCC, Cad, Ce1 , Ce2, DDB, Fia, Ing, LCa, LMe, LRo, LSG, Lag, Lel, MRe, Mug, PLa, PMa, PT1, PT2, PTe, PTr, RM1, Ron, So2, Ste, Tov, Tre, VD1, VD2, VG1, VG3, VN2, VP1, VP2, Va1, ViT;

Group 2: Bed, Can, Ech, LPo, MBa, MP1, MP2, Mar, Mon, PLC, PR1, PR2, Pos;

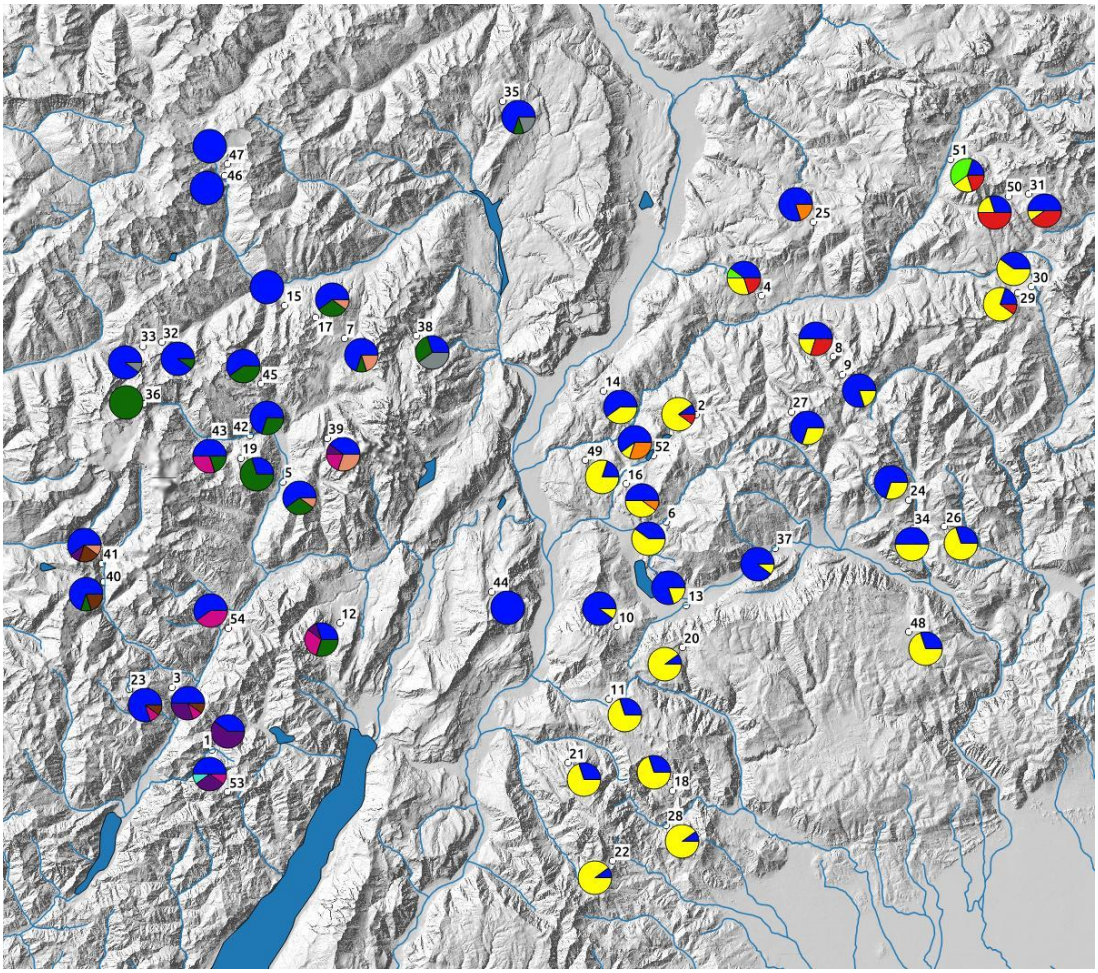
Group 3: PS1, PS2.

	Amp	Bed	Bon	Bro	CCC	Cad	Can	Ce1	Ce2	DDB	Ech	Fia	Ing	LCa	LMe	LPo	LRo	LSG	Lag	Lel	MBa	MP1	MP2	MRe	Mar	Mon	Mug	PLC	PLa	PMa	PR1	PR2	PS1	PS2	PT1	PT2	PTe	PTr	Pos	RM1	Ron	So2	Ste	Tov	Tre	VD1	VD2	VG1	VG3	VN2	VP1	VP2	Va1	ViT
Amp		+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Bed	0.67		+	-	+	+	-	-	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bon	0.05	0.63		-	-	-	+	-	-	-	+	-	-	-	-	+	-	+	-	+	+	+	+	-	+	-	+	-	+	-	-	+	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
Bro	0.23	0.14	0.17		+	+	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	-
CCC	0.40	0.66	0.11	0.22		-	+	+	-	-	+	-	-	-	-	+	-	+	-	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Cad	0.40	0.65	0.15	0.22	-0.02		+	+	-	-	+	-	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Can	0.56	0.03	0.48	0.05	0.51	0.51		-	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Ce1	0.24	0.22	0.20	-0.09	0.25	0.25	0.16		-	-	-	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	+	+	-
Ce2	0.28	0.44	0.09	0.05	0.10	0.13	0.21	0.12		-	-	-	-	-	-	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
DDB	0.30	0.56	0.05	0.12	0.03	0.07	0.37	0.17	-0.07		+	-	-	-	-	+	-	+	-	-	+	+	+	+	-	+	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Ech	0.65	-0.03	0.59	0.11	0.62	0.62	-0.09	0.22	0.34	0.49		+	-	+	+	-	+	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fia	0.29	0.63	0.07	0.21	0.10	0.02	0.49	0.24	0.14	0.11	0.59		-	-	-	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
Ing	0.28	0.44	0.09	0.05	0.10	0.13	0.21	0.12	-0.11	-0.07	0.34	0.14		-	-	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
LCa	0.56	0.69	0.13	0.23	0.07	0.17	0.56	0.26	0.11	0.00	0.67	0.19	0.11		-	+	-	+	-	-	+	+	+	-	+	+	-	+	-	-	+	+	+	-	-	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-
LMe	0.40	0.65	0.15	0.22	-0.02	-0.11	0.51	0.25	0.13	0.07	0.62	0.02	0.13	0.17		+	+	-	+	+	+	+	+	-	+	+	-	+	-	-	+	+	+	+	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
LPo	0.65	-0.03	0.59	0.11	0.62	0.62	-0.09	0.22	0.34	0.49	-0.11	0.59	0.34	0.67	0.62		+	+	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LRo	0.47	0.67	0.15	0.24	0.22	0.25	0.53	0.27	0.17	0.15	0.63	0.03	0.17	0.33	0.25	0.63		+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	-	+	-	+	+	-	+	-	-	-	-	+	-	+	-	-	-
LSG	0.62	0.69	0.44	0.30	0.41	0.17	0.58	0.32	0.34	0.39	0.67	0.18	0.34	0.67	0.17	0.67	0.55		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	+	+	+	+
Lag	0.38	0.21	0.27	-0.01	0.30	0.31	-0.03	0.09	-0.01	0.13	0.07	0.30	-0.01	0.33	0.31	0.07	0.33	0.42		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Lel	0.29	0.10	0.21	-0.08	0.25	0.25	-0.04	-0.01	0.02	0.12	0.03	0.25	0.02	0.26	0.25	0.03	0.27	0.34	-0.07		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	+
MBa	0.75	-0.06	0.70	0.20	0.73	0.72	-0.01	0.29	0.48	0.62	-0.08	0.69	0.48	0.78	0.72	-0.08	0.74	0.76	0.21	0.12		-	-	+	-	-	-	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MP1	0.65	-0.03	0.59	0.11	0.62	0.62	-0.09	0.22	0.34	0.49	-0.11	0.59	0.34	0.67	0.62	-0.11	0.63	0.67	0.07	0.03	-0.08		-	+	-	-	-	+	-	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MP2	0.85	-0.05	0.81	0.30	0.84	0.83	0.13	0.39	0.62	0.76	0.02	0.79	0.62	0.89	0.83	0.02	0.84	0.86	0.37	0.24	-0.07	0.02		+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MRe	0.42	0.65	0.04	0.20	0.04	0.11	0.50	0.23	0.06	-0.03	0.62	0.09	0.06	0.00	0.11	0.62	0.10	0.52	0.28	0.23	0.73	0.62	0.85		+	+	-	+	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mar	0.65	-0.03	0.59	0.11	0.62	0.62	-0.09	0.22	0.34	0.49	-0.11	0.59	0.34	0.67	0.62	-0.11	0.63	0.67	0.07	0.03	-0.08	-0.11	0.02	0.62		-	-	-	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mon	0.85	-0.05	0.81	0.30	0.84	0.83	0.13	0.39	0.62	0.76	0.02	0.79	0.62	0.89	0.83	0.02	0.84	0.86	0.37	0.24	-0.07	0.02	-0.11	0.85	0.02		+	-	+	+	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mug	0.32	0.32	0.18	0.00	0.20	0.21	0.07	0.09	-0.08	0.02	0.20	0.21	-0.08	0.22	0.21	0.20	0.25	0.36	-0.09	-0.04	0.34	0.20	0.49	0.17	0.20	0.49		-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	+	-
PLC	0.65	-0.03	0.59	0.11	0.62	0.62	-0.09	0.22	0.34	0.49	-0.11	0.59	0.34	0.67	0.62	-0.11	0.63	0.67	0.07	0.03	-0.08	-0.11	0.02	0.62	-0.11	0.02	0.20		+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PLa	0.13	0.42	0.06	0.01	0.11	0.12	0.30	-0.01	0.07	0.07	0.38	0.12	0.07	0.11	0.12	0.38	0.14	0.24	0.15	0.06	0.48	0.38	0.57	0.09	0.38	0.57	0.10	0.38		-	+	+	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PMa	0.32	0.32	0.18	0.00	0.20	0.21	0.07	0.09	-0.08	0.02	0.20	0.21	-0.08	0.22	0.21	0.20	0.25	0.36	-0.09	-0.04	0.34	0.20	0.49	0.17	0.20	0.49	-0.11	0.20	0.10		-	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PR1	0.57	-0.09	0.52	0.06	0.56	0.55	-0.04	0.14	0.32	0.45	-0.07	0.53	0.32	0.59	0.55	-0.07	0.57	0.60	0.10	0.01	-0.06	-0.07	-0.01	0.55	-0.07	-0.01	0.20	-0.07	0.33	0.20		-	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PR2	0.56	0.03	0.48	0.05	0.51	0.51	-0.11	0.16	0.21	0.37	-0.09	0.49	0.21	0.56	0.51	-0.09	0.53	0.58	-0.03	-0.04	-0.01	-0.09	0.13	0.50	-0.09	0.13	0.07	-0.09	0.30	0.07	-0.04		+	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PS1	0.42	0.29	0.40	0.04	0.44	0.44	0.31	-0.04	0.34	0.39	0.34	0.43	0.34	0.46	0.44	0.34	0.45	0.48	0.29	0.14	0.40	0.34	0.46	0.44	0.34	0.46	0.30	0.34	0.14	0.30	0.23	0.31		-	+	+	-	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PS2	0.30	0.31	0.27	-0.02	0.32	0.32	0.27	-0.09	0.22	0.26	0.33	0.31	0.22	0.34	0.32	0.33	0.34	0.38	0.21	0.08	0.40	0.33	0.48	0.31	0.33	0.48	0.20	0.33	0.02	0.20	0.24	0.27	-0.07		-	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PT1	0.48	0.68	0.11	0.22	0.01	0.02	0.54	0.25	0.10	0.00	0.65	0.10	0.10	0.00	0.02	0.65	0.27	0.49	0.32	0.26	0.76	0.65	0.87	0.00	0.65	0.87	0.21	0.65	0.11	0.21	0.57	0.54	0.46	0.33		-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PT2	0.48	0.68	0.11	0.21	0.06	0.13	0.54	0.24	0.10	0.00	0.65	0.17	0.10	0.00	0.13	0.65	0.27	0.58	0.32	0.25	0.76	0.65	0.87	0.00	0.65	0.87	0.21	0.65	0.09	0.21	0.57	0.54	0.45	0.32	0.00		-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PTe	0.47	0.11	0.37	0.01	0.40	0.41	-0.09	0.11	0.09	0.24	-0.02	0.39	0.09	0.44	0.41	-0.02	0.43	0.49	-0.09	-0.07	0.09	-0.02	0.24	0.39	-0.02	0.24	-0.02	-0.02	0.22	-0.02	0.02	-0.09	0.29	0.23	0.43	0.43		+	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PTr	0.40	0.66	0.11	0.20	0.04	0.05	0.51	0.23	0.10	0.03	0.62	0.10	0.10	0.07	0.05	0.62	0.22	0.41	0.30	0.24	0.73	0.62	0.84	0.04	0.62	0.84	0.20	0.62	0.08	0.20	0.55	0.51	0.43	0.30	0.01	-0.05	0.40		+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pos	0.85	-0.05	0.81	0.30	0.84	0.83	0.13	0.39	0.62	0.76	0.02	0.79	0.62	0.89	0.83	0.02	0.84	0.86	0.37	0.24	-0.07	0.02	-0.11	0.85	0.02	-0.11	0.49	0.02	0.57	0.49	-0.01	0.13	0.46	0.48	0.87	0.87	0.24	0.84		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RM1	0.83	0.74	0.71	0.40	0.75	0.58	0.67	0.41	0.56	0.67	0.74	0.48	0.56	1.00	0.58	0.74	0.81	0.22	0.56	0.44	0.82	0.74	0.91	0.83	0.74	0.91	0.53	0.74	0.37	0.53	0.66	0.67	0.54	0.46	0.89	0.91	0.60	0.75	0.91		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ron	0.30	0.56	0.05	0.12	0.03	0.07	0.37	0.17	-0.07	-0.11	0.49	0.11	-0.07	0.00	0.07	0.49	0.15	0.39	0.13	0.12	0.62	0.49	0.76	-0.03	0.49	0.76	0.02	0.49	0.07	0.02	0.45	0.37	0.39	0.26	0.00	0.00	0.24	0.03	0.76	0.67		-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
So2	0.20	0.24	0.14	-0.08	0.18	0.19	0.13	-0.06	0.05	0.10	0.21	0.18	0.05	0.19	0.19	0.21	0.20	0.27	0.04	-0.02	0.29	0.21	0.39	0.16	0.21	0.39	0.03	0.21	-0.01	0.03	0.16	0.13	0.07	-0.01	0.18	0.17	0.08	0.16	0.39	0.37	0.10		-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ste	0.21	0.30	0.17	-0.03	0.22	0.22	0.22	-0.07	0.13	0.16	0.28	0.21	0.13	0.23	0.22	0.28	0.24	0.29	0.14	0.02	0.36	0.28	0.45	0.20	0.28	0.45	0.12	0.28	-0.06	0.12	0.22	0.22	0.01	-0.06	0.22	0.21	0.17	0.20	0.45	0.38	0.16	-0.02		-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tov	0.41	0.64	0.22	0.21	0.17	0.09	0.50	0.23	0.18	0.17	0.60	0.13	0.18	0.29	0.09	0.60	0.30	0.25	0.32	0.25	0.70	0.60	0.81	0.22	0.60	0.81	0.24	0.60	0.10	0.24	0.54	0.50	0.41	0.29	0.19	0.17	0.41	0.00	0.81	0.55	0.17	0.17	0.20		+	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tre	0.07	0.63	-0.07	0.19	0.08	0.14	0.49	0.22	0.13	0.08	0.59	0.04	0.13	0.15	0.14	0.59	0.07	0.43	0.29	0.23	0.70	0.59	0.80	0.06	0.59	0.80	0.20	0.59	0.08	0.20	0.53	0.49	0.41	0.28	0.13	0.13	0.39	0.12	0.80	0.68	0.08	0.16	0.19	0.22		-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VD1	0.40	0.63	0.06	0.17	0.04	0.05	0.47	0.21	0.04	-0.02	0.59	0.10	0.04	0.07	0.05	0.59	0.22	0.41	0.25	0.20	0.71	0.59	0.82	-0.04	0.59	0.82	0.14	0.59	0.08	0.14	0.52	0.47	0.42	0.29	0.01	0.06	0.36	0.04	0.82	0.75	-0.02	0.14	0.19	0.17	0.12		-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VD2	0.27	0.62	-0.01	0.16	0.01	0.09	0.46	0.19	0.04	-0.02	0.57	0.13	0.04	0.06	0.09	0.57	0.19	0.44	0.24	0.19	0.69	0.57	0.81	-0.03	0.57	0.81	0.13	0.57	0.06	0.13	0.51	0.46	0.40	0.27	0.04	0.04	0.35	0.06	0.81	0.73	-0.02	0.13	0.17	0.20	0.03	-0.06		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VG1	0.44	0.67	0.17	0.23	0.05	-0.09	0.53	0.26	0.14	0.08	0.63	0.02	0.14	0.22	-0.09	0.63	0.29	0.20	0.32	0.26	0.74	0.63	0.85	0.13	0.63	0.85	0.22	0.63	0.12	0.22	0.56	0.53	0.45	0.33	0.02	0.17	0.42	0.05	0.85	0.67	0.08	0.19	0.23	0.11	0.17	0.05	0.13		-	-	-	-	+	-
VG3	0.38	0.65	0.10	0.22	0.09	0.05	0.50	0.25	0.13	0.09	0.61	-0.09	0.13	0.18	0.05	0.61	-0.03	0.30	0.30	0.25	0.71	0.61	0.82	0.05	0.61	0.82	0.21	0.61	0.12	0.21	0.55	0.50	0.44	0.32	0.09	0.15	0.40	0.09	0.82	0.63	0.09	0.19	0.22	0.16	0.06	0.09	0.12	0.05		-	-	-	-	-
VN2	0.47	0.67	0.22	0.24	0.12	-0.07	0.53	0.27	0.17	0.15	0.63	0.03	0.17	0.33	-0.07	0.63	0.33	0.07	0.33	0.27	0.74	0.63	0.84	0.22	0.63	0.84	0.25	0.63	0.14	0.25	0.57	0.53	0.45	0.34	0.13	0.27	0.43	0.12	0.84	0.56	0.15	0.20	0.24	0.11	0.22	0.12	0.19	-0.09	0.08		-	-	-	+	-
VP1	0.56	0.69	0.13	0.23	0.07	0.17	0.56	0.26	0.11	0.00	0.67	0.19	0.11	0.00	0.17	0.67	0.33	0.67	0.33	0.26	0.78	0.67	0.89	0.00	0.67	0.89	0.22	0.67	0.11	0.22	0.59	0.56	0.46	0.34	0.00	0.00	0.44	0.07	0.89	1.00	0.00	0.19	0.23	0.29	0.15	0.07	0.06	0.22	0.18	0.33		-	-	-
VP2	0.56	0.69	0.13	0.23	0.07	0.17	0.56	0.26	0.11	0.00	0.67	0.19	0.11	0.00	0.17	0.67	0.33	0.67	0.33	0.26	0.78	0.67	0.89	0.00	0.67	0.89	0.22	0.67	0.11	0.22	0.59	0.56	0.46	0.34	0.00	0.00	0.44	0.07	0.89	1.00	0.00	0.19	0.23	0.29	0.15	0.07	0.06	0.22	0.18	0.33	0.00		-	-
Va1	0.28	0.63	0.06	0.21	-0.01	0.10	0.49	0.24	0.13	0.08	0.59	0.07	0.13	0.15	0.10	0.59	0.07	0.43	0.29	0.24	0.70	0.59	0.80	0.06	0.59	0.80	0.20	0.59	0.11	0.20	0.53	0.49	0.43	0.30	0.13	0.13	0.39	0.12	0.80	0.68	0.08	0.18	0.21	0.22	-0.02	0.12	0.03	0.17	0.06	0.22	0.15	0.15		-
ViT	0.56	0.69	0.13	0.23	0.07	0.17	0.56	0.26	0.11	0.00	0.67	0.19	0.11	0.00	0.17	0.67	0.33	0.67	0.33	0.26	0.78	0.67	0.89	0.00	0.67	0.89	0.22	0.67	0.11	0.22	0.59	0.56	0.46	0.34	0.00	0.00	0.44	0.07	0.89	1.00	0.00	0.19	0.23	0.29	0.15	0.07	0.06	0.22	0.18	0.33	0.00	0.00	0.15	

Table S4 Below diagonal: matrix of pairwise PhiST values. Abobe diagonal: significant values ($p < 0.05$ after FDR correction for multiple comparisons) are marked with "+"; non-significant values with "-"

Supplementary Figures

Figure S1 Spatial distribution of *Rana temporaria* COI haplotypes in Trentino



Haplotype legend:

Hapl.		Lineage
CA2		Alp. 1
TN1		
TN2		
TN3		
TN4		
TN5		
TN6		
DE10		
MT5		
VC6		Alp. 2
PR4		Alp. 4
SA1		

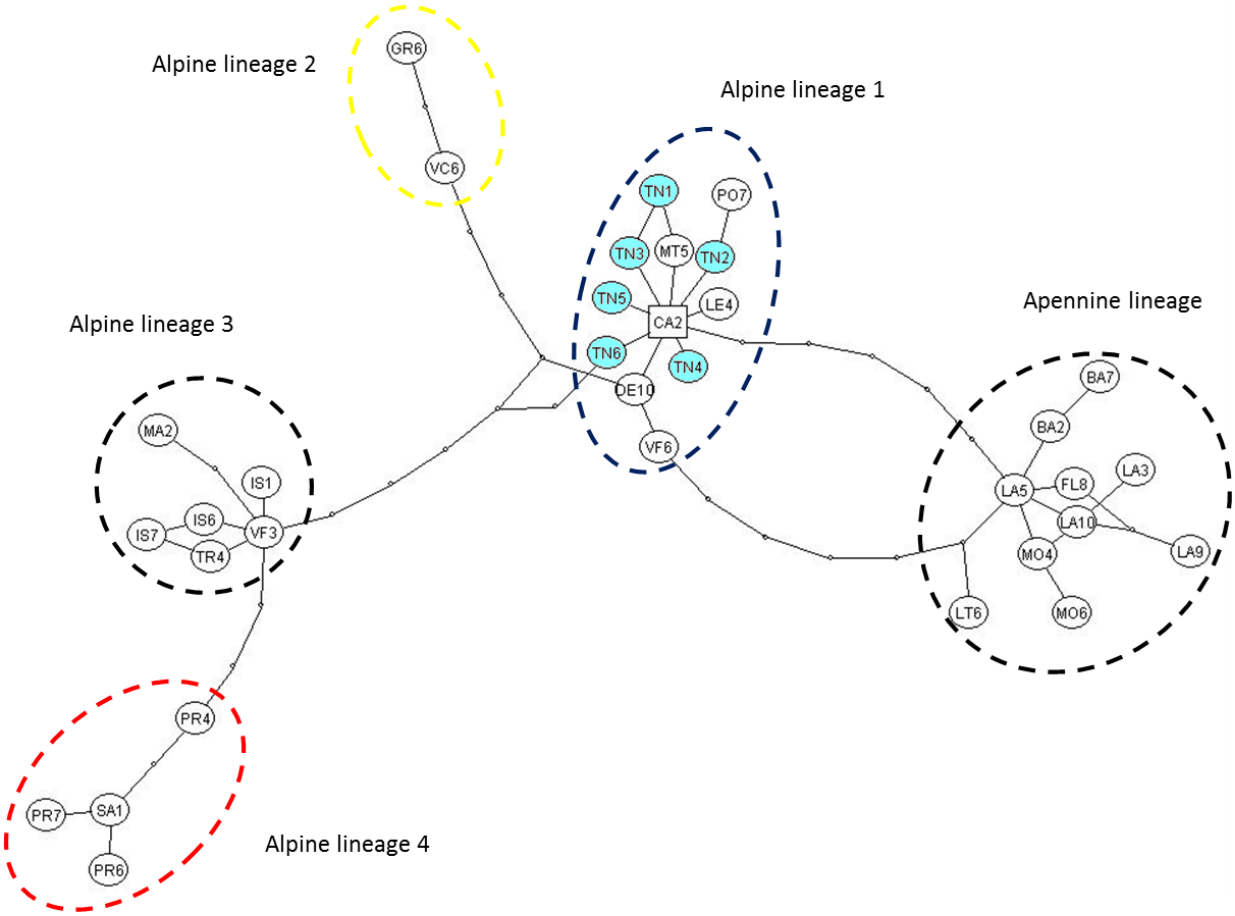


Figure S2 Phylogenetic network of *Rana temporaria* COI haplotypes, based on the statistical parsimony procedure implemented in TCS. All the haplotypes known for the Italian Peninsula and available from public repositories have been included in the analysis (Stefani *et al.* 2012; EMBL codes FN813783-FN813810).
Light blue circles represent newly discovered haplotypes.

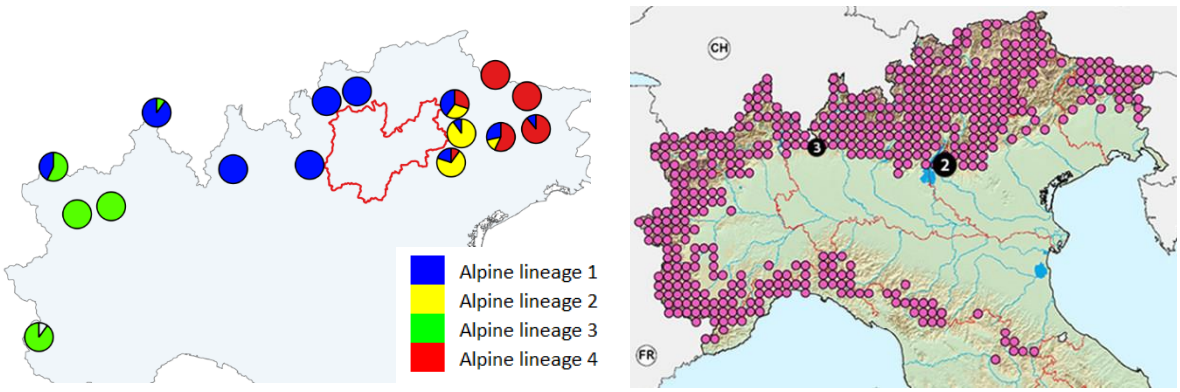


Figure S3 a) Distribution of *Rana temporaria* COI lineages across the whole Italian Alps (adapted from Stefani *et al.* 2012); b) Fossil records (black circles) for the species in the Italian Alps. Pink dots depict the current distribution of the species (adapted from Bartolini *et al.* 2014)

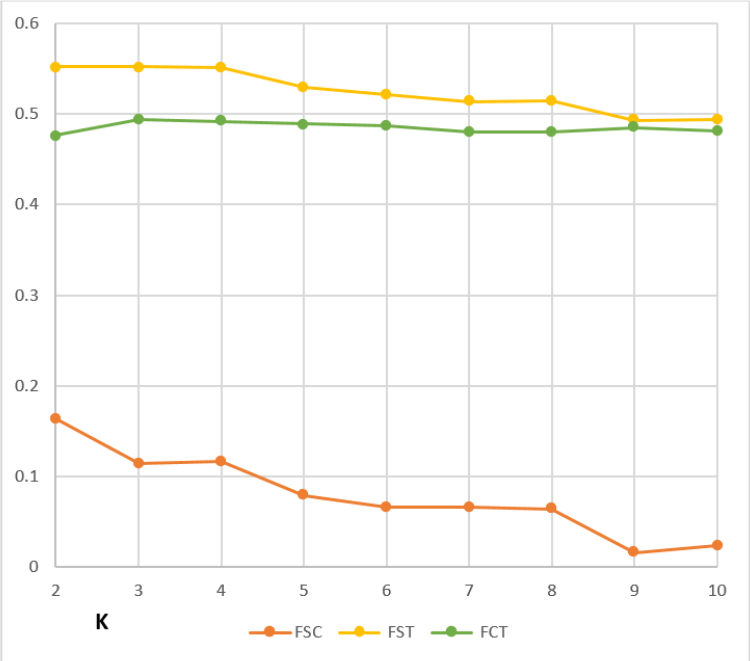


Figure S4 Spatial analysis of molecular variance (SAMOVA)

For each value of K (= hypothesized number of groups), the proportion of genetic variability found among populations (FST), among populations within groups (FSC) and among groups (FCT) are reported in the chart

Chapter 3.

STUDY 2:

Population and landscape genetics of the common frog (*Rana temporaria*) in the Trentino region: assessing current levels of intra-population genetic variability and present connectivity.

Population and landscape genetics of the common frog (*Rana temporaria*) in the Trentino region: assessing current levels of intra-population genetic variability and present connectivity.

Alexis Marchesini, Andrea Battisti, Cristiano Vernesi

Abstract

Amphibians are facing a dramatic crisis worldwide, with increasing reports of population declines and extinctions, A general common threat for many amphibian species is represented by habitat loss and fragmentation, leading to a reduction of gene flow and genetic variability, which in turn negatively affects the survival of populations. Alpine areas may be particularly prone to the effects of fragmentation, due to their complex topography, characterized by high mountain peaks and broad valleys, and ongoing climate change is predicted to affect the distribution of species, determining range shits and contractions. In this context, monitoring levels of genetic diversity and current patterns of connectivity play a crucial role in the development of effective long-term conservation strategies. Here, we used a model organism, the common frog, for a fine-scale analysis (79 sites; 1522 individuals) of patterns of genetic diversity at microsatellite markers, both intra- and inter-population, in a south-eastern Alpine region. We detected heterogeneous levels of genetic variability, with opposite latitudinal trends in two different sub-areas (i.e. eastern and western part of the region), potentially reflecting past evolutionary processes, although current isolation seems to play an important role for the considerably low genetic diversity of some specific populations. Genetic differentiation was generally high, and a main barrier was detected, corresponding to a broad valley in the middle of the region. Moreover, genetic differentiation showed different spatial patterns in the two sub-areas, reflecting different overall levels of connectivity, or different past evolutionary processes. Our intense, detailed genetic survey, performed using a common species, allowed us to highlight broad-scale and fine-scale spatial patterns of genetic diversity in the study region. The outcome of this study can be used as term of comparison with more endangered species, or for testing specific hypothesis in further investigations.

Keywords: amphibians, *Rana temporaria*, Alps, Trentino, genetic diversity, population structure, connectivity

Introduction

Amphibians are considered the most endangered group of vertebrate: they are facing a dramatic decline worldwide (Wake 1991; Houlahan *et al.* 2000; Gardner 2001; Stuart *et al.* 2004; IUCN 2016), characterized by global and local extinctions involving many different species in all continents. Numerous different factors have been invoked as potential explanations (Collins & Storfer 2003), and several scientists now argue that amphibian declines are complex phenomena, often driven by multiple abiotic and biotic stressors acting synergistically and giving rise to a vast array of complicated local interaction (Blaustein & Kiesecker 2002; Blaustein & Bancroft 2007; Blaustein *et al.* 2011). Due to this ‘‘context dependency’, general conservation strategies are difficult to develop and often inadequate: there is a strong need for local studies in order to find effective local solutions (Grant *et al.* 2016). Despite the complexity characterizing amphibian declines, a general common threat for many amphibian species is represented by habitat loss and fragmentation. Indeed, amphibians often have a patchy distribution and low dispersal rates, and connectivity plays an essential role in regulating demographic and evolutionary processes of their populations. Therefore, the effects of habitat loss and fragmentation seem to be particularly severe for this group of animals (Marsh & Trenham 2001; Cushman 2006). Fragmentation can lead to a decrease in intra-population genetic diversity due to intense drift. A loss of genetic diversity, in turn, may have detrimental effects on populations, increasing the risk of extinction (Frankham 2005). Indeed, genetic diversity has a crucial evolutionary importance, being required for populations to adapt to a changing environment and to develop resistance to diseases (Booy *et al.* 2000; Reusch *et al.* 2005; Höglund 2009); moreover, a loss of genetic diversity is often associated with inbreeding depression and fitness reduction (Frankham et al., 2002; Hansson & Westerberg 2002; Reed & Frankham 2003). In the face of amphibian declines, it is therefore of great relevance to monitor genetic diversity and understand the underlying processes.

In the context of global change and biodiversity decline, Alpine environments are considered of great concern, being heavily affected by both the ongoing increase in temperature (Cannone *et al.* 2008; Brunetti *et al.* 2009; Gobiet *et al.* 2014) and habitat alteration (Chemini & Rizzoli 2003; Lassen & Savoia 2005; Vanham *et al.* 2009): these fragile habitats deserve therefore a special attention.

In Alpine landscapes, mountains may be perceived as islands for cold-adapted species, isolated by low elevation valleys and main rivers, which represent strong migration barriers

for many vertebrate species, limiting gene flow among populations (Lomolino & Davis 1997; Li *et al.* 2009; Zhan *et al.* 2009). On the other hand, the highest mountain ridges may act as important physiological barriers, limiting the distribution and dispersal of other species (Funk *et al.* 2005; Zhang *et al.* 2011). As a result, Alpine animal species often experience a complex mosaic of fragmented, suitable habitat patches, within an unsuitable landscape matrix, and the scenario may be further complicated by human-driven habitat alterations and climate change (Barry *et al.* 1995; Watson *et al.* 1998). Understanding how landscape features are perceived by the different species is therefore of crucial importance; however; it is not always straightforward, particularly for elusive animals such as amphibians. Genetic methods can provide valuable information in this regard (Storfer *et al.* 2009), and sometimes even revealing counterintuitive patterns (e.g. Spear *et al.* 2005).

In this study, we chose the common frog (*Rana temporaria*) as a model organism for investigating spatial patterns of genetic diversity, both within- and among populations, with a fine-scale analysis in a south-eastern Italian Alpine region (Trentino, Autonomous Province of Trento). Specifically, using 12 microsatellites markers, we aimed at: (1) assessing current levels of genetic variability, identifying genetic diversity "hotspots" and situations of criticality; (2) estimating genetic differentiation among populations and population structure; (3) evaluating patterns of present connectivity and identifying potential barriers to gene flow. Moreover, since a previous phylogeographic study highlighted a complex evolutionary history for the species in the study region (see Chapter 2 of the present thesis), we investigated the potential effects of past processes on current patterns of genetic diversity measured at microsatellite loci.

The common frog is one of the most widespread and abundant amphibian species in Europe (Gasc 1997); in addition, this anuran is characterized by high adaptability to different ecological conditions, showing local adaptations even for short geographic distance (Muir *et al.* 2014; Richter-Boix *et al.* 2010): it is therefore a perfect candidate for evolutionary (e.g. Miaud 1999; Laugen *et al.* 2003), and genetic studies (e.g. Hitchings & Beebee 1997; Palo *et al.* 2004; Johansson *et al.* 2006). The importance of studies focusing on common species is well recognized in conservation genetics, since fine-scale, detailed studies are usually difficult to implement for rare organisms. Genetic patterns detected in model organisms can be used as term of comparison with more endangered species, in order to highlight similarities and important differences reflecting peculiar evolutionary features, or for testing specific hypothesis in further investigations (Whiteley *et al.* 2006). Lastly, it should be recognized that, although not considered endangered (due to its wide distribution), the

common frog is experiencing local declines in several areas, particularly at the southern limit of its distribution (Lanza *et al.* 2009). Moreover, range shifts and reductions (leading to increased fragmentation) are predicted for this species in the near future, in response to ongoing global warming (Bartolini *et al.* 2014). Since local declines of widespread, abundant species may have important consequences at the ecosystem level (Gaston & Fuller 2008; Gaston 2010; Redforf *et al.* 2013), we stress the conservation relevance of monitoring the genetic status of the common frog, a key component of many low-productivity alpine habitats (Luiselli *et al.* 1995; Lodé 1996; Sztatecsny *et al.* 2013), together with other dominant species.

Materials and Methods

Ethics Statement

All conducted experiments complied with the current laws of Italy. Sampling and monitoring procedures were approved by the Italian Ministry of Environment and the Environmental Unit of the Autonomous Province of Trento (DPN/2D/2003/2267 and 4940-57/B-09-U265-LS-fd). Samples from Veneto were collected thanks to a collaboration with University of Padova (Dept. of Biology).

Sampling

Our study area is Trentino (Autonomous Province of Trento, Italy), a mountainous region of 6212 km^2 belonging to the eastern Italian Alps. The region is characterized by a complex terrain (elevation range: 65-3764 m above sea level; more than 70% of surface lies above 1000 m a.s.l), including part of the Dolomites and Prealps as well as low elevation valleys. The Adige river valley (130–270 a.s.l.) represents the major discontinuity, dividing the area into western and eastern halves, with a north-south orientation. This central valley is also characterized by intensive agriculture and a high level of urbanization, while the rest of the region is mostly covered by forests (> 56%).

We selected sampling areas in order to cover the whole geographic and altitudinal distribution of the species in the study region, as well as different ecological environments. In years 2009-2012, the selected areas were screened for common frog spawn during the breeding season. We collected eggs and larvae: one fertilized egg from each distinct clutch, and tadpoles coming from separate ponds, therefore minimizing the probability of gathering full-sibs. This sampling procedure has been widely used in earlier studies with the common

frog and other pond breeding amphibians (e.g. Rowe *et al.* 1998; Brede & Beebee 2004, 2006; Stefani *et al.* 2012; Van Buskirk 2012). Tadpoles were stored in 95% ethanol until DNA extraction, while eggs were brought to the laboratory, were allowed to hatch and larvae were harvested at Gosner stage 23 (active swimming, Gosner 1960), following indications in previous studies (e.g. Brede & Beebee 2004; Stevens *et al.* 2006; Johansson *et al*. 2013). GPS coordinates of each sample were recorded, and samples coming from different ponds within the same 1 km^2 area were considered belonging to the same sampling site (Johansson 2005, 2007). Three additional areas (LPo, MP2, Pos), located outside of the political borders of the Autonomous Province of Trento were included in the study, because of their particular geographic position (at the southern margin of *Rana temporaria* distribution range in the considered part of the Alps). Specimens from these additional sites were collected during field surveys focused on other amphibian species.

A total of 1522 samples were collected from 79 different sites (Fig. 1).

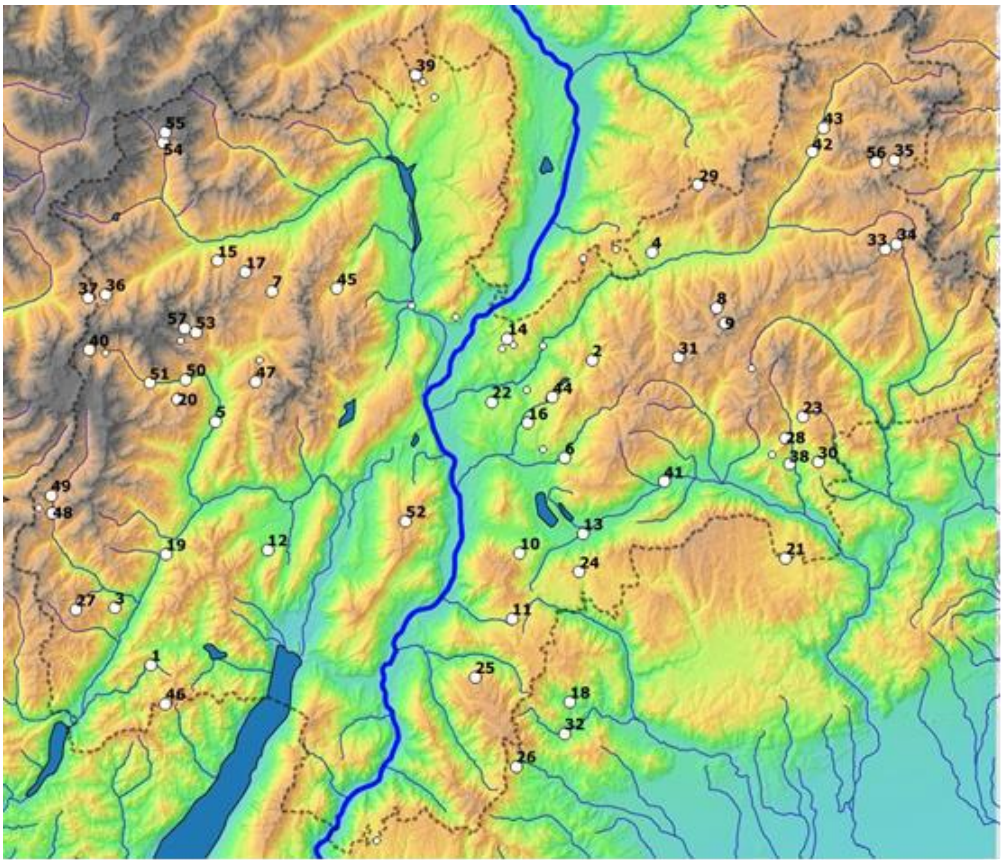


Figure 1 Sampling sites of *Rana temporaria* in the Trentino region. Small white points represent sites with N < 9; big white points, with associated numbers, represent sites with N ≥ 9. Sites are numbered according to Table 1 and Table S1 in Supporting Information). The blue line in the middle of the region represents the Adige river.

DNA extraction and microsatellite genotyping

Total genomic DNA was extracted using the Qiagen Dneasy 96 Well Plate Kit (QIAGEN Inc., Hilden, Germany), following the manufacturer's protocol. 21 tetranucleotide microsatellite markers (SSR) originally developed for *Rana temporaria* by Matsuba & Merilä (2009) were initially tested on a subset of samples, and the 13 SSR that successfully amplified in all samples were selected for subsequent genotyping (Table S2a). The selected loci were amplified in 4 multiplex PCR reactions under the conditions described in Table S2b (Supporting Information). Contamination and repeatability were rigorously checked by means of negative and positive controls, respectively. PCR products were run on ABI Prism 310 Genetic Analyzer (Applied Biosystems) and 2 reference samples were included in each run, in order to avoid errors due to different electrophoretic conditions. Amplified fragment lengths were scored using GeneMapper 3.7 software (Applied Biosystems).

Statistical analysis

Each SSR locus was tested for the presence of null alleles, allele drop-out and scoring errors using MicroChecker (Van Oosterhout *et al.* 2004) and FreeNa (Chapuis & Estoup 2007). Test of departure from Hardy-Weinberg was performed for each locus in every population, by means of permutation tests with the software Arlequin 3.5 (Excoffier & Lischer 2010). Linkage disequilibrium (LD) for each pair of loci was checked using Genepop 4.1.4 (Rousset 2008). For the purposes of further analyses, we will refer to three different datasets: dataset A, including all genotyped samples (1522); dataset B, including all sampling sites (hereafter populations) with sample size (N) $\geq$ 9 (57 sites, for a total of 1444 samples); dataset C, including all sites with N $\geq$ 18 (47 sites, for a total of 1320 samples). This distinction was made since the accuracy of the different analytical methods is affected in different ways by sample size (Kalinowski 2005; Sinclair *et al.* 2009; Hale *et al.* 2012). We therefore found a compromise between data availability and analytical requirements, with the aim of both covering the whole considered geographical area and relying on accurate estimates.

Standard genetic diversity measures, including observed heterozygosity (H_o), expected heterozygosity (H_e) and mean number of alleles (Na), were computed for each population, using dataset C (N $\geq$ 18), using Arlequin 3.5 (Excoffier & Lischer 2010). In addition, rarefied allelic richness (AR) and locally common alleles (LCA) were also computed for populations in dataset C, using the software FSTAT 2.9.3.2 (Goudet 2001), and GenAlEx 6.5 (Peakall & Smouse 2006), respectively. Allelic richness provides an unbiased estimate of the mean number of alleles, corrected for differences in sample size

using rarefaction (El Mousadik & Petit 1996). Locally common alleles are defined as those that are present locally with frequency > 5%, but occur in less than 25% of populations (LCA_{25}), or in less than 50% of populations ($LCA_{50;}$ Maguire *et al.* 2002). We tested for the presence of geographic patterns in the recorded genetic diversity levels, assessing the correlations with latitude and longitude by means of Pearson coefficient in R statistical environment (R Core Team 2016). Correlation analyses were performed for the whole study area, as well as for the different subsets identified by STRUCTURE (see below).

Differentiation between pairs of populations was assessed using both traditional F_{ST} and Jost's D. G_{ST}-values and its relatives (F_{ST}) have been traditionally the most used indices for assessing genetic differentiation among populations. However, since the proposal of the new index D (Jost 2008), there has been a lot of debate over the validity of F_{ST} as a measure of population genetic differentiation. Particularly, F_{ST} has been found to underestimate differentiation when heterozygosity is high (Jost 2008; Heller & Siegismund 2009). This limitation is overcome by Jost's D, which partition genetic diversity into pure and independent within- and between-group components (Jost 2008). Actually, simulations showed that neither F_{ST} nor D operates satisfactorily in all situations (e.g. Jost's D is very sensitive to mutation models; Leng & Zhang 2011): in empirical studies, both indexes should be calculated and compared for a more accurate assessment of population differentiation (Meirmans & Hedrick 2011; Leng & Zhang 2011; Ma *et al.* 2015). Pairwise F_{ST} values (Weir & Cockerham 1984) were computed with Arlequin 3.5 (Excoffier & Lischer 2010); Jost's D values (corrected for small sample size, D_{est}) with GenAlex 6.5 (Peakall & Smouse 2012). Significance of both F_{ST} and D_{est} values was tested with 10,000 permutations and associated P values were adjusted for multiple comparisons using false discovery rate method (FDR; Benjamini & Hochberg 1995), as implemented in "p.adjust" R function (R Development Core Team 2006). Correlation between F_{ST} and D_{est} matrices was tested through a Mantel test with 10,000 permutations, using the Ecodist R Package. Correlation analyses were carried out both on the global dataset and within the single main clusters identified by Bayesian analyses (STRUCTURE; see below). In order to graphically visualize genetic relationships among populations, we used both F_{ST} and D_{est} matrices to perform principal coordinates analyses (PCoA) using GenAlEx.

Population structure was further investigated using the Bayesian clustering approach implemented in the software STRUCTURE 2.3.4 (Pritchard *et al.* 2000). STRUCTURE's algorithm uses individual multilocus genotype data to cluster individuals into groups (K), based on based on minimization of gametic phase and Hardy-Weinberg disequilibrium. We

applied the "admixture" ancestry model and the "correlated allele frequency" model, which have proved to be more powerful with populations sharing recent ancestry (Falush *et al.* 2003). We performed 10 independent runs for each K to check consistency across runs. A first exploratory analysis was performed with K max set to 57 (potential maximum number of different clusters), using 100,000 iterations after a burn-in period of 50,000 for each run. Then, a second analysis were performed with K max set to 10, according to the outcome of previous analysis, using 1,000,000 iterations after a burn-in period of 250,000 for each run. The most likely value of K was selected by means of Structure Harvester (Earl & vonHoldt 2012), following Evanno's method (Evanno *et al.* 2005). Since STRUCTURE relies on an individual-based method, all runs were performed using dataset A (all genotyped samples). Different studies have shown the tendency for the optimal K inferred using Evanno's method to capture only the higher-level of subdivision (Evanno *et al.* 2005; Vaha *et al.* 2007: Pisa *et al.* 2014), in the presence of hierarchical population structure (a common feature of fragmented habitats with the combination of locally divergent populations and major barriers to gene flow). Therefore, we performed separate STRUCTURE re-analyses of the groups identified in the first step analysis, using the same parameters as above. Geographical patterns in the average population assignment probabilities (Qp) for the different identified clusters were evaluated testing the correlations with latitude and longitude, by means of Pearson correlation coefficient in R statistical environment (R Core Team 2016). In addition, an analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) was carried out with the software ARLEQUIN 3.5 (Excoffier & Lischer 2010), using the higher-level grouping pattern inferred by STRUCURE. The statistical significance of the variance components was tested by means of 10,000 permutations.

For a more detailed understanding of spatial patterns of genetic differentiation, we tested for isolation by distance (IBD; Wright 1943) among populations, performing a linear regression analysis of genetic distances, estimated as Slatkin's (1995) linearized F_{ST} (F_{ST} /1 - F_{ST}), and the natural log of geographic distances (Rousset 1997). Euclidean distances between populations were calculated in R statistical environment (R Core Team 2016). Significance of the correlation was tested by means of a Mantel test, performed in Ecodist R package using 10,000 permutations (Goslee & Urban 2007). Using Ecodist package, we also computed Mantel correlograms (Smouse *et al.* 1986; Goslee & Urban 2007). In Mantel correlogram, the dataset is partitioned into spatial lags, which include only the pair-wise comparisons that fall within a certain class of geographic distance. A Mantel test is performed on each distance class and a correlogram is generated, with distance classes on

the x-axis and corresponding Mantel r on the y-axis. In other words, this approach decomposes the relationship between geographic and genetic distances, allowing to determine the exact spatial scale at which IBD occurs (Epperson 2003). The optimal number of distance classes was determined using Sturge's rule. IBD analysis and Mantel correlograms were computed for both the whole dataset (dataset B), and the separate subsets identified by STRUCTURE analysis (see below), and repeated considering D_{est} as genetic distance.

Results

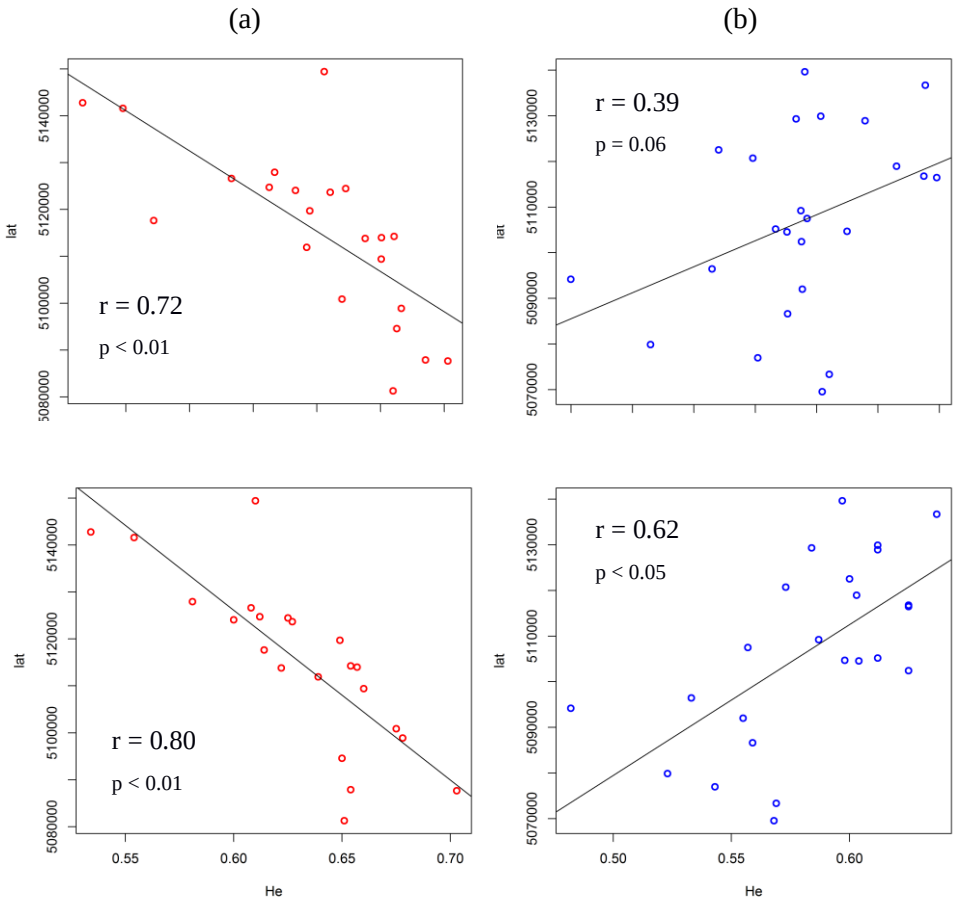
A total of 1522 samples from 79 populations were successfully genotyped at the 13 selected loci. MicroChecker excluded the presence of allelic drop-out or scoring errors. FreeNA detected evidence for null alleles at locus BFG072, in most of the populations. We therefore excluded BFG072 from further analyses. Neither loci nor populations showed systematic deviations from HWE, and only 15 of 564 were significant for $P < 0.05$, after adjustment for multiple comparisons using false discovery rate method (FDR; Benjamini & Hochberg 1995). No significant evidence of linkage disequilibrium was observed between the selected loci (only 3/78 significant values after FDR correction). Despite all loci were claimed to be tetranucleotides, BFG131 showed an unexpected dinucleotide allelic pattern. After sequencing by means of non-marked primers, we concluded that the recorded allelic pattern was due to a deletion in the flanking region, and not to mutations in the repeat motif (which proved to be a perfect tetranucleotide microsatellite). Due to this deletion, allele size was not proportional to number of repeats. However, since all further analyses didn't assume the stepwise mutation model (SMM; Kimura & Otha 1978), i.e. all our results are not affected by differences in allele size, we decided to retain the considered locus (see Appendix I in Supporting Information, for a detailed discussion). All the 12 ultimately selected loci were polymorphic, with a total number of alleles of 191 (average across loci: 14.7).

Intra-population genetic diversity

We detected heterogeneous levels of intra-population genetic diversity among populations (Table 1). For example, expected heterozygosity (H_e) ranged from 0.48 to 0.70 (mean = 0.60); allelic richness (AR) from 3.5 to 7 (mean = 5.87). ViT (n° 52 on map in Fig. 1) and DDB (n° 10) showed the lowest levels at both H_e and AR. Other populations showing low levels, for both measures, were: MP1, VP1, VP2, RM1 and Ing (see Tab.1 for their n° on map). The populations showing the highest levels of H_e ($H_e > 0.65$; 9 populations) belong to the Western part of the region (hereafter West Trentino); populations with high levels of AR

(AR > 6.5; 12 populations) were detected in both West (8) and East (4) Trentino. Considering the whole study area, a moderate negative correlation was observed between H_e and longitude (r =0.40; $p < 0.01$). Correlation analyses for evaluating geographic patterns of genetic diversity were repeated in the two separated sub-areas: West Trentino and East Trentino (this separation has both a geographic and a genetic meaning; see STRUCTURE results below) and interesting patterns emerged. In West Trentino, all measures of genetic diversity showed a strong, negative correlation with latitude (H_e: r = 0.80; AR: r = 0.72; LCA_{50}: r = 0.69; all $p < 0.01$; Fig. 2a). By contrast, in East Trentino, H_e and AR showed a moderate-low positive correlation with latitude (Fig. 2b), although only marginally significant for AR (H_e: r = 0.62, $p < 0.01$; AR: r = 0.39, p = 0.06). In this sub-area, a low positive correlation was also detected between H_e and longitude (r = 0.42; $p < 0.05$; Fig. 2b). Taken together, these results indicate a strong decline in genetic diversity with latitude in West Trentino; while for East Trentino, the highest values seem to be located in the north-eastern corner, although patterns are more complex.

Figure 2 Correlation between latitude and standard measures of genetic diversity (AR = allelic richness; H_e = expected heterozygosity), in West (a) and East (b) Trentino



code	site	N	H_e	H_o	Na	AR	LCA_{25}	LCA_{50}
1	Amp	30	0.651	0.622	7.417	6.599	1.750	3.333
2	Bed	20	0.625	0.587	7.167	6.979	1.667	2.917
3	Bon	24	0.654	0.649	7.417	6.855	1.667	3.583
4	Bro	21	0.612	0.599	6.667	6.395	1.500	3.083
5	Cad	44	0.660	0.669	7.917	6.507	1.750	3.583
6	Can	18	0.612	0.593	5.667	5.667	1.083	2.417
7	CCC	26	0.625	0.601	6.917	6.227	1.333	3.000
8	Ce1	29	0.600	0.621	5.750	5.202	1.083	2.667
9	Ce2	24	0.573	0.604	5.833	5.481	1.083	2.250
10	DDB	20	0.482	0.488	4.083	3.998	0.750	1.417
11	Ech	24	0.559	0.563	6.250	5.764	1.583	2.667
12	Fia	31	0.650	0.637	7.417	6.629	1.583	3.083
13	Ing	33	0.533	0.508	5.833	5.149	1.250	2.417
14	Lag	29	0.603	0.580	7.500	6.652	2.000	3.667
15	LCa	31	0.581	0.610	6.250	5.668	1.667	2.833
16	Lel	24	0.587	0.566	6.333	5.872	1.250	2.750
17	LMe	36	0.608	0.623	6.083	5.330	1.083	2.250
18	LPo	19	0.543	0.526	5.583	5.521	1.250	2.417
20	LSG	53	0.639	0.646	7.417	5.921	1.750	3.250
24	Mon	24	0.555	0.576	6.333	5.883	1.333	3.000
25	MP1	37	0.523	0.493	5.000	4.647	0.917	1.917
26	MP2	27	0.568	0.565	6.750	6.046	1.500	2.917
27	MRe	52	0.703	0.703	8.583	7.031	1.917	3.750
28	Mug	28	0.557	0.554	6.500	5.922	1.667	2.750
29	PLa	23	0.637	0.655	7.333	6.886	1.750	3.250
30	PLC	26	0.598	0.606	6.833	6.249	1.667	3.083
31	PMa	42	0.625	0.605	8.750	6.873	2.500	4.083
32	Pos	19	0.569	0.513	6.167	6.103	1.417	3.000
33	PR1	18	0.584	0.505	5.833	5.833	1.000	2.167
34	PR2	19	0.612	0.618	6.083	6.033	0.833	2.250
35	PS2	22	0.597	0.629	6.167	5.903	1.000	2.333
36	PT1	18	0.600	0.574	5.833	5.833	1.083	2.500
37	PT2	30	0.627	0.602	6.833	6.106	1.667	3.083
38	PTe	23	0.604	0.554	6.167	5.759	1.250	2.667
39	PTr	20	0.610	0.579	6.250	6.059	1.250	2.667
40	RM1	36	0.614	0.602	5.167	4.719	1.000	2.083
41	Ron	31	0.625	0.599	6.500	5.877	1.500	2.833
45	Tov	26	0.612	0.625	6.083	5.627	1.250	2.500
47	Va1	21	0.657	0.687	6.750	6.508	1.083	2.500
48	VD1	40	0.678	0.671	7.833	6.664	2.000	3.667
49	VD2	20	0.675	0.679	6.333	6.199	1.250	2.500
50	VG1	42	0.654	0.647	8.000	6.608	2.083	3.750
51	VG3	29	0.622	0.629	7.167	6.380	1.250	3.000
52	ViT	20	0.506	0.508	3.500	3.465	0.167	1.333
53	VN2	37	0.649	0.646	6.750	5.946	1.167	2.750
54	VP1	33	0.554	0.598	4.917	4.477	1.000	1.833
55	VP2	21	0.534	0.540	4.250	4.161	0.667	1.417

Table 1 Genetic variability in *Rana temporaria* populations (dataset C; N≥_18). N = number of genotyped samples; H_e = expected heterozygosity; H_o = observed heterozygosity; Na = mean number of alleles; AR = rarefied allelic richness; LCA_{25} and LCA_{50} = least common alleles, found in < 25% and < 50% of the populations, respectively. Populations are numbered according to Table S1 in Supporting Information.

Genetic differentiation and population structure

Overall, pairwise F_{ST} values highlighted a high level of genetic differentiation among populations (F_{ST} max = 0.293; F_{ST} mean = 0.093; Table S3 in Supporting Information). Comparisons yielding the highest F_{ST} values involved populations at opposite side of the Adige valley (i.e. West vs East Trentino). Indeed, considering the two separate sub-areas, the maximum recorded F_{ST} values were 0.15 for West Trentino (mean F_{ST} = 0.05), 0.17 for East Trentino (mean F_{ST} = 0.06). Despite the fine-scale sampling design, only 20/1596 comparisons (1.25 %) yielded non-significant values ($p > 0.05$, after adjustment for multiple comparisons using false discovery rate method; Benjamini & Hochberg 1995). Most of these comparisons involved populations separated by less than 10 km, but with notable exceptions. In particular, in West Trentino, 2 comparisons between populations separated by 20 and 25 km, respectively (LRo-VG1; VD1; VN1) yielded non-significant F_{ST} values; in East Trentino, even a comparison between populations separated by 30 km yielded non-significant F_{ST} values. By contrast, significant F_{ST} were found for populations separated by a few km, in some cases (e.g. Ce1-Ce2: F_{ST} = 0.034; geographic distance = 2 km). ViT, a population laying on an isolated mountain massif in the middle of the study region (Monte Bondone; n° 52 on map in Fig. 1), yielded high F_{ST} values with all other populations (F_{ST} max = 0.27; min = 0.12; mean = 0.17). A similar pattern was found for another populations located in central part of the area, DDB (F_{ST} max = 0.27; min = 0.06; mean = 0.14; n° 10 in Fig. 1). Other populations showing strong patterns of differentiation, although not so generalized, were VP1 (F_{ST} max = 0.27; min= 0.009; mean = 0.15) and VP2 (similar values). These two populations are situated on a mountain chain in the north-western corner of the region (n° 54 and 55 in Fig. 1).

Pairwise D_{est} values were always higher than corresponding F_{ST} (D_{est} max = 0.461; mean = 0.157), but the two measures were highly correlated, for both the global dataset (Mantel R = 0.976; $p < 0.01$), and the two subsets corresponding to Western and Eastern Trentino, considered separately (Mantel R = 0.972 and 0.985; all $p < 0.01$). D_{est} values will be therefore not discussed here.

Principal coordinate analysis (PCoA), based on pairwise F_{ST} values, pointed to a clear separation between populations lying on the western and eastern side of the Adige Valley (West Trentino and East Trentino, respectively; Fig. 3). ViT (n° 52 in Fig. 1) was plotted in the middle of the chart, although slightly closer to the East Trentino group. PCoA based on pairwise D_{est} led to the same population grouping (not shown).

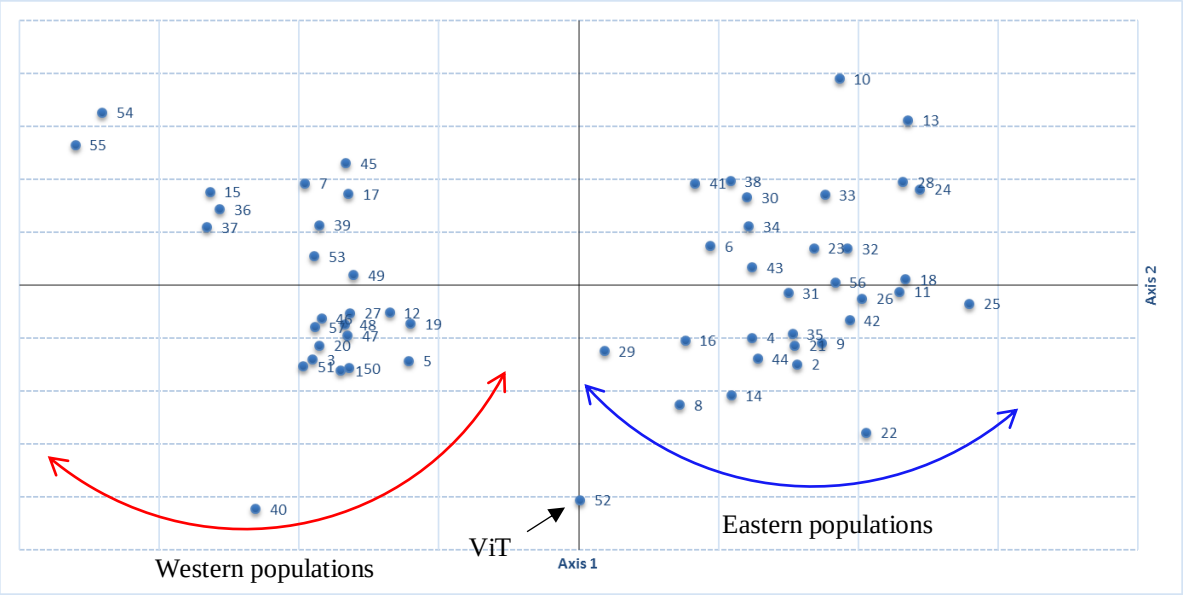


Figure 3 PCoA plot constructed using pairwise F_{ST} values for the 57 common frog populations (dataset B). Each point represents a population. Populations are numbered according to Table S1 in Supporting Information. The first and the second axis explained 40% and 8% of the variation, respectively.

The first step of the Bayesian clustering analysis, performed with STRUCTURE software, suggested K = 2 as the most likely higher-level subdivision, according to the Evanno method (Evanno et al., 2005) (Fig. S1 in Supporting Information). The two clusters clearly correspond to West and East Trentino, with the Adige river as separation line (Fig. 4). The only exception to this pattern is represented by ViT: although being geographically located in the western side of the Adige river (n° 52 in Fig. 1), STRUCTURE grouped this population within the cluster corresponding to East Trentino. The average assignment probabilities for each population (Qp) indicated low admixture between the two clusters, suggesting a relatively high level of differentiation. In the second level of Structure analysis, with the Bayesian clustering analysis separately conducted for the two previously identified clusters, K = 2 was again identified as most likely grouping, for both subsets. In this case, however, the average assignment probabilities for each population (Qp) showed variable levels of admixture between the 2 clusters, in each subset. Interestingly, Qp showed a strong correlation with latitude, in both subsets ($r = 0.84$ and $r = 0.8$; both $p < 0.05$). This may be interpreted as an evidence that the detected sub-structure, both in East and West Trentino, may be better explained as a north-south cline in allele frequencies, rather than two clearly separated clusters.

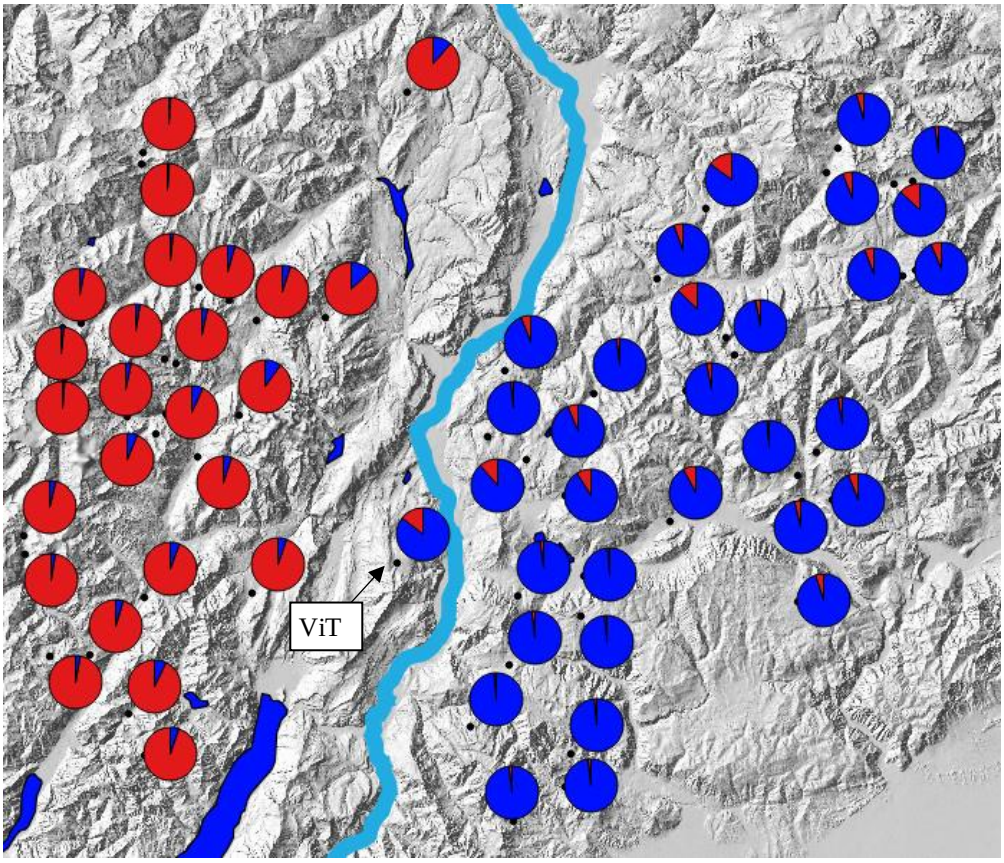


Figure 4 Average assignment probability (Qp) for each *Rana temporaria* population, according to K = 2 (STRUCTURE; step 1, i.e. analysis performed on the whole dataset). The light blue line, in the middle of the region, depict the Adige river. Note the position of ViT (see text).

The AMOVA analysis, performed using the population grouping inferred by STRUCTURE (K = 2; whole dataset analysis), showed a significant partitioning of genetic variation ($P<0.001$) for the tested structure (Table S4 in Supporting Information). However, the assumed grouping explained only the 6.75 % of the total variation, and high genetic variation within populations was revealed (explaining 87.5 % of the total variation).

Spatial analysis

Both IBD and Mantel correlogram analyses yielded identical results regardless of the selected genetic distance (linearized F_{ST} and Dest): we will therefore report only results based on linearized F_{ST}. Considering the whole study region, we detected a moderate significant relationship between genetic distance and the logarithm of geographic distance

(Mantel r = 0.469; p < 0.01). When we conducted separate IBD analysis for the West and East Trentino populations, ViT was excluded, due to its central, isolated position, the high F_{ST} values showed with all other populations, and its ambiguous collocation (geographically located in West Trentino, but genetically closer to the East Trentino group; see above). Different IBD patterns were detected for the two sub-area (Fig. 5a, 5b). Specifically, in West Trentino the correlation remained moderately high (Mantel r = 0.416; p < 0.01), while in East Trentino a considerably lower correlation was detected, although still significant (Mantel r = 0.228; p < 0.01). The two sub-areas showed differences also in the slope of the regression line, which in West Trentino was twice as big as in East Trentino.

In order to further investigate the underlying spatial structure, we compared the shape of Mantel correlograms in the two sub-areas. Eight distance classes were identified for both sub-areas, with a class distance interval (cdi) of 9.6 (West Trentino) and 10.1 (East Trentino) km. Total distance range were 77 km for West Trentino, and 81 km for East Trentino. The two separate analyses are therefore highly comparable, reflecting a symmetrical sampling design. However, the shape of the correlograms were considerably different in the two sub-regions (Fig. 6a, 6b). We will discuss here only the first part of the correlogram (i.e. lower distance classes), since in all-directional correlograms, as distances become larger, correlation coefficients tend to become less significant and less interpretable due to the blurring effect caused by diagonal comparisons (Legendre & Legendre 1998). In West Trentino, a significant correlation between genetic and geographic distances was found for the first two distance classes, therefore for distances up to 19.2 km (2 x cdi), then the correlation decreased following a gradual, linear pattern. By contrast, in East Trentino only the first class yielded a significant positive correlation, therefore for distances up to 10.1 km. Then the curve rapidly approached 0, showing a steeper slope than in West Trentino. In other words, spatial autocorrelation of genetic distances is relatively pronounced even at medium scale in West Trentino, while is limited to fine scale in East Trentino. Integrating IBD results with the output of Mantel correlograms, the lower correlation between genetic and geographic distances detected for East Trentino seems therefore to be driven by a more pronounced spatial "patchiness" of populations, even for relatively short geographic distances.

Figure 5 Isolation by distance (IBD): genetic distances (Slatkin linearized F_{ST}) are plotted against the logarithm of geographic distances, for West Trentino (a) and East Trentino (b). Manter r are reported (all $p < 0.01$), together with the slope of the regression line.

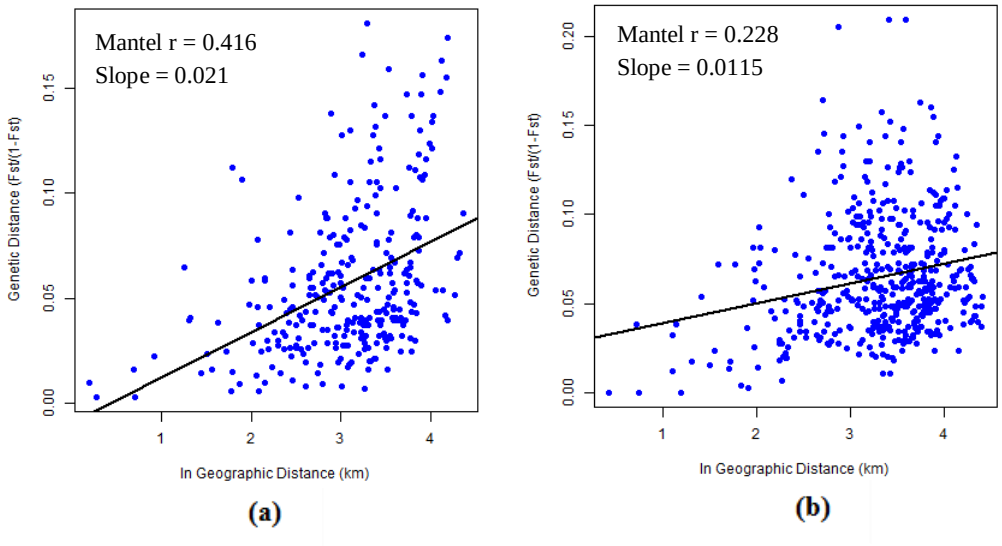
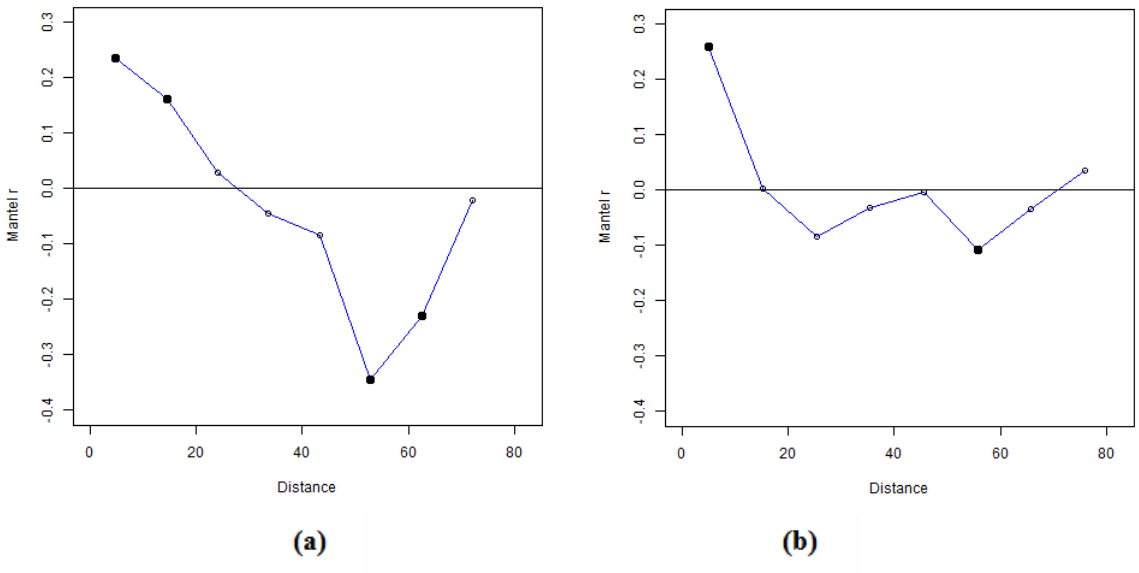


Figure 6 Mantel correlograms based on genetic distances (Slatkin's linearized F_{ST}), for West Trentino (a) and East Trentino (b). Similarly to Pearson's coefficient, Mantel r ranges from -1 to 1: negative values indicate negative autocorrelation (populations are genetically less similar than expected by chance), positive values indicate positive autocorrelation (populations are genetically more similar than expected by chance). For each distance class, a black filled dot represents a significant Mantel r value ($p < 0.05$; 10,000 permutations), empty dot non-significant value. Horizontal line: Mantel r = 0.



Discussion

Complex patterns of genetic diversity and differentiation were highlighted for the common frog in the Trentino region. Overall, the recorded levels of intra-population genetic variability seem to be in line with data from other European areas. For example, Brede & Beebee (2006), in a genetic survey on common frog populations from across Europe, reported a mean He of 0.687 (range: 0.615-0.745) and mean allelic richness of 5.47 (range 4.61-6.12). Palo *et al.* (2004), for 29 European populations of the species reported a mean H_e of 0.535 (range 0.35-0.72) and a mean allelic richness of 3.9 (range: 3.5-7.3). Both the cited studies were performed using 8 microsatellite loci, however characterized by high levels of polymorphism (21 and 25 mean n° of allele/locus, respectively), considerably higher than our microsatellite set (mean n° of allele/locus = 15). Anyway, direct comparisons among studies based on different markers must be considered with extreme caution, and we prefer to focus on the differences and peculiarities highlighted within the study region.

Indeed, we detected heterogeneous levels of genetic diversity among different populations and areas. Two main findings emerged from the globally intricate scenario: (1) populations which showed the lowest genetic diversity levels (e.g. ViT, DDB; discussed above) were also found to be characterized by the highest levels of genetic differentiation, and (2) latitudinal trends were detected for genetic diversity levels, in both the two sub-regions considered separately (West and East Trentino), but, interestingly, with contrasting patterns. Regarding point (1), this was markedly evident for two populations located on two isolated, calcareous, Prealpine mountain massifs in the central part of the region (Monte Bondone and Vigolana). We provided important, quantitative evidence that a reduced connectivity leads to a substantial loss of genetic diversity in the common frog. This aspect is of particular relevance, since ongoing climate warming is predicted to cause latitudinal and altitudinal range shifts in cold-adapted species (including *Rana temporaria*; Bartolini *et al.* 2014), which in complex mountain landscape translates into an increase in fragmentation (cold-adapted species will be confined to mountain tops and therefore highly fragmented in space). Monitoring the entity and the biological consequences of this process deserves a high priority under a conservation perspective. However, connectivity does not seem to be the only factor affecting current levels of genetic diversity in the study species. The contrasting latitudinal patterns observed in the two sub-regions (point 2) are indeed difficult to explain considering only differences in connectivity or any other ongoing ecological process. West

and East Trentino don't seem to vary so widely in climate or anthropic impact: both are predominantly mountainous areas and, except for a few narrow valleys (where the species can be found, although sometimes at lower abundance), levels of urbanization are low. Interestingly, a previous phylogeographic study highlighted an analogous pattern for the species at mitochondrial DNA (see Chapter 2). Specifically, number of alleles showed a decrease towards the north in West Trentino ($r = -0.42$, $p < 0.05$), while in East Trentino an opposite trend was highlighted ($r = 0.59$, $p < 0.05$). These findings were interpreted as the effects of Pleistocene evolutionary history: sequential founder events, linked to the re-colonization process, were hypothesized to be the driver of the negative latitudinal trend in West Trentino), while addictive effect due to admixture among different lineages seemed to be responsible for the positive correlation found in East Trentino. Although we cannot completely exclude current factors (e.g. local, subtle differences in microclimate) as potential drivers for the mirroring pattern detected at microsatellite loci, the most parsimonious explanation might therefore be found in the legacy of past evolutionary history. Notably, in East Trentino we also detected a low positive correlation between He and longitude, and even in this case a correspondent pattern was previously found at mtDNA: low positive correlation between haplotype diversity and longitude, due to the presence of different mtDNA lineages in the north-eastern corner of the region.

These large correspondences seem to indicate that, even at regional spatial scale, current levels of genetic diversity are largely affected by past processes in these low-vagility vertebrates.

Considering population structure, the most relevant finding is the broad-scale genetic subdivision among East and West Trentino. Our fine-scale sampling led to the identification of the Adige river valley as clear-cut separation line, with only one exception to this pattern, which will be discussed above. The above mentioned broad valley, located in the middle of the region, is characterized by low elevation (and a correspondent warmer climate), high anthropization (high urban density, intensive agriculture, highways, etc.) and the presence of a large river (Adige). Understanding which of these factors is responsible for the strong barrier effect is therefore not easy, since the common frog is negatively affected by all these ecological and landscape features, and ultimately each one may contribute. In this regard, however, it might be worth assessing the effect of the Adige river on other amphibian species, less influenced by other factors. Indeed, in this valley some relict, small wetlands still persist, in a degraded landscape matrix, and some more thermophiles amphibian species find here their ecological optimum (i.e. *Bombina variegata*, *Rana dalmatina*; Caldonazzi et

al. 2002). Determining whether the Adige river is perceived as uncrosssable barrier for these species is a matter of conservation relevance, as their distribution is mostly limited to the lowlands flanking the river and both species are not common, suggesting a potential ongoing decline mainly due to habitat alteration (ascertained for *Bombina variegata*; Caldonazzi *et al.* 2002). We demonstrated that the two sub-regions, resulting from the detected barrier, host genetically differentiated common frog populations: two main conservation units are therefore suggested for the species. This consideration is reinforced by the different spatial patterns identified for intra-population genetic diversity.

As already anticipated, a single population stands as a (partial) exception to this geographical subdivision. Indeed, ViT (n° 52 in Fig. 1), although being located in the western side of the river, appeared to be genetically closer to eastern populations (Bayesian clustering analysis). Actually, it must be observed that the considered population showed high levels of differentiation with all other populations, as already discussed. This population corresponds to an isolated mountain massif (Monte Bondone), laying in the immediate proximity of the Adige river valley. The position of this mountain, relative to the Adige river, has changed throughout history: indeed, before Würm glaciation the river flowed following a different course, more precisely through a valley located at the western side of the mountain (Caldonazzi & Avanzini 2011). We cannot exclude, therefore, that the anomaly found for ViT might be due to a past connection with eastern populations; however, in this case we don't have any further evidence in support to this hypothesis.

Finally, different fine-scale spatial structures were highlighted in West and East Trentino (IBD and Mantel correlograms). Common frog populations in the two sub-regions, therefore, not only have distinct gene pools, but are also characterized by different spatial processes, both at intra- and inter-population genetic diversity. Further investigations are needed to disentangle the effects of present (e.g. difference in orographic morphology, leading to different connectivity patterns) and past (e.g. complex pattern of admixture among different evolutionary lineages) factors in explaining the recorded differences in fine-scale population structure. Indeed, both present limitations to gene flow, leading to a true process of isolation by distance, as originally defined by Wright (1943), and past processes (e.g. post-glacial recolonization) may lead to fine-scale patterns of spatial autocorrelation in genetic data (de Campos Telles & Diniz-Filho 2005; Meirmans 2011; Meirmans et al. 2011). Potentially, due to this "structural" difference, the species in the two sub-regions may be affected in different ways by the same stressors (e.g. climate change, but also the spread of emerging diseases). A different management seems, thus, to be strongly recommended.

Again, ascertaining whether these differences extend to other small vertebrate species, including more endangered taxa, is critical for developing an effective conservation planning, focused on the preservation of genetic diversity as well as genetic differences (potentially linked to local adaptation; Teske *et al.* 2008; Moritz *et al.* 2012), and for identifying the most critical conservation actions, in the face of complex, context-dependent biodiversity declines.

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Supporting Information

Appendix I

We selected 13 tetranucleotide microsatellite, which had been developed by Matsuba & Merila (2009) and tested by the authors on 46 *Rana temporaria* individuals.

However, the marker BFG131 didn't showed a tetranucleotide allele pattern in our dataset, but instead a dinucleotide allele pattern (i.e. the different alleles separated by 2 b.p.). We therefore decided to sequence the considered locus using non-marked primers, for an exact determination of the allelic state. Samples were selected in order to cover the majority of the alleles, according to the availability of homozygous individuals. All sequenced samples showed a tetranucleotide repeat motif in the microsatellite region, as stated by the authors (Matsuba & Merila 2009). No interruption in the repeat motifs was detected, and the microsatellite region consisted of perfect repeats in all samples. However, in several sequences, corresponding to specific alleles, we detected a 10 b.p. deletion in the flanking region (before the microsatellite region). Therefore, two allele sets were present in our dataset for locus BFG131: (1) carrying the "normal" microsatellite sequence and (2) carrying the deletion. Both sets consisted of alleles carrying the same tetranucleotide repeat, but, since the 10 b.p. deletion was responsible for a shift in allele size, this led to an apparent global dinucleotide allele pattern. Therefore, allele size is not proportional to number of repeats in locus BFG131. The alleles carrying the deletion were recorded in the whole study region, with a widespread distribution. Further tests (Microchecker, FreeNA) showed no evidence of allele drop-out or null alleles for the considered locus; no departure from Hardy-Weinberg equilibrium was detected (Arlequin).

Therefore, we decided to retain locus BFG131 for further analysis. It must be noted that no analysis was performed assuming the stepwise mutation model (SMM; Kimura & Otha 1978), i.e. all our results are not affected by differences in allele size.

Supplementary Tables

Table S1 List of sampling sites with geographic coordinates (UTM 32N), average elevation and number of genotyped samples (N). Sites with N ≥ 9 are numbered (n°) according to map in Fig.1.

n°	code	site name	long	lat	elev	N
1	Amp	Lago d'Ampola	628417.60	5081295.41	795	30
	Avi	Torrente Avisio	673684.52	5118162.14	398	5
2	Bed	Bedollo	679305.15	5116474.12	1183	20
3	Bon	Palù di Boniprati	624179.64	5087838.14	1206	24
	Bre	Pozza Brez	661126.63	5146801.84	1196	3
4	Bro	Brozin	686222.98	5128925.19	999	21
5	Cad	Caderzone	635910.74	5109449.63	741	44
6	Can	Canezza	676177.78	5105191.05	653	18
7	CCC	Campo Carlo Magno	642432.50	5124095.78	1649	26
8	Ce1	Alpe Cermis 1	693736.49	5122545.33	2204	29
9	Ce2	Alpe Cermis 2	694680.75	5120738.12	2329	24
10	DDB	Dos del Bue	671032.74	5094213.99	1014	20
11	Ech	Torbiera Echen	670105.21	5086611.27	1273	24
12	Fia	Fiavé	641854.05	5094566.97	665	31
13	Ing	Inghiaie	678193.60	5096406.92	444	33
14	Lag	Lagabrun	669606.82	5118968.18	1115	29
	Las	Palù Redont - Lases	671799.07	5113036.24	682	3
15	LCa	Lago dei Caprioli	636083.84	5127929.61	1385	31
16	Lel	Laghestel	671929.29	5109185.19	876	24
	Les	Monti Lessini	654529.75	5060974.39	1353	2
	LLa	Laghetti di Lasteati	697765.31	5115529.09	2167	3
17	LMe	Laghetti di Mezzana	639387.90	5126646.46	2061	36
	LNe	Lago Nero	678350.75	5128193.53	1790	2
18	LPo	Lago di Posina	676805.59	5076971.15	578	19
19	LRo	Lago di Roncone	630176.33	5094077.47	861	15
	LSa	Lago Santo	670259.53	5118236.08	1194	3
20	LSG	Laghi di S.Giuliano	631434.04	5111673.29	1974	53
	Mad	Maderlina	669013.68	5117793.82	1030	2
21	Mar	Marcesina	701657.05	5093641.50	1362	13
22	MBa	Monte Barco	667755.17	5111671.55	869	16
23	MCa	Masi Carretta	703677.00	5109929.54	1429	11
	Mez	Monte Mezzocorona	663633.26	5121420.71	914	7
24	Mon	Monterovere	677903.42	5091992.61	1240	24
25	MP1	Monte Pasubio 1	665544.16	5079695.49	1622	37
26	MP2	Monte Pasubio 2	670587.22	5069572.44	1004	27
27	MRe	Monte Remà	619688.13	5087671.65	1846	52
28	Mug	I Mughi	701666.64	5107535.63	1269	28
	PBr	Passo Brocon	705302.18	5110584.02	1612	3

n°	code	site name	long	lat	elev	N
29	PLa	Passo Lavazé	691637.15	5136707.18	1802	23
30	PLC	Parco La Cascatella	705422.11	5104710.20	965	26
	PLg	Palù Longa	682114.05	5129295.08	1435	1
	PLo	Palù Longia	659893.78	5148600.75	1572	5
31	PMa	Passo Manghen	689325.93	5116716.50	2083	42
32	Pos	Posina	676177.88	5073354.41	563	19
33	PR1	Passo Rolle 1	713133.95	5129291.31	1947	18
34	PR2	Passo Rolle 2	714373.36	5129504.97	2039	20
	PR3	Passo Rolle 3	715879.04	5130995.00	2170	3
	Pra	Pradellano	700105.77	5105584.46	910	4
56	PS1	Passo S. Pellegrino 1	712113.99	5139349.83	1838	9
35	PS2	Passo S. Pellegrino 2	714227.16	5139648.66	1940	22
	PS3	Passo S. Pellegrino 3	715655.07	5140255.29	1790	7
36	PT1	Passo Tonale 1	623180.22	5124088.48	1856	18
37	PT2	Passo Tonale 2	621165.25	5123673.77	1873	30
38	PTe	Pieve Tesino	702104.78	5104555.24	833	23
39	PTr	Palù Tremole	659254.64	5149321.39	1738	20
	Rip	Riposo	673760.14	5106183.30	730	8
40	RM1	Rifugio Mandrone 1	621113.72	5117638.14	2405	36
	RM2	Rifugio Mandrone 2	617691.23	5117656.05	2374	2
	Roc	Rocchetta	658591.25	5122720.04	252	5
41	Ron	Palude di Roncegno	687646.13	5102432.35	401	31
42	So1	Soraga 1	704802.38	5140609.57	1205	14
43	So2	Soraga 2	706035.52	5143301.19	1346	11
44	Ste	Palude di Sternigo	674812.61	5112122.57	1010	15
45	Tov	Lago di Tovel	649873.14	5124726.62	1210	26
46	Tre	Monte Tremalzo	630130.99	5076829.09	1668	11
47	Va1	Valagola 1	640508.73	5113981.17	1689	21
	Va2	Valagola 2	640996.68	5116498.59	1323	3
	VAm	Val d'Amola	631877.82	5118687.01	2362	2
48	VD1	Val Daone 1	617070.10	5098849.11	1651	40
49	VD2	Val Daone 2	616896.37	5100881.24	1847	20
	VD3	Val Daone 3	615553.75	5099434.12	1925	2
50	VG1	Val di Genova 1	632329.52	5114214.29	1053	42
51	VG3	Val di Genova 3	628270.87	5113827.68	1233	29
	VG4	Val di Genova 4	623191.74	5117261.38	1006	2
52	ViT	Torbiera delle Viote	657847.29	5097919.00	1570	20
57	VN1	Val Nambrone 1	632391.63	5120133.39	2339	9
53	VN2	Val Nambrone 2	633619.20	5119630.28	2170	37
54	VP1	Val di Peio 1	629704.98	5141635.24	2155	33
55	VP2	Val di Peio 2	630096.63	5142697.97	2577	21

Table S2a List of microsatellite markers selected for the study (Matsuba & Merila 2009). BFG072 were excluded from final analysis due to the presence of null alleles.

Locus	*Primer sequence*	*Dye*	T_a *(°C)*	*Repeat*
BFG239	F: GGAACCCTATAACCGTACCTCC	NED	54	AACT
	R: CTTGGGCAAACACATAAAAGGT			
BFG202	F: AAAACACAGCAACCCTCAAGAC	6FAM	54	CTAA
	R: TCCCTTGTCTCTTCTCTCATCC			
BFG237	F: GGATTCTACGGATCTTTGGACA	PET	54	GATA
	R: CCTTCCATTCTGTTTGTTAGGC			
BFG129	F: GCATGACAGATAAGCATAAG	6FAM	54	CTAT
	R: AAGCTGTTAAATCACTAGGC			
BFG072	F: AACTTTGCCACACCTGAAATG	VIC	56	TGTA
	R: AATGTTTGTCATCAGAGAGACCTG			
BFG099	F: CAGTAAGGAATGGATACTAAGC	PET	56	ACTC
	R: TCCAGTGTAGCATAACAGAGT			
BFG155	F: GATGCTTGCACTTGTCTCC	6FAM	56	TACT
	R: GTCAGCACGGATTCATAAAA			
BFG050	F: TAAGGGAAATTGTGTAATGCCC	PET	58	GAGT
	R: CTTGAGGCGATTTAGTTTGCAT			
BFG053	F: TTTAGTGAGCATTGTGGTGGAG	VIC	58	GTGA
	R: TGTTGAGGAGATTAAGTTCGCA			
BFG161	F: TCTCCAATGAACAGGAAGCAC	PET	58	AGAA
	R: GCAGCAACAACCTGATTAGAAA			
BFG131	F: CAGTACGTCAGCCATATCGTGT	6FAM	58	TACA
	R: GTGAAAGGAGGCAGCAAAGT			
BFG130	F: GCAGTTTTATAGAGGTGGGG	6FAM	56	TCTT
	R: ATATCTCCATCCGGTCCA			
BFG250	F: CCTGTTAGAGAAGCCGATCATT	VIC	56	GATA
	R: TTGGACTGGAAGTATTGGGAGT			

Table S2b PCR reaction mix and thermal profiles for the 4 multiplex panels.

PANEL 1		***PCR thermal profile***		
Reagents	**µl**	**T°**	**Time**	**Cycles**
H_2O	6.57	95°C	2’	
Buffer 10X	1	94°C	1’	
BFG239 F/R 10 ρmol/ µl	0.18	54°C	30’’	25
BFG202 F/R 10 ρmol/ µl	0.08	70°C	30’’	
BFG237 F/R 10 ρmol/ µl	0.2	4°C		
BFG129 F/R 10 ρmol/ µl	0.22			
dNTP 10 mM	1			
HotMaster Taq 5 U/µl	0.25			
DNA	0.5			

PANEL 2		***PCR thermal profile***		
Reagents	**µl**	**T°**	**Time**	**Cycles**
H2O	6.93	95°C	2’	
Buffer 10X	1	94°C	1’	
BFG072 F/R 10 ρmol/ µl	0.08	56°C	45’’	25
BFG099 F/R 10 ρmol/ µl	0.12	70°C	45’’	
BFG155 F/R 10 ρmol/ µl	0.12	4°C		
dNTP 10 mM	1			
HotMaster Taq 5 U/µl	0.25			
DNA	0.5			

PANEL 3		***PCR thermal profile***		
Reagents	**µl**	**T°**	**Time**	**Cycles**
H_2O	6.19	95°C	2’	
Buffer 10X	1	94°C	1’	
BFG050 F/R 10 ρmol/ µl	0.3	58°C	30’’	25
BFG053 F/R 10 ρmol/ µl	0.07	70°C	45’’	
BFG161 F/R 10 ρmol/ µl	0.12	60°C	10’	
BFG131 F/R 10 ρmol/ µl	0.07	4°C		
dNTP 10 mM	1			
HotMaster Taq 5 U/µl	0.25			
DNA	1			

PANEL 4		***PCR thermal profile***		
Reagents	**µl**	**T°**	**Time**	**Cycles**
H_2O	6.53	95°C	2’	
Buffer 10X	1	94°C	1’	30
BFG130 F/R 10 ρmol/ µl	0.07	56°C	45’’	
BFG250 F/R 10 ρmol/ µl	0.15	60°C	10’	
dNTP 10 mM	1	4°C		
HotMaster Taq 5 U/µl	0.25			
DNA	1			

Table S3 Matrix of pairwise F_{ST} values. Above diagonal, non-significant values are highlighted. All other values are significant ($p < 0.05$ after FDR correction for multiple comparisons)

	Amp	Bon	Cad	Fia	LCa	LMe	LRo	LSG	MRe	PT1	PT2	PTr	RM1	Ste	Tov	Tre	Va1	VD1	VD2	VG1	VG3	VN2	VP1	VP2	VN1	CCC	ViT	PS1	So2	So1	Ron	PTe	PS2	PR2	PR1	Pos	PMe	PLC	PLa	Mug	MP2	MP1	Mon	MCa	MBe	Mar	LPo	Lel	Lag	Ing	Ech	DDB	Ce2	Ce1	Can	Bro	Bed
Amp	*																																																								
Bon	0.01	*					-																																																				
Cad	0.02	0.02	*				-																																																			
Fia	0.03	0.03	0.02	*																																																						
LCa	0.08	0.06	0.07	0.07	*					-																																																		
LMe	0.08	0.06	0.06	0.04	0.06	*																																																					
LRo	0.03	0.01	0.01	0.01	0.06	0.05	*													-																																						
LSG	0.06	0.05	0.04	0.05	0.07	0.05	0.04	*																																																			
MRe	0.03	0.02	0.02	0.03	0.08	0.06	0.03	0.05	*																																																		
PT1	0.06	0.04	0.06	0.07	0.01	0.05	0.04	0.05	0.06	*	-																																															
PT2	0.06	0.04	0.05	0.06	0.01	0.05	0.04	0.05	0.05	0.00	*																																															
PTr	0.07	0.05	0.05	0.05	0.04	0.04	0.05	0.04	0.06	0.03	0.03	*																																														
RM1	0.09	0.07	0.08	0.09	0.12	0.11	0.08	0.05	0.10	0.10	0.10	0.10	*																																													
Ste	0.10	0.11	0.06	0.09	0.14	0.12	0.07	0.09	0.08	0.13	0.13	0.10	0.14	*																																													-
Tov	0.10	0.07	0.07	0.06	0.06	0.05	0.05	0.07	0.07	0.04	0.06	0.06	0.12	0.12	*																																												
Tre	0.02	0.03	0.02	0.03	0.08	0.10	0.03	0.07	0.04	0.07	0.07	0.08	0.13	0.12	0.10	*																																											
Va1	0.03	0.03	0.01	0.02	0.06	0.04	0.02	0.02	0.02	0.05	0.04	0.04	0.07	0.07	0.05	0.04	*			-																																							
VD1	0.03	0.02	0.03	0.03	0.06	0.04	0.03	0.04	0.02	0.04	0.04	0.04	0.07	0.09	0.05	0.05	0.02	*							-																																	
VD2	0.04	0.03	0.03	0.03	0.07	0.05	0.03	0.04	0.02	0.05	0.05	0.04	0.08	0.10	0.06	0.05	0.03	0.02	*																																								
VG1	0.03	0.02	0.01	0.02	0.06	0.05	0.01	0.02	0.02	0.04	0.04	0.04	0.06	0.07	0.06	0.04	0.01	0.02	0.03	*																																							
VG3	0.03	0.03	0.03	0.04	0.06	0.08	0.02	0.04	0.04	0.04	0.04	0.05	0.07	0.11	0.09	0.04	0.03	0.03	0.03	0.01	*																																							
VN2	0.04	0.04	0.03	0.04	0.06	0.04	0.04	0.04	0.03	0.04	0.04	0.04	0.09	0.10	0.06	0.06	0.03	0.03	0.03	0.02	0.04	*			-																																			
VP1	0.13	0.11	0.12	0.12	0.05	0.07	0.11	0.08	0.11	0.04	0.05	0.05	0.14	0.18	0.08	0.14	0.09	0.08	0.10	0.09	0.10	0.08	*																																						
VP2	0.14	0.12	0.13	0.13	0.07	0.10	0.13	0.10	0.11	0.06	0.08	0.07	0.15	0.19	0.09	0.15	0.11	0.10	0.12	0.11	0.12	0.08	0.01	*																																						
VN1	0.03	0.03	0.03	0.04	0.06	0.04	0.03	0.03	0.03	0.03	0.05	0.04	0.08	0.08	0.06	0.05	0.02	0.01	0.04	0.02	0.03	0.01	0.07	0.07	*																																						
CCC	0.05	0.04	0.03	0.04	0.05	0.04	0.03	0.05	0.03	0.04	0.04	0.03	0.11	0.11	0.06	0.05	0.02	0.04	0.03	0.04	0.05	0.03	0.07	0.10	0.04	*																																						
ViT	0.16	0.14	0.14	0.15	0.18	0.17	0.13	0.14	0.15	0.17	0.16	0.17	0.16	0.13	0.17	0.20	0.13	0.14	0.16	0.13	0.16	0.17	0.25	0.27	0.18	0.17	*																																						
PS1	0.10	0.11	0.07	0.08	0.15	0.11	0.08	0.12	0.09	0.14	0.14	0.11	0.18	0.05	0.12	0.12	0.09	0.09	0.09	0.09	0.12	0.10	0.21	0.24	0.10	0.10	0.15	*		-			-		-																																
So2	0.12	0.11	0.07	0.08	0.14	0.08	0.08	0.09	0.08	0.12	0.13	0.10	0.15	0.05	0.08	0.14	0.08	0.07	0.08	0.08	0.11	0.08	0.17	0.18	0.08	0.10	0.17	0.03	*	-																																				
So1	0.11	0.11	0.08	0.09	0.15	0.11	0.08	0.10	0.10	0.14	0.14	0.11	0.16	0.04	0.12	0.14	0.09	0.09	0.09	0.09	0.11	0.11	0.20	0.23	0.11	0.12	0.14	0.02	0.01	*																																				
Ron	0.10	0.09	0.07	0.07	0.14	0.09	0.06	0.10	0.07	0.12	0.12	0.10	0.17	0.08	0.11	0.11	0.09	0.08	0.06	0.09	0.10	0.08	0.17	0.20	0.09	0.08	0.20	0.06	0.05	0.05	*																																				
PTe	0.10	0.10	0.07	0.07	0.11	0.08	0.06	0.09	0.08	0.09	0.10	0.08	0.17	0.05	0.08	0.10	0.08	0.08	0.07	0.08	0.09	0.09	0.15	0.17	0.08	0.08	0.16	0.03	0.04	0.03	0.03	*						-							-																						
PS2	0.11	0.10	0.08	0.08	0.13	0.11	0.07	0.11	0.11	0.12	0.13	0.10	0.16	0.06	0.11	0.13	0.09	0.09	0.09	0.09	0.11	0.11	0.20	0.22	0.10	0.11	0.13	0.00	0.04	0.02	0.07	0.04	*																																		
PR2	0.11	0.10	0.08	0.07	0.13	0.10	0.06	0.10	0.10	0.12	0.12	0.09	0.15	0.06	0.08	0.12	0.09	0.09	0.07	0.09	0.10	0.10	0.18	0.20	0.11	0.09	0.14	0.02	0.03	0.03	0.06	0.04	0.03	*	-																																	
PR1	0.12	0.12	0.09	0.09	0.15	0.12	0.09	0.13	0.11	0.14	0.15	0.12	0.18	0.06	0.11	0.14	0.11	0.11	0.09	0.10	0.13	0.11	0.20	0.22	0.13	0.11	0.17	0.03	0.04	0.03	0.06	0.04	0.04	0.00	*																																		
Pos	0.12	0.12	0.10	0.10	0.15	0.12	0.09	0.13	0.12	0.14	0.15	0.12	0.19	0.07	0.12	0.14	0.11	0.10	0.11	0.10	0.12	0.11	0.21	0.23	0.11	0.13	0.17	0.05	0.05	0.04	0.06	0.03	0.05	0.05	0.05	*					-																												
PMe	0.11	0.11	0.08	0.09	0.13	0.10	0.08	0.10	0.10	0.12	0.13	0.09	0.16	0.03	0.10	0.12	0.09	0.08	0.09	0.08	0.11	0.10	0.17	0.19	0.09	0.11	0.15	0.03	0.02	0.01	0.05	0.03	0.03	0.04	0.04	0.04	*																																	
PLC	0.10	0.09	0.07	0.07	0.12	0.08	0.06	0.10	0.08	0.10	0.11	0.09	0.17	0.05	0.09	0.10	0.07	0.08	0.08	0.08	0.10	0.09	0.17	0.19	0.09	0.08	0.15	0.03	0.03	0.03	0.03	0.00	0.05	0.04	0.04	0.04	0.04	*							-																										
PLa	0.08	0.07	0.06	0.06	0.09	0.08	0.06	0.07	0.08	0.08	0.09	0.05	0.11	0.07	0.09	0.10	0.06	0.06	0.07	0.06	0.07	0.08	0.14	0.15	0.06	0.09	0.14	0.05	0.05	0.04	0.08	0.05	0.03	0.04	0.06	0.07	0.04	0.06	*																																	
Mug	0.13	0.14	0.11	0.11	0.17	0.14	0.09	0.15	0.12	0.16	0.16	0.15	0.22	0.06	0.14	0.14	0.12	0.13	0.13	0.12	0.14	0.14	0.23	0.25	0.15	0.13	0.17	0.04	0.07	0.05	0.05	0.03	0.06	0.04	0.03	0.04	0.05	0.02	0.09	*																																	
MP2	0.13	0.12	0.10	0.11	0.16	0.12	0.10	0.12	0.12	0.14	0.16	0.12	0.17	0.05	0.12	0.15	0.11	0.10	0.12	0.10	0.13	0.12	0.22	0.23	0.11	0.14	0.16	0.05	0.04	0.04	0.07	0.05	0.05	0.06	0.06	0.00	0.03	0.05	0.07	0.06	*					-																												
MP1	0.16	0.17	0.13	0.15	0.23	0.18	0.14	0.17	0.15	0.21	0.22	0.19	0.22	0.07	0.19	0.19	0.15	0.14	0.16	0.15	0.17	0.17	0.27	0.29	0.16	0.18	0.21	0.07	0.09	0.06	0.09	0.07	0.09	0.09	0.08	0.05	0.07	0.06	0.12	0.05	0.05	*																																	
Mon	0.14	0.14	0.10	0.12	0.18	0.13	0.10	0.14	0.12	0.18	0.17	0.15	0.21	0.05	0.15	0.14	0.12	0.13	0.12	0.12	0.15	0.13	0.23	0.26	0.15	0.13	0.18	0.04	0.06	0.04	0.05	0.05	0.06	0.05	0.04	0.05	0.05	0.04	0.10	0.02	0.05	0.06	*																																	
MCa	0.11	0.10	0.08	0.09	0.14	0.10	0.08	0.10	0.10	0.12	0.12	0.09	0.18	0.04	0.11	0.12	0.09	0.09	0.11	0.09	0.11	0.10	0.19	0.21	0.10	0.10	0.15	0.04	0.03	0.01	0.06	0.02	0.04	0.04	0.05	0.03	0.03	0.01	0.05	0.04	0.03	0.08	0.04	*																																	
MBe	0.16	0.16	0.14	0.16	0.21	0.17	0.14	0.15	0.15	0.20	0.20	0.16	0.19	0.07	0.19	0.21	0.14	0.14	0.17	0.13	0.17	0.15	0.25	0.26	0.14	0.18	0.20	0.10	0.10	0.08	0.13	0.11	0.10	0.12	0.14	0.09	0.09	0.11	0.11	0.11	0.09	0.10	0.09	0.08	*																																	
Mar	0.11	0.11	0.09	0.10	0.14	0.12	0.09	0.10	0.11	0.13	0.13	0.10	0.16	0.04	0.13	0.13	0.10	0.09	0.11	0.09	0.11	0.12	0.19	0.21	0.09	0.12	0.16	0.05	0.05	0.03	0.07	0.03	0.05	0.07	0.08	0.05	0.02	0.04	0.05	0.06	0.03	0.06	0.07	0.03	0.08	*																																	
LPo	0.14	0.14	0.11	0.12	0.18	0.13	0.11	0.13	0.13	0.16	0.17	0.14	0.20	0.05	0.14	0.16	0.12	0.12	0.14	0.12	0.15	0.12	0.23	0.24	0.12	0.14	0.18	0.06	0.04	0.05	0.08	0.05	0.07	0.07	0.06	0.02	0.05	0.04	0.08	0.04	0.01	0.05	0.05	0.04	0.07	0.04	*																																	
Lel	0.10	0.10	0.08	0.09	0.13	0.10	0.08	0.10	0.10	0.12	0.13	0.09	0.15	0.05	0.11	0.11	0.08	0.08	0.11	0.08	0.12	0.08	0.17	0.17	0.07	0.10	0.17	0.07	0.06	0.07	0.08	0.07	0.06	0.08	0.09	0.08	0.06	0.07	0.07	0.10	0.07	0.11	0.08	0.06	0.07	0.05	0.06	*																																	
Lag	0.10	0.09	0.07	0.09	0.12	0.12	0.07	0.09	0.10	0.11	0.13	0.09	0.13	0.05	0.12	0.12	0.09	0.08	0.09	0.07	0.09	0.10	0.18	0.19	0.09	0.12	0.14	0.05	0.05	0.03	0.09	0.06	0.02	0.05	0.06	0.06	0.04	0.07	0.03	0.09	0.05	0.09	0.07	0.06	0.07	0.05	0.07	0.05	*																																	
Ing	0.17	0.17	0.13	0.16	0.20	0.17	0.14	0.17	0.14	0.20	0.19	0.18	0.24	0.09	0.18	0.17	0.16	0.15	0.15	0.15	0.18	0.15	0.25	0.28	0.18	0.15	0.21	0.08	0.11	0.09	0.07	0.08	0.10	0.09	0.08	0.09	0.09	0.07	0.14	0.05	0.09	0.09	0.02	0.08	0.13	0.11	0.09	0.12	0.11	*																																	
Ech	0.13	0.13	0.10	0.12	0.18	0.13	0.10	0.12	0.12	0.17	0.17	0.13	0.19	0.05	0.15	0.15	0.11	0.11	0.13	0.11	0.14	0.13	0.22	0.25	0.13	0.14	0.17	0.04	0.05	0.02	0.07	0.05	0.06	0.06	0.06	0.05	0.03	0.04	0.07	0.05	0.03	0.06	0.03	0.04	0.09	0.05	0.04	0.08	0.05	0.07	*																																	
DDB	0.16	0.16	0.14	0.14	0.19	0.13	0.14	0.16	0.16	0.18	0.18	0.15	0.25	0.12	0.16	0.20	0.15	0.15	0.15	0.15	0.18	0.16	0.24	0.28	0.18	0.15	0.21	0.10	0.12	0.09	0.11	0.09	0.10	0.09	0.09	0.09	0.10	0.09	0.14	0.09	0.09	0.13	0.06	0.09	0.17	0.14	0.11	0.14	0.12	0.08	0.08	*																																	
Ce2	0.13	0.12	0.09	0.10	0.17	0.11	0.09	0.12	0.12	0.15	0.16	0.12	0.18	0.06	0.14	0.16	0.11	0.11	0.12	0.11	0.13	0.13	0.21	0.22	0.12	0.13	0.18	0.04	0.03	0.03	0.08	0.05	0.03	0.05	0.07	0.07	0.03	0.05	0.04	0.07	0.07	0.10	0.08	0.04	0.09	0.05	0.06	0.07	0.06	0.13	0.06	0.13	*																																	
Ce1	0.11	0.11	0.09	0.10	0.15	0.12	0.09	0.12	0.11	0.14	0.14	0.12	0.17	0.08	0.14	0.14	0.11	0.11	0.12	0.10	0.12	0.12	0.19	0.20	0.09	0.13	0.17	0.08	0.07	0.06	0.10	0.08	0.07	0.09	0.10	0.10	0.06	0.09	0.05	0.11	0.09	0.13	0.12	0.09	0.13	0.08	0.10	0.10	0.08	0.17	0.10	0.17	0.04	*																																	
Can	0.11	0.10	0.07	0.09	0.13	0.11	0.07	0.09	0.08	0.11	0.12	0.08	0.15	0.05	0.10	0.11	0.08	0.08	0.08	0.08	0.11	0.09	0.17	0.19	0.10	0.09	0.16	0.04	0.05	0.05	0.06	0.04	0.05	0.05	0.06	0.08	0.05	0.05	0.06	0.07	0.08	0.10	0.05	0.06	0.11	0.07	0.09	0.07	0.04	0.08	0.05	0.10	0.08	0.12	*																																	
Bro	0.10	0.10	0.07	0.07	0.13	0.09	0.06	0.10	0.10	0.12	0.13	0.09	0.15	0.05	0.11	0.12	0.09	0.09	0.09	0.07	0.10	0.10	0.18	0.21	0.09	0.11	0.15	0.03	0.04	0.02	0.06	0.04	0.02	0.04	0.04	0.05	0.03	0.05	0.03	0.07	0.06	0.09	0.06	0.06	0.10	0.05	0.07	0.06	0.02	0.11	0.05	0.12	0.03	0.05	0.06	*																																	
Bed	0.11	0.10	0.07	0.09	0.14	0.11	0.08	0.09	0.09	0.13	0.13	0.10	0.15	0.00	0.12	0.12	0.08	0.08	0.09	0.07	0.11	0.10	0.19	0.20	0.08	0.11	0.14	0.04	0.03	0.02	0.07	0.04	0.04	0.06	0.06	0.04	0.02	0.04	0.05	0.06	0.03	0.06	0.04	0.02	0.03	0.02	0.04	0.03	0.02	0.08	0.04	0.12	0.05	0.08	0.04	0.05	*																																	

Table S4 Analysis of molecular variance (AMOVA) computed for the grouping inferred by STRUCTURE (K = 2; whole dataset analysis), and relative FST statistics

Source of variation	d.f.	Sum of squares	Variance components	Variation (%)	F statistics	P value
Among groups	1	423.2	0.28	6.75	FCT: 0.067	0.0001
Among poputions within groups	55	869.1	0.24	5.79	FSC: 0.062	0.0001
Within populations	2831	10320.1	3.65	87.46	FST: 0.125	0.0001
Total	2887	11612.4	4.17			

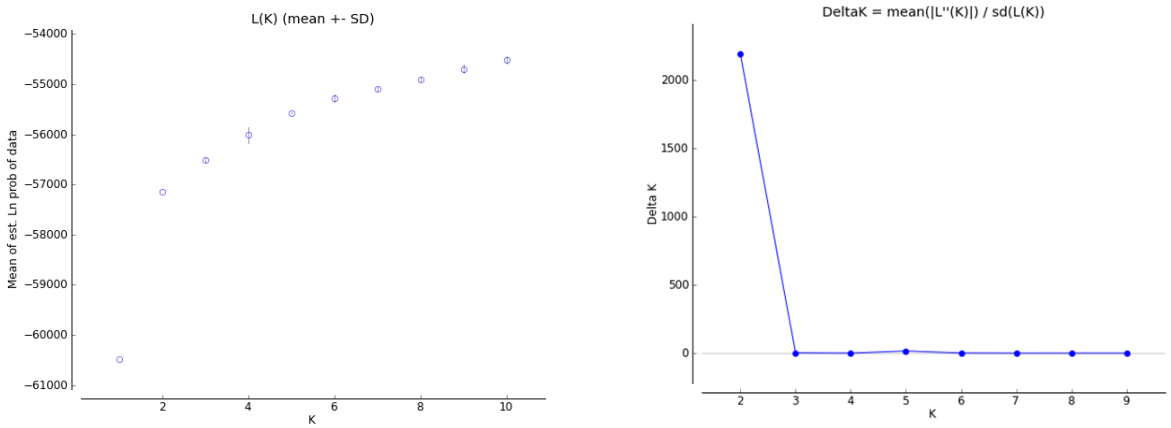
Supplementary Figures

Figures S1 STRUCTURE analysis: Step 1; dataset = whole study area.

(a) plot of mean likelihood L(K) and variance per K value;

(b) plot of the second-order rate of change of the likelihood function (ΔK) with respect to K.

(a) (b)



Chapter 4.

STUDY 3:

Species-genetic diversity correlation: the case study of the common frog (*Rana temporaria*) and amphibian communities in an Alpine region.

Species-genetic diversity correlation: the case study of the common frog (*Rana temporaria*) and amphibian communities in an Alpine region.

Alexis Marchesini, Cristiano Vernesi, Andrea Battisti, Gentile Francesco Ficetola

Abstract

The evolutionary and ecological importance of genetic diversity is widely recognized. Nevertheless, genetic diversity assessment is rarely included in conservation strategies and species diversity is taken as a surrogate, supposing a general positive correlation. We tested whether intrapopulation genetic diversity and species richness co-vary in the amphibian communities of a southern Alpine region (Trentino, Italy), computing the so-called species-genetic diversity correlation (SGDC) for a dataset consisting of 26 wetland sites. We chose a widespread amphibian, the common frog (*Rana temporaria*), as focal species for the evaluation of genetic diversity. Here, we show that species richness and genetic diversity are negatively correlated in our study system. Moreover, different amphibian species exhibit different effects on the genetic diversity of the focal species. We demonstrate that the recorded patterns are most likely due to the opposite influence of environmental factors on the two levels of biodiversity, ruling out the potential role of interspecific competition. These findings have important implications for conservation planning, suggesting that species richness cannot be universally used as proxy for genetic diversity.

Keywords: alpine amphibians, *Rana temporaria*, species-genetic diversity correlation, biodiversity conservation

Introduction

Worldwide biodiversity is threatened by a severe crisis, caused directly or indirectly by human activities (Myers 1993; Pimm *et al.* 1995; Ceballos *et al.* 2015). According to the widely-accepted definition included in the text of the Convention of Biological Diversity (CBD), biodiversity embraces three fundamental levels: "diversity within species, between species and of ecosystems" (UNEP, 1992). The importance of preserving all the three levels of biological diversity is explicitly stressed in the new Strategic Plan for Biodiversity, the so-called "Aichi Targets", which claim the urgent need to improve the status of biodiversity "by safeguarding ecosystems, species and genetic diversity" (Strategic Goal C; SCBD 2010).

The most fundamental level of biodiversity, i.e. genetic diversity, has become a topic of increasing interest in the scientific community during the last decades, and its crucial role for the evolution and persistence of species is now universally recognized. Indeed, genetic diversity is required for populations to adapt to a changing environment (Booy *et al.* 2000; Reusch *et al.* 2005; Höglund 2009), and thus it can be viewed as the basis for "evolutionary potential" (Conner & Hartl 2004). Moreover, a loss in genetic diversity is often associated with inbreeding depression and reduction in fitness, with detrimental consequences for the persistence of populations (Frankham *et al.* 2002; Hansson & Westerberg 2002; Reed & Frankham 2003). In addition, recent studies are now extending the importance of genetic diversity at the ecosystem level, suggesting it may be involved in determining community structure, ecosystem resilience and productivity (see Hughes *et al.* 2008 for a review). Nevertheless, genetic diversity is often neglected in conservation practice: the implementation of genetic monitoring is rarely considered in the identification of "biodiversity hotsposts" (Vandergast *et al.* 2008) as well as in species conservation rankings (Willoughby *et al.* 2015), and direct actions to protect genetic diversity are still largely lacking (Laikre *et al.* 2009; Hoban *et al.* 2013). Species richness, the most widely used biodiversity proxy (Gaston 1996), is often taken as a surrogate, supposing a positive correlation with genetic diversity.

However, despite the potential connections among population genetics and community ecology have long been recognized (e.g. Antonovics 1976, 2003; Huston 1994; Amarasekare 2000; Bell 2001; Hubbell 2001), only in the last 15 years specific attempts have been made to elucidate the potential relationships between these two levels of biodiversity. In 2003, Vellend suggested the hypothesis of a general positive correlation between genetic diversity in single species and species richness in communities, introducing

the term species-genetic diversity correlation (SGDC) for identifying such a relationship. This seminal idea was later refined, and Vellend & Geber (2005) developed a more complex theoretical framework for explaining SGDCs patterns, identifying three different types of potential interactions (which are not mutually exclusive): parallel effects of locality characteristics on the two levels of diversity (Case I); causal effects of genetic diversity on species diversity (Case II); causal effects of species diversity on genetic diversity (Case III). According to the authors, in Case I, positive SGDCs are generally assumed to arise, while in Case II and Case III, the sign and entity of SGDC may vary largely, depending on the relative role of the different underlying processes and to the properties of the study system (e.g. focal species and ecological community, molecular markers, etc.). In particular, the authors hypothesized that negative SGDCs may arise due the causal effects of species diversity on genetic diversity (Case III), even under neutral assumptions (it must be noted that the vast majority of traditional SGCs studies employs neutral genetic markers). More specifically, under limited resources interspecific competition may lead to a decrease in population size (particularly of the inferior competitors), which in turn may reduce genetic diversity due to genetic drift (Vellend & Geber 2005; Wehenkel *et al.* 2006).

Recently, Laroche *et al.* (2015) built a neutral model and tested its predictions under different scenarios using a simulation approach, aiming at providing a basis for predicting and interpreting SGDCs. The outcome showed that negative SGDCs may indeed frequently arise, particularly when using molecular markers characterized by high mutation rates (such as microsatellites). Therefore, modern theories are highlighting a high degree of complexity in the potential relationships between species and genetic diversity.

From an empirical perspective, a specific interest has grown for explicitly testing SGDCs in plant and animal communities over the past decade (reviewed by: Vellend 2003; Vellend & Geber 2005; Vellend *et al.* 2014). Analyzing 40 different empirical studies, Vellend *et al.* (2014) concluded claiming again a general prevalence of positive SGDCs. Interestingly, according to these authors, positive SGDCs are expected to be more frequent and stronger in studies focusing on discrete habitats (such as islands, forest fragments, ponds), rather than in arbitrarily delineated areas (in continuous habitats), due to the strongest (parallel) influence of nonselective factors (e.g. habitat area and isolation) on both levels of diversity (Vellend *et al.* 2014). However, empirical data show a high variability in the sign and magnitude of correlations, as well as in the suggested ecological drivers (Vellend & Geber 2005; Vellend *et al.* 2014). Indeed, negative (e.g. Karlin *et al.* 1984; Marshall & Camp 2006; Wehenkel *et al.* 2006; Puşcaş *et al.* 2008; Silvertown *et al.* 2009; Taberlet *et al.*

2012) and non-significant (e.g. Odat *et al.* 2004, Derry *et al.* 2009, Helm *et al.* 2009, Silvertown *et al.* 2009; Fridley & Grime 2010; Chang & Smith 2012; Taberlet *et al.* 2012; Wei & Jiang 2012; Avolio & Smith 2013; Xu *et al.* 2016) SGDCs are not so rare in natural systems. Interestingly, in some cases the detected negative correlation was found to be driven by an opposite effect of site characteristics on the two levels of diversity (Silvertown *et al.* 2009; Avolio & Smith 2013), although Vellen & Geber (2005) supposed a general prevalence of SGDCs due to the parallel effects of locality characteristics (Case I; see above).

Taberlet *et al.* (2012) warned about the indiscriminate use of species richness as a proxy for genetic diversity in conservation strategies, highlighting the risk of failing to preserve genetic diversity, in the presence of negative or non-significant SGDCs. Kahilainen *et al.* (2014), though not excluding the potential use of SGDC for deriving one level of diversity from the other, expressed caution due to the high variability of reported correlations and suggested that its usefulness may also depends on its (case-specific) drivers, stressing the importance of a proper knowledge of the underlying processes.

In this study, we performed an empirical test of SGDC in amphibian communities of a southern Alpine region (Provincia Autonoma di Trento, Italy), choosing a widespread amphibian, the common frog (*Rana temporaria*), as focal species for the evaluation of genetic diversity. Specifically, we aimed at answering the following questions: (1) do species richness of amphibian communities and genetic diversity of *Rana temporaria* populations co-vary in space? (2) do the different species in the community have the same influence on *Rana temporaria* genetic diversity? (3) which is the most likely ecological explanation for the observed patterns?

Finally, our results will be discussed from a conservation perspective. At this regard, our survey may be viewed as a highly informative case study, since the spatial scale we chose corresponds to the political borders of an Italian Autonomous Province, and therefore to the fundamental unit of conservation planning: the regional scale. Moreover, our study system is of particular conservation concern, since (1) the Alps are heavily affected by ongoing climate change (Cannone *et al.* 2008; Brunetti *et al.* 2009; Gobiet *et al.* 2014) and habitat alteration (Chemini & Rizzoli 2003; Lassen & Savoia 2005; Vanham *et al.* 2009), with a wide range of detrimental consequences on biodiversity (Bragazza 2008; Leonelli *et al.* 2011; Nagy *et al.* 2012; Maiorano *et al.* 2013; Tafani *et al.* 2013); (2) amphibians are considered the most endangered group of vertebrates and they are facing a dramatic decline worldwide (Wake 1991; Houlahan *et al.* 2000; Gardner 2001; Stuart *et al.* 2004); and (3)

amphibians are also considered biological indicators of general ecosystem health (Vitt *et al.* 1990; Hopkins 2007).

Materials and Methods

Study system

Our study area is Trentino (Autonomous Province of Trento, Italy), a mountainous region of 6212 km^2 in the eastern Italian Alps. The region is characterized by a complex terrain (elevation range: 65-3764 m above sea level; more than 70% of it lies above 1000 m a.s.l), including part of the Dolomites and Prealps as well as low elevation valleys. As a consequence, the climate is also heterogeneous, varying from the typical Alpine climate in the most elevated areas, to the sub-continental moderate climate of the minor valleys, and the sub-Mediterranean conditions approaching the southernmost part of the region, which is characterized by the mitigating effect of Lake Garda.

We chose amphibians as study system for investigating SGDC because accurate community composition data were available for this group of animals in several wetlands of the region. Communities of alpine amphibians are species-poor, and a total of 12 native species are known for the Trentino region (Caldonazzi *et al.* 2002).

Following Vellend (2003) and Vellend & Geber (2005), we chose one focal species to assess genetic diversity levels: the common frog (*Rana temporaria*). This frog is the most widespread amphibian in Europe (Sillero *et al.* 2014), and is characterized by high adaptability to different ecological conditions. Being often abundant, it is an important component of many ecological communities (Luiselli *et al.* 1995; Lodé 1996). Hence, *Rana temporaria* is a model organism for ecological (e.g. Loman 2004; Vos 2007; Decout *et al.* 2012), evolutionary (e.g. Miaud 1999; Laugen *et al.* 2003; Muir *et al.* 2014), and genetic studies (e.g. Hitchings & Beebee 1997; Palo *et al.* 2004; Johansson *et al.* 2006). Common species are widely used in empirical studies reporting SGDCs, due to practical sampling reasons (Laroche *et al.* 2015; e.g. He *et al.* 2008; Derry *et al.* 2009; Odat *et al.* 2010; Struebig *et al.* 2011; Taberlet *et al.* 2012; Wei & Jiang 2012; Lamy *et al.* 2013; Xu *et al.* 2016). It is also worth to note that Vellend (2005), based on simulation models, claimed a tendency for stronger positive SGDCs in more common species (Vellend 2005; Gugerli *et al.* 2008; Taberlet *et al.* 2012). In the study region, the common frog can be found in a wide variety of habitats, alone or in syntopy with other amphibians, ranging from valley bottoms up to the vegetation limit (approximate elevation range: 200-2600 m a.s.l; Caldonazzi *et al.* 2002).

Species richness and composition of amphibian communities

Data for species richness (SR) and composition of amphibian communities were taken from the amphibian monitoring program performed by the regional environmental agency in several wetland sites of the region, and complemented by personal monitoring surveys (for 7 sites). All monitoring surveys were coordinated by a group of experts, performed using multiple monitoring methods (barriers and pitfall traps, eggs searches, larval netting, vocalizations, etc.), often repeated in different years and with detailed reports available (PAT). For these reasons, and also considering the relative low number of potentially present species, they can be assumed to represent presence/absence data and were used to compute SGDCs. Species were considered present only when reproduction within the site was ascertained, in order to exclude occasional presences (e.g. dispersing individuals).

Genetic diversity data

We derived our dataset from a previous genetic survey on *Rana temporaria* populations in the study region (see Chapter 3). We selected a subset of 26 sites, according to the availability of correspondent amphibian community data, and in order to cover the whole study region and different ecological environments (e.g. elevation range: 401 - 2083 m a.s.l.; see Fig. 1 and Table S2 in Supporting Information). Intrapopulation levels of genetic diversity in *Rana temporaria* were investigated using 12 microsatellite DNA markers, a common choice in SGDCs studies (e.g. He *et al.* 2008; Struebig *et al*. 2011; Blum *et al.* 2012; Wei & Jiang 2012; Lamy *et al.* 2013; Xu *et al.* 2016). Microsatellites are assumed to be generally neutral (Goldstein & Schlotterer 1999; Ellegren 2004): consequently, local genetic diversity levels in our study are expected to predominantly reflect neutral processes, and this aspect will be considered in the evaluation of different potential drivers for the recorded correlation patterns. We chose two standard measures of genetic diversity: allelic richness (AR) and mean expected heterozygosity (H_e). Allelic richness was estimated using the rarefaction formula described by El Mousadik & Petit (1996) and implemented in the program FSTAT 2.9.3.2 (Goudet 2001); it was recomputed for this study according to the minimum sample size in our subset (15 individuals). Mean expected heterozygosity was computed using the unbiased method implemented in GenAlEx 6.5 (Peakall & Smouse 2012).

Species distribution and environmental data

For both habitat suitability modeling (Maxent) and niche overlap analysis (ecospat; see below), species occurrences were derived from a public WebGIS database implementing

amphibian observational records for the whole Trentino region (LIFE+T.E.N. WebGis; http://webgis.muse.it/), complemented with data derived from ACE-SAP research project (http://www.ace-sap.it/).

We obtained a total of 2534 presence records for the 7 amphibian species (see Table S4 in Supporting Information). We considered 8 environmental variables: mean annual temperature (proxy for energy availability), annual precipitation, 4 land cover types (anthropized areas, i.e. urban + agricultural areas; coniferous forests; broad-lived and mixed forest; coniferous forest; water areas, i.e. lakes, rivers and wetlands), slope and geological types (crystalline vs non-crystalline rocks). Details on variable selection, processing and extraction are provided in Appendix S1 in Supporting Information. The selected variables were tested for multicollinearity using Pearson correlation coefficient. Correlation coefficient was < 0.7 for all cross-tests (R max= 0.50), therefore all the variables were retained.

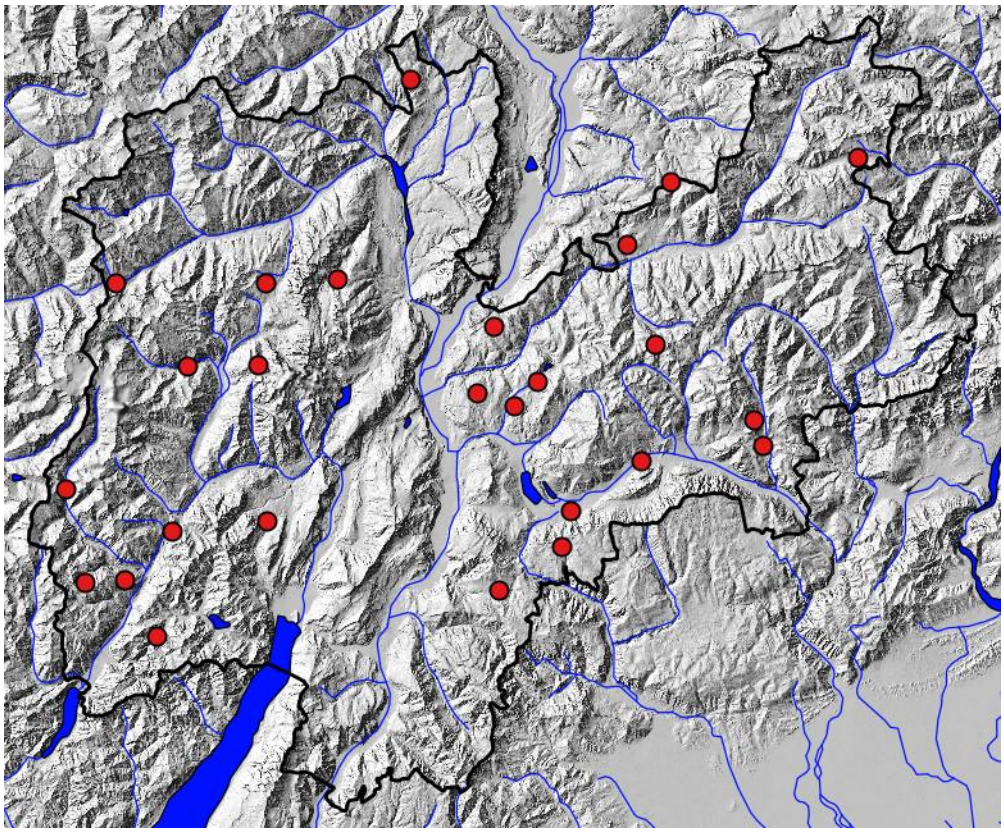


Figure 1 Map of the study region showing the selected wetland sites (red points), for which both information on species richness and composition of amphibian communities, as well as data on *Rana temporaria* genetic diversity were available.

Species-genetic diversity correlation (SGDC) and effect of community composition on genetic diversity

To investigate whether amphibian species richness and genetic diversity in *Rana temporaria* populations were correlated across sites (SGDC), we used Pearson Product Moment correlation tests. Correlation tests were performed using both measures of genetic diversity: allelic richness (AR) and expected heterozygosity (H_e).

A previous phylogeographic study for the common frog in the study region (using the mitochondrial cytochrome oxidase I gene, COI) revealed a complex scenario, with different evolutionary lineages (COI lineages Alp1, Alp2 and Alp4; see Chapter 2). As phylogeographic history and post-glacial colonization can potentially influence current levels of genetic diversity in different ways (Widmer & Lexer 2001; Petit *et al.* 2003; Ficetola *et al.* 2007; Roberts & Hamann 2015), we decided to test the significance of SGDC taking into account also the past evolutionary history of populations. In order to do this, we tested two different models, corresponding to different evolutionary scenarios: (1) model 1: including the frequency of the COI lineage Alp1 as proxy for evolutionary history (see Appendix S1 in Supporting Information for a justification of the choice), under the assumption that different evolutionary lineages carry different levels of genetic diversity; and (2) model 2: including the number of COI lineages as proxy for evolutionary history, under the assumption that admixture among different lineages increases genetic diversity (addictive effect in admixture zones; Petit *et al.* 2003). COI data for the selected populations were derived from Chapter 2 of the present thesis.

We analyzed the factors related to genetic diversity using generalized least squares (GLS). GLS are regression models that successfully incorporate spatial structure in the error term, and are thus suitable to analyze spatially-explicit data, controlling for potential issues of spatial autocorrelation. Specifically, we tested the influence of past evolutionary history and species richness (predictors) on genetic diversity (response variable) of the considered common frog populations. A total of 4 GLS models were tested, implementing the two above described evolutionary scenarios, and considering both H_e (model 1a, 2a) and AR (model 1b, 2b) as response variables. We fit GLS by maximizing the restricted loglikelihood.

Afterwards, we tested the relationships between community composition and *Rana temporaria* genetic diversity, with the aim of assessing in which way the presence of each species was related to levels of He and AR in *Rana temporaria*. Again, we used GLS for

taking into account past evolutionary history and spatial autocorrelation, and 4 different GLS models were tested, as described above.

All statistical analyses were performed in R statistical environment (R Core Team 2016).

Unravelling the underlying processes: niche overlap analysis and habitat suitability modeling

We tested two different hypotheses for explaining the detected negative SGDCs and the heterogenous effect of the different amphibian species on *R. temporaria* genetic diversity: (1) interspecific competition; (2) different ecological optima between *Rana temporaria* and the other amphibian species.

For testing "interspecific competition hypothesis", we used niche overlap analysis. This approach is commonly used to evaluate competition potential among species (e.g. Zhou *et al.* 2010; Volmer *et al.* 2016; Čuda *et al.* 2015). Indeed, according to classic niche theory, the strength of competition between species depends on the degree to which their niches overlap (Hutchinson 1957; MacArthur & Levins 1967; Begon *et al.* 1996): two species with highly similar niches are predicted to compete strongly, and vice versa. It must be noted that we are not interest in detecting actual competition, but to assess the relative potential of different amphibians to compete with our focal species, *Rana temporaria*. Therefore, we estimated niche overlap for all amphibian species with *R. temporaria*. In order to accept "interspecific competition hypothesis", species with higher niche overlap with *R. temporaria* should have stronger (negative) influence on its genetic diversity, while species with lower niche overlap should have no effect.

For comparing the niches (sensu Grinnel 1917) of *Rana temporaria* and other amphibian species, we used the "PCA-env" method developed by Broennimann *et al.* (2012, 2014). PCA-env measures niche overlap on the basis of occurrence and environmental data, and has shown to outperform other techniques for niche comparison (Broennimann *et al.* 2012). Specifically, the method performs a PCA for translating the multivariate environmental space available for the species into a two-dimensional space, where the occurrences of the species are projected. Measurement of niche overlap is then performed along the gradients of this multivariate analysis. PCA-env uses a kernel density function to compute the density of occurrences in the multivariate PCA space, in order to take into account potential bias caused by unequal sampling effort (Broennimann *et al.* 2012). Niche overlap was then computed by means of the Schoener´s D metric (Schoener 1970; Warren *et al*. 2008).

Schoener's D ranges between 0 (lack of overlap) and 1 (complete overlap), and is particularly suitable to analyze overlap in Grinellean niches (Warren *et al.* 2008). We further performed pairwise tests of niche similarity between *Rana temporaria* and the other species. Niche similarity test evaluates if the niche occupied by one species is more similar to the niche of the other species than expected by chance, while taking into account the level of background environmental heterogeneity, i.e. the differences in available habitat between two species (Warren *et al.* 2008; Broennimann *et al.* 2012). Specifically, niche similarity is tested by comparing the observed niche overlap (Schoener´s D) to the expected distribution of overlaps obtained by randomizing the occurrences of one species across its range of occupancy, while keeping constant the occurrences distribution of the other species. Similarity tests were performed in both directions (i.e. species 1 > species 2; species 2 > species 1), and significance was assessed with 1000 replications. Rejection of the null hypothesis indicates that the niches of the considered species are more similar than expected by chance. All analyses were performed using the "ecospat" package (Broennimann *et al.* 2014) in R 3.1.3 (R Core Team 2016).

For testing "different ecological optima hypothesis", we build habitat suitability models for all amphibian species, and then performed pairwise correlation tests between the habitat suitability map of *R. temporaria* and those of other amphibian species. Correlations were computed using Pearson's coefficients and significance was tested with Dutilleul's correction for spatial autocorrelation, as implemented by the program SAM (Dutilleul *et al.* 1993; Rangel *et al.* 2010). "Different ecological optima hypothesis" predicts that species with low habitat suitability correlation with *Rana temporaria* should exhibit stronger (negative) influence on its genetic diversity: their presence in syntopy with *R. temporaria* may indeed be viewed as indicative of sub-optimal ecological conditions for our focal species. Habitat suitability modeling was implemented using Maxent software (version 3.3.3; https://www.cs.princeton.edu/~schapire/maxent/; Phillips *et al.* 2004, 2006; Elith *et al.* 2011). Maxent is based on the maximum-entropy approach and estimates the probability distribution for a species' occurrence based on presence-only data and environmental variables. This method has been found to yield robust predictions, outperforming alternative approaches (Elith *et al.* 2006; Hernandez *et al.* 2006, 2008). Model validation was performed with the commonly used approach which consist in randomly partitioning the data into "training" and "test" sets (Fielding & Bell 1997; Guisan *et al.* 2005). Model performance was evaluated using AUC ("area under the curve"), which measures the probability that a

randomly chosen presence site will be ranked above a randomly chosen pseudoabsence site (Phillips & Dudik 2008). Models with AUC > 0.75 are considered "fair" predictors of observed data (Landis & Koch 1977; Fielding & Bell 1997; Elith *et al.* 2006). Habitat suitability maps were generated using a logistic link function, to yield a suitability index between 0 and 1 for all cells in the study region (Phillips & Dudik, 2008).

Results

Species genetic diversity correlation (SGDC)

Species richness varied from 1 to 7. The following amphibian species were recorded in the study sites (reported according to their frequency of occurrence): common frog (*Rana temporaria*; focal species for genetic diversity), common toad (*Bufo bufo*), Alpine newt (*Ichthyosaura alpestris*), fire salamander (*Salamandra salamandra*), green frog (*Pelophylax* synkl. *esculentus*), yellow-bellied toad (*Bombina variegata*) and agile frog (*Rana dalmatina*).

Allelic richness varied among populations from 4.83 to 6.68, and expected heterozygosity varied from 0.50 to 0.70 (Table S3, Supporting Information).

We found a significant negative correlation between species richness of amphibian communities and genetic diversity in *Rana temporaria* populations, for both expected heterozygosity ($R = -0.738$; $p < 0.05$) and allelic richness ($R = -0.583$; $p < 0.05$). All correlations remained significant also taking into account spatial autocorrelation and the evolutionary history of populations using Generalized Least Squares (GLS) models, considering both evolutionary scenarios (model 1a, 1b and model 2a, 2b; see Table S1 in Supporting Information).

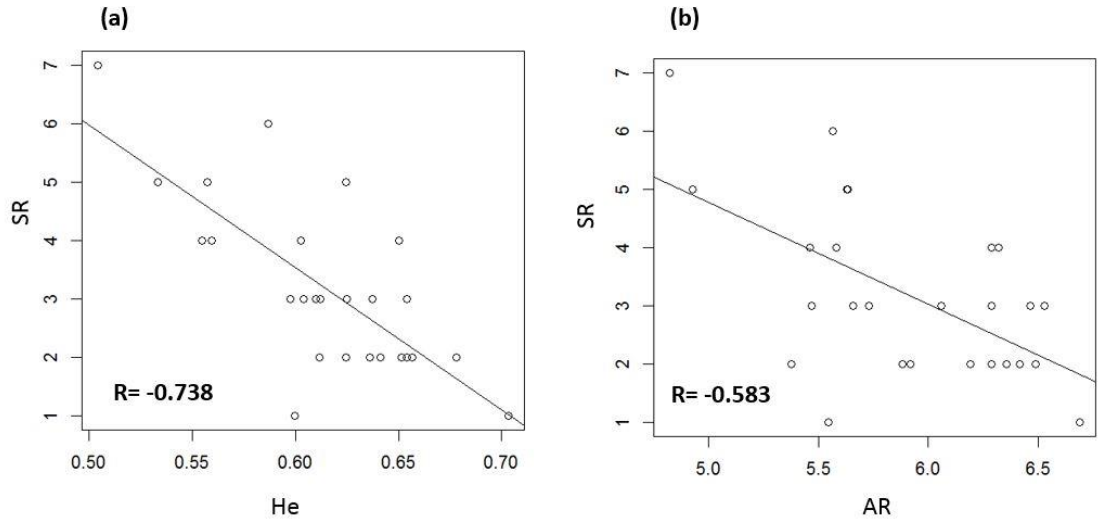


Figure 2 Species-genetic diversity correlation (SGDC). Plots of the relationship between amphibian species richness (SR) and *Rana temporaria* genetic diversity: (a) expected heterozygosity; (b) allelic richness. Pearson's correlation values (R) are reported. (all $P < 0.05$; all correlations remained significant also taking into account the evolutionary history of populations and spatial autocorrelation with GLS)

Influence of the different amphibian species on R. temporaria genetic diversity

Generalized Least Squares (GLS) models for evaluating the individual effect of the different amphibian species highlighted a heterogeneous scenario, with different species showing different influences (negative or no effect) on *Rana temporaria* genetic diversity. Overall, models considering different proxy for evolutionary history of populations (model 1a, 1b: proxy for history = frequency of Alp1 lineage; model 2a, 2b: proxy for history = n° of lineages) yielded similar results (Table 1). The only relevant difference was detected between models 1a and 2a (therefore considering H_e as response variable), with model 2a finding significant negative effects for 2 additional species (*Pelophylax* synkl. *esculentus* and *Ichthyosaura alpestris*), compared to model 1a. A third species, *Bombina variegata*, showed a marginally significant effect in model 2a. *Salamandra salamandra* was found to have a significant negative influence in all four models, while *Bombina variegata*, *Pelophylax* synkl. *esculentus* and *Rana dalmatina* were found to have a significant negative influence in at least two of the four models. However, the limited number of occurrences of these two species must be noted (Table 1). By contrast, *Bufo bufo* was found to have no significant effect in all 4 models, while *Ichthyosaura alpestris* being significant in only one case (already discussed), despite these two species were the most represented (25 and 14 occurrences, respectively).

(1)

Model	Predictor	n	Response	Coefficient	p-value	t-value	AIC
1a	*Ichthyosaura alpestris*	15	He	-0.0260	0.0929	-1.7530	-78.6316
1b			AR	-0.1158	0.5697	-0.5767	45.0944
1a	*Bufo bufo*	25	He	-0.0111	0.6642	-0.4398	-76.9360
1b			AR	-0.0142	0.9677	-0.0410	44.3429
1a	*Rana dalmatina*	2	He	-0.0291	0.2806	-1.1050	-78.0657
1b			AR	-0.7656	**0.0383**	-2.1983	40.1127
1a	*Pelophylax* synkl. *esculentus*	6	He	-0.0274	0.0897	-1.7715	-78.8292
1b			AR	-0.4683	**0.0418**	-2.1554	40.8711
1a	*Bombina variegata*	2	He	-0.0603	**0.0108**	-2.7741	-82.5529
1b			AR	-0.5271	0.1396	-1.5303	42.3186
1a	*Salamandra salamandra*	9	He	-0.0301	**0.0356**	-2.2326	-80.1899
1b			AR	-0.4579	**0.0119**	-2.7312	39.5144

(2)

Model	Predictor	n	Response	Coefficient	p-value	t-value	AIC
2a	*Ichthyosaura alpestris*	15	He	-0.0410	**0.0174**	-2.5639	-70.4511
2b			AR	-0.1828	0.3798	-0.8956	48.1427
2a	*Bufo bufo*	25	He	-0.0210	0.4897	-0.7021	-66.1854
2b			AR	-0.0811	0.8167	-0.2344	47.7837
2a	*Rana dalmatina*	2	He	-0.0611	***0.0594***	-1.9834	-69.5215
2b			AR	-0.8196	**0.0236**	-2.4250	42.4195
2a	*Pelophylax* synkl. *esculentus*	6	He	-0.0390	**0.0476**	-2.0932	-68.6735
2b			AR	-0.5140	**0.0302**	-2.3103	43.8607
2a	*Bombina variegata*	2	He	-0.0654	**0.0006**	-3.9479	-75.6917
2b			AR	-0.4451	0.1580	-1.4593	46.7057
2a	*Salamandra salamandra*	9	He	-0.0390	**0.0225**	-2.4471	-69.7698
2b			AR	-0.4720	**0.0131**	-2.6903	42.6295

Table 1 Influence of the single amphibian species on *Rana temporaria* genetic diversity. Significance of the effect of amphibian species were tested using GLS, for taking into account spatial autocorrelation and past evolutionary history of populations. 1) models considering the frequency of COI lineage Alp1 as proxy for past evolutionary history; 2) models considering the number of COI lineages as proxy for past evolutionary history (see Appendix S1 in Supporting Information for details on model choice and phylogeographic background). Both expected heterozygosity (H_e; model 1a, 2a) and allelic richness (AR, model 1b, 2b) were considered as response variables. Significant p values ($p < 0.05$) are shown in bold; marginally significant p values are shown in bold italic. Number of occurrences of the different amphibian species is reported (n).

Niche overlap analysis: R. temporaria vs other amphibian species

The first two PCA axes generated in PCA-env analysis explained 31,43 % and 20.78 % of the original environmental variation, respectively (Figure S1b). The most important explanatory variables for axis 1 were mean annual temperature and crystalline rocks, followed by annual precipitation and slope; the most important explanatory variables for axis 1 were precipitation, anthropized areas, coniferous forests and slope. The contribution of each variable to the two PCA axes is shown in Figure S1B-C.

The pairwise comparisons between *Rana temporaria* and the other 6 amphibian species yielded different values of niche overlap (D), ranging from 0.108 to 0.532 (Table 2; Fig. S1, in in Supporting Information). The higher niche overlap values were recorded for *Ichthyosaura alpestris* and *Bufo bufo,* while *Rana dalmatina*, *Pelophylax* synkl. *esculentus* and *Bombina variegata* showed the lower niche overlaps with the focal species.

Niche similarity tests showed that the niche of *Rana temporaria* is not more similar to the niches of the other 6 amphibian species than expected by chance (all $p > 0.05$; Fig. S1, o-t in Supporting Information). Therefore, we can conclude that *Rana temporaria* occupy a sufficiently different environmental space compared to all other amphibians in the considered area, although exhibiting a certain degree of niche overlap with some species (particularly *Ichthyosaura alpestris* and *Bufo bufo*).

Habitat suitability modeling and habitat suitability maps correlation

In general, habitat suitability models produced with Maxent for the different amphibian species showed fair predictive power (training AUC ranging from 0.77-0.94; test AUC ranging from 0.75-0.92; average AUC standard deviation: 0.035; see Table S4 in Supporting Information). *Bufo bufo* and *Rana temporaria* yielded the models with lowest performance, although these are the species with the higher number of occurrences (454 and 1287, respectively). However, this is not surprising, since *Bufo bufo* and *Rana temporaria* are the more generalist among the considered amphibian species. The slightly lower predictive power of the models may be the result of their capability to persist in a wide range of environmental conditions, that are not easily to define and model. Indeed, there is a general agreement that generalist species are more difficult to model than specialists (Segurado & Araujo 2004; Evangelista *et al.* 2008).

For the focal species, *Rana temporaria*, the best predictor variables based on the percent contribution was temperature, followed by water areas (Table S5 in Supporting

Information). In general, temperature was recognized as an important environmental variable, being among the top 2 variables in 5 species. However, it must be noted that variable response curves for temperature differ among the species, reflecting different temperature optima (Fig. S2 in Supporting Information). For example, the temperature response curve for *R. temporaria* exhibited an opposite shape, compared to the curves of *Bombina variegata*, *Pelophylax* synkl. *esculentus*, *Rana dalmatina* and *Salamandra salamandra*. Other important variables were anthropized areas (which include agricultural areas) for *Bombina variegata* and *Pelophylax* synkl. *esculentus*, broad-leaved forests for *Rana dalmatina* and *Salamandra salamandra* and crystalline rock (negative influence) for *Ichthyosaura alpestris*.

The correlation tests between the habitat suitability map of *Rana temporaria* and those of other amphibian species yielded different outcomes (Table 2), from positive (*Ichthyosaura alpestris*, *Bufo bufo*) to negative (all other amphibian species, and particularly *Salamandra salamandra*) correlations. All correlations were significant after Dutilleul's correction for spatial autocorrelation ($p < 0.05$).

For a graphical comparison of the habitat suitability maps for the different species, see Fig. S3 in Supporting Information.

Table 2 Table showing, for each amphibian species, the correlation in habitat suitability map with *Rana temporaria*, together with the degree of niche overlap (Schoener´s D) with *R. temporaria*

n	Amphibian species	Habitat suitability correlation* *(with *Rana temporaria*)	*Niche overlap (D)**
15	*Ichthyosaura alpestris*	0.2235	0.5325
25	*Bufo bufo*	0.2118	0.3535
2	*Rana dalmatina*	-0.1883	0.1079
6	*Pelophylax* synkl. *esculentus*	-0.2447	0.1478
2	*Bombina variegata*	-0.2844	0.1335
9	*Salamandra salamandra*	-0.3502	0.2881

All habitat suitability correlations were significant after Dutilleul's correction for spatial autocorrelation ($p < 0.05$). All niche similarity tests were not significant ($p > 0.05$; Fig. S1, o-t in Supporting Information), i.e. the niche of *Rana temporaria* is not more similar to the niches of the other 6 amphibian species than expected by chance.

Niche overlap, habitat suitability correlation, and the effect of the single species on Rana temporaria genetic diversity: a synthesis

Niche similarity tests, showing niche differentiation, should exclude strong interspecific competition between *Rana temporaria* and the other considered amphibian species.

Moreover, comparing the results of niche overlap analysis (Table 2) with the outcome of GLS models assessing the effect of the single amphibian species on *Rana temporaria* genetic diversity (Table 1), we can note that the species characterized by the higher niche overlap with *Rana temporaria* (i.e. *Ichthyosaura alpestris* and *Bufo bufo)* seem to have low or no effect on its the genetic diversity levels (only *Ichthyosaura alpestris* show a significant effect in 1/4 models). On the other side, the species with low niche overlap (i.e. *Rana dalmatina*, *Pelophylax* synkl. *esculentus* and *Bombina variegata*) exhibit a more frequent negative effect. *Salamandra salamandra*, characterized by an intermediate value of niche overlap, displays a negative effect on genetic diversity in all the 4 models. The power of our analysis might be limited by the low number of occurrences of some species; however, it should be noted that *Ichthyosaura alpestris* and *Bufo bufo,* the best candidate for being strong competitors with *Rana temporaria* based on the value of niche overlap, are present in syntopy with the focal species in 15 and 25 sites, respectively: assuming that these species negatively affect the genetic diversity levels in *Rana temporaria*, via competition, their effect should be evident in our dataset. Therefore, our data don't seem to support a role for interspecific competition in shaping the genetic diversity levels in the focal species ("interspecific competition hypothesis").

Considering instead the habitat suitability maps, the species that exhibit positive (although low) correlations with *Rana temporaria* (i.e. *Ichthyosaura alpestris* and *Bufo bufo)* have low or no (negative) effect on its the genetic diversity. This may be interpreted assuming that *Rana temporaria*, when occurring in syntopy with these species, is not far from its ecological optimum and therefore experiences relatively favorable conditions, allowing the persistence of medium-sized populations, which in turn prevent the loss of genetic diversity due to drift. As already mentioned, *Ichthyosaura alpestris* and *Bufo bufo* are the species with the higher number of syntopyic occurrences with the focal species; in addition, in 9/26 wetland sites, these two species are the only amphibians found, besides *Rana temporaria* (see Table S3 in Supporting Information), giving rise with the latter to species poor communities. On the contrary, all the other 4 species showing a negative

correlation, exhibit a more frequent negative effect. In particular, *Salamandra salamandra*, which is characterized by the highest negative correlation, negatively affects genetic diversity of the focal species in all the 4 models. The presence of these species, characterized by different habitat requirements, may thus indicate sub-optimal ecological conditions for *Rana temporaria*. In other words, when *R. temporaria* occurs in syntopy with one or more of these amphibian species, it is far from its ecological optimum, and this may lead to a reduction in population size and consequently of genetic diversity due to intense drift.

All these considerations together provide support for "different ecological optima hypothesis", stating that the negative SGDC and the heterogeneous effect of the different amphibian species are due to differences in ecological optima between *Rana temporaria* and the (majority) of other amphibian species.

Discussion

Our data revealed a clear negative species-genetic diversity correlation (SGDC) between amphibian communities and genetic diversity in the focal species, *Rana temporaria*. The correlation remained significant also taking into account the past evolutionary history of populations, a factor which is often neglected in SGDCs studies, although its importance in shaping genetic diversity is well recognized and may potentially affect SGDC, too (e.g. Taberlet *et al.* 2012). It should be noted that the features of our study system mirror those of most empirical studies reporting SGDCs (Vellend 2003; Vellend & Geber 2005; Laroche *et al.* 2015), i.e. (1) species diversity measured as species richness in a single taxonomic level; (2) genetic diversity measured at microsatellite markers, (3) within one focal species, and (4) choosing a common, abundant organism. Moreover, our sampling units (wetland sites) represent discrete habitat patches, and according to Vellend *et al.* (2014), SGDCs are predicted to be generally positive and significantly stronger in studies focusing on discrete sampling units rather than in continuous habitats, given the greater potential for strong drift and limited dispersal*;* however, this was not the case in our study.

Vellend *et al.* (2014) claimed for a prevalence of positive SGDCs in empirical studies, although numerous examples of negative and non-significant SGDCs have been found (see Introduction). Given this large variation in the observed correlations, we stressed the importance of assessing not only SGDC's patterns, but also to analytically unravel the underlying processes, for evaluating the usefulness of SGDC in conservation planning (i.e. for using one level of diversity as surrogate for the other), both from a general and local

perspective. In particular, despite the potential important implications of negative SGDCs, their ecological drivers are rarely investigated analytically (Kahilainen *et al.* 2014). In order to shed light on the processes underlying the detected negative SGDC, we evaluated the effect of each single species, finding a heterogeneous response of the genetic diversity in the focal species to the presence of different amphibians.

Combining habitat suitability modeling and niche overlap analysis, we provide evidence that differences in ecological optima, between the focal species and (most) of the other amphibians present in the region, seem to be the most parsimonious explanation for our findings. Niche overlap analysis undoubtedly ruled out interspecific competition as a potential cause for the recorded patterns. Indeed, although we cannot exclude potential competition dynamics in the considered amphibian communities, which may occur at small spatial scale (e.g. the pond scale), we didn't find evidence for a role of interspecific competition in shaping the genetic diversity of *Rana temporaria* populations, which probably depend on processes occurring at larger spatial scales (i.e. the wetland, or landscape scale).

The parallel effect of locality characteristics on the two levels of diversity is generally supposed to lead to a prevalence of positive SGDC (Vellend & Geber 2005); our study, however, represents a concrete example of the opposite outcome: the same environmental variables seem to have an opposite effect on genetic diversity in our focal species, *Rana temporaria*, and species richness of amphibian communities. Such a conclusion is not counter-intuitive for the considered study system. Our focal species, *Rana temporaria*, although being generally found in a wide variety of habitats, showing high adaptability to different ecological conditions (Gasc *et al.* 1997; Lanza *et al.* 2009), is more frequent at high elevation in the Trentino region (1500-2000 m a.s.l; Caldonazzi *et al.* 2002). Indeed, the species is well adapted to cold climates, showing physiological and behavioral adaptations that allow the species to cope with extreme environments (Ludwig *et al.* 2015). On the other side, the species appears to be very sensitive to high temperatures, particularly when associated with low relative humidity: this environmental constraint limits its distribution at the lowest elevation and at the southern limit of its range. As a consequence, this anuran is less abundant and more localized in the Prealps (Lanza *et al.* 2009), and rare in the Adige valley river, a broad, low elevation area located in the middle of the study region (Sartori 2012). The species is known to exhibit large variation in local population sizes (Johansson *et al.* 2006), and this has been specifically ascertained also for the study region (PAT). As population size determines the magnitude of random genetic drift (Wright 1931; Frankham

1996), genetic variability also is expected to be lower in these populations persisting outside the species optimum.

By contrast, among the other amphibian species present in the region, only a few of them can persist at higher elevations (> 1500 m a.s.l.): *Ichthyosaura alpestris*, *Salamandra atra* (the only Alpine amphibian not linked to water for reproduction; absent in the considered wetland sites) and, to a lesser extent, *Bufo bufo* (Caldonazzi *et al.* 2002). The lower temperature at higher elevation, together with higher snow cover and short favorable seasons, results in a progressively more hostile and less energy-rich environment for most species, generally leading to a decline in species richness (e.g. Boone & Krohn 2000). Not surprisingly, in the considered alpine region the highest amphibian richness is found in the Adige river valley (Caldonazzi *et al.* 2002), exactly where *Rana temporaria* is rarer. Thus, when found in species-rich communities, *Rana temporaria* is probably far from its ecological optimum, persisting at lower density and with lower population sizes: this in turn negatively affect genetic diversity, leading to a negative SGDC.

Our habitat suitability models confirm these intuitive considerations, highlighting an important role for temperature, both for *R. temporaria* and most of the other amphibians, and, notably, with a response curve showing an opposite shape (in the focal species vs the other amphibians). In addition, other environmental factors and stressors may be perceived differently in *Rana temporaria* compared to the other species, e.g. agricultural pollution and water pH (which is influenced by geological bedrock; see Appendix S1 in Supporting Information, for a more detailed discussion on the effects of both stressors on different amphibian species).

Although not excluding the potential implication of other factors, this study showed that local environmental characteristics may be important drivers of SGDCs, not necessarily leading to positive correlations, confirming the outcome of other recent studies (e.g. Xu *et al.* 2016). Under these assumptions, the sign and entity of SGDCs may depend on the particular ecological requirements of the focal species (selected for the assessment of genetic diversity), compared to the other species in the community, as well on other context-dependent factors. In systems where biodiversity patterns are governed by environmental gradients (e.g. altitude), understanding the effects of these gradients on both the species and genetic level of biological diversity may provide an opportunity for predicting the sign of SGDCs. However, caution is needed, since these effects vary depending on the considered functional level, are often species-specific even within the same taxonomic group, and may be influenced by other processes in complex ways (e.g. Wei & Jiang 2011, for the effect of

altitude on species and genetic diversity in natural and disturbed forest tree communities). We must specify that our considerations are limited to the case of neutral genetic variation, i.e. genetic diversity measured using neutral markers, the most frequent choice in empirical studies reporting SGDCs so far. The rapid spread of later-generation molecular technologies and the consequent "genomics revolution" will offer the opportunity for investigating the effects of local adaptation on SGDCs in the near future.

To conclude, the outcome of this study provides a clear and relevant exception to the general assumption that species diversity and genetic diversity co-vary with a prevalence of positive correlations (Vellend 2005; Vellend & Geber 2005). Given that (1) modern theoretical models are showing that the relationships between species and genetic diversity may vary with complex dynamics, driven by different neutral and non-neutral forces, potentially involved in context-dependent interactions; and (2) empirical studies are indeed highlighting a wide variety of outcome, both in terms of correlations and supposed drivers, we agree with Taberlet *et al.* (2012) in warning against a general use of SGDCs in conservation planning. Specifically, conservation prioritization which focus only on species richness, the most widely used biodiversity proxy (Gaston 1996), assuming a positive correlation with genetic diversity, may fail to preserve the latter, in the presence of negative or non-significant SGDCs. This is a matter of great concern, since the importance of genetic diversity for the persistence of species and ecosystems is well recognized, both scientifically and politically (see Introduction). Generalizations about SGDCs, ignoring local processes and context-dependent interactions should be therefore avoided in the development of long-term conservation strategies, particularly under the consideration that complexity and context dependency are common features of ecological communities and, consequently, also of biodiversity declines (Wellnitz & Poff 2001; Blaustein & Kiesecker 2002; Green *et al.* 2005; Blaustein *et al.* 2011). Nevertheless, we stress the importance of empirical studies focusing on the different levels of biological diversity, with the aim of unravelling not only patterns but also the underlying processes and interactions, believing that conservation science must account for the dynamic nature of biological communities.

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Supporting Information

Appendix S1

Proxy for past evolutionary history of populations: background and model choice

A previous fine-scale phylogeographic study (using the mitochondrial cytochrome oxidase I gene, COI) revealed a complex past evolutionary history for the common frog (*Rana temporaria*) in the Trentino region (Chapter 2 of the present thesis). Specifically, three different evolutionary lineages (COI haplogroups) were detected in the study region: Alpine lineage 1 (Alp1), Alpine lineage 2 (Alp2) and Alpine lineage 4 (Alp4). These three lineages, previously identified for the central and eastern Alps by a recent large-scale study (Stefani et al. 2012), most likely originated in different Pleistocene glacial refugia and harbor different levels of genetic diversity at mtDNA (Stefani *et al.* 2012).

Therefore, the recorded current levels of genetic diversity (measured at microsatellite loci) may be potentially influenced by the complex phylogeographic patterns highlighted for the species, and we decided to further test the significance of SGDC and the effects of amphibian species on *Rana temporaria* genetic diversity taking into account this historical factor. As highlighted in text, we built generalized least squares (GLS) models testing two different proxy for evolutionary history, which correspond to different evolutionary scenarios (with different potential influence on current levels of genetic diversity): (1) Model 1: considering the frequency of the COI lineages as proxy for evolutionary history, under the assumption that different evolutionary lineages carry different levels of genetic diversity; and (2) Model 2: considering the number of COI lineages as proxy for evolutionary history, assuming that admixture among different lineages increase genetic diversity (addictive effect in admixture zones; Petit *et al.* 2003). COI data for the selected populations were derived from Chapter 2 of the present thesis. Considering the sites included in the present study, the presence of the lineage Alp4 is marginal (only in 5 sites, with frequency always < 0.4), while both Alp1 and Alp2 are widely distributed and their frequency is complementary (Table S3): indeed, they exhibit a high negative correlation ($R = -0.926$; p value < 0.001). Thus, we used the frequency of Alp1 as proxy of evolutionary history in Model 1.

Ecological variables included in habitat suitability models and niche overlap analysis

We used the same environmental variables for both habitat suitability modeling and niche overlap analysis, in order to enable a meaningful and fair comparison among the results of the two analyses. We considered the following variables: mean annual temperature, annual precipitation, 4 land cover types (anthropized environments, i.e. urban and agricultural area; coniferous forests; broad-lived and mixed forest; coniferous forest; water areas, i.e. lakes, rivers and wetlands), slope and geological type (crystalline vs non-crystalline rocks).

Temperature and precipitation are recognized as crucial factors governing the distribution and diversity of species (Boone & Krohn 2000; Evans et al. 2005; McCain & Grytnes 2010), particularly for amphibians (Buckley & Jetz 2007; Ortiz-Yusty *et al.* 2013). The adopted land cover classification is supposed to reflect the different habitat preferences of the considered species, and their different sensitivity to anthropic disturbance. For example, in its Italian distribution range, *R. temporaria* seems to prefer forested areas as terrestrial habitat, particularly coniferous forests, while *Rana dalmatina* and *Salamandra salamandra* generally prefers broad-leaved forests (Lanza *et al.* 2009). Moreover, *Pelophylax* synkl. *esculentus* and *Bombina variegata* are more tolerant to low-quality and polluted water environments (Ortiz-Santaliestra *et al.* 2010; IUCN 2016) and often depend on anthropic aquatic habitat for reproduction (e.g. ditches, artificial ponds, drinking troughs, agricultural cisterns, etc.), in Italy (Lanza *et al.* 2009) and particularly in the study area (Caldonazzi *et al.* 2002). Slope is a terrain parameter which is commonly used in analyzing the spatial distribution of amphibians (e.g. Gage *et al.* 2006; Bartelt *et al.* 2010; Liang & Stohlgren 2011; Block & Morrison 2008). Some amphibians tend to prefer flat regions, particularly the species which are linked to temporary ponds for reproduction (e.g. Dayton & Fitzgerald 2006), while others, e.g. those reproducing in stream ponds in mountainous regions, are associated with habitats that have steep slopes (Diller & Wallace, 1996; Diller & Wallace, 1999). Slope may also influence microclimatic patterns (Peterman & Semlitsch 2013), as well as the availability of daytime and overwintering refugia for some species (e.g. Apodaca & Godwin 2015).

The implemented geological classification has a double meaning for amphibian biology. Indeed, alpine lakes and other waterbodies differ markedly in pH according to bedrock composition, and crystalline rocks are known to be particularly vulnerable to acidification (Lerman *et al.* 1995). High sensitivity to acidification in alpine lakes have been reported also in the study region (Cantonati *et al.* 2002a,b). Amphibians exhibit high intraspecific

variation in acid tolerance (Pierce 1985). Among European amphibians, *Rana temporaria* and *Rana arvalis* show the highest acid tolerance (Andren et al. 1988; Pasanen et al 1998; Dolmen et al. 2010), while newts (e.g. *Triturus cristatus* and *Lissotriton vulgaris*; Skei & Dolmen 2006) are known to be particularly sensitive to low pH, probably because of the external gills (Brunelli & Tripepi 2005).

Moreover, crystalline and non-crystalline rocks also differ in the availability of surface water, which in turn affect the density of breeding habitats, particularly of temporary ponds (Skidds & Golet 2005; Batzer *et al.* 2012).

Slope was derived from the digital elevation model (DEM; 10 m resolution); land cover types and geological types were derived from the land cover (1:10,000) and geological map (1:100,000) of the region. DEM, land cover and geology GIS layers were retrieved from the public geodatabase of the Autonomous Province of Trento (http://dati.trentino.it/). Annual precipitation was obtained from WorldClim (Hijmans *et al.* 2005), while mean annual temperature was derived from reconstructed MODIS land surface temperature (LST) data (https://modis.gsfc.nasa.gov/data). More specifically, the original MODIS LST products were reconstructed at 250 m resolution, i.e. gap-filled to remove void pixels due to clouds (Neteler *et al.* 2010). Environmental data preprocessing, layers reclassification and variable extraction was performed in GRASS GIS (Neteler *et al.* 2012).

Table S1 Relationship between amphibian species richness and *Rana temporaria* genetic diversity: GLS models considering past evolutionary history of populations.

Model	Predictor	Response	Coefficient	p-value	t-value	AIC
1a	*species richness*	He	-0.0153	0.0021	-3.4558	-82.3507
1b		AR	-0.1535	0.0225	-2.4460	42.2453
2a	*species richness*	He	-0.0192	0.0004	-4.1528	-75.3648
2b		AR	-0.1745	0.0094	-2.8345	44.3547

Model 1: proxy for past evolutionary history of populations = frequency of COI lineage Alp1

Model 2: proxy for past evolutionary history of populations = number of COI lineages

Both expected heterozygosity (He; model 1a, 2a) and allelic richness (AR, model 1b, 2b) were considered as response variables. The effect of species richness is always significant ($p < 0.05$).

Table S2 List of wetland sites included in the study. Geographical coordinates (UTM 32N) and average elevation are reported, together with a brief ecological description. For sites belonging to Natura 2000 Network, the relative code is reported.

Code	Protected area	Site name	Long	Lat	Elev	Site description
Amp	IT3120076	Lago d'Ampola	628257.7	5080990.1	794.9	Lake, bogs
Bon	IT3120066	Palu' di Boniprati	624240.0	5087833.4	1205.7	Bogs, pond
Bro	local reserve	Brozin	641572.6	5124276.8	998.9	Bogs, pond
CCC	IT3120167	Campo Carlo Magno	641670.9	5095108.7	1648.7	Bogs, bog woodland
Ech	IT3120078	Torbiera Echen	630107.3	5093903.6	1272.5	Bogs, dystrophic pond
Fia	IT3120068	Fiave'	619430.9	5087524.0	664.9	Bogs, ponds
Ing	IT3120038	Inghiaie	623260.5	5124283.9	444.4	Lowland alluvial forest, bogs, ponds
Lag	IT3120045	Lagabrun	659256.4	5149302.6	1115.4	Bogs
Lel	IT3120035	Laghestel di Pine'	650390.6	5124742.5	876.2	Bogs, dystrophic pond
LRo	local reserve	Lago di Roncone	640675.9	5114260.0	861.2	Lake, riparian wetland
MBa	IT3120170	Monte Barco - Le Grave	617016.0	5099100.1	869.2	Mires, bogs, pond, bog woodland
Mon	IT3120088	Palu' di Monte Rovere	631954.7	5114059.0	1240.2	Pond, humid grasslands
MRe	IT3120067	Paludi di Malga Clevet	670173.7	5086653.0	1846.0	Bogs, bog woodland
Mug	IT3120032	I Mughi	678919.9	5096375.0	1268.9	Bogs, bog woodland
PLa	IT3120169	Torbiere del Lavaze'	669525.1	5119054.0	1802.1	Bogs
PMa	IT3120021	Lago delle Buse	667537.3	5110859.9	2082.7	Alpine lake, bogs
PS2	local reserve	Passo S.Pellegrino	677938.9	5091972.0	1939.8	Alpine lake, bogs
PT1	IT3120064	Torbiera del Tonale	701500.4	5107510.3	1856.3	Bogs
PTe	local reserve	Arboreto di Pieve Tesino	691289.8	5136694.3	833.3	Bogs, pond
PTr	IT3120057	Palu' Tremole	689410.3	5116817.3	1737.6	Bogs
Ron	IT3120033	Palude di Roncegno	702549.5	5104372.3	401.5	Lowland alluvial forest, pond
Ste	IT3120034	Paludi di Sternigo	687578.1	5102537.0	1010.1	Lake, riparian wetland
Tov	IT3120063	Lago di Tovel	685913.2	5129069.6	1210.3	Alpine lake
Va1	SIC IT3120177	Lago di Valagola	672052.7	5109190.5	1688.5	Alpine lake, bogs
VD1	local reserve	Nudole-Val Daone	714135.8	5139654.1	1651.5	Bogs
VG1	SIC IT3120175	Val Genova	674857.6	5112178.4	1053.0	Brooks, temporary ponds

Table S3 Species diversity and composition of amphibian communities; genetic diversity and phylogeographic history of *Rana temporaria* populations.

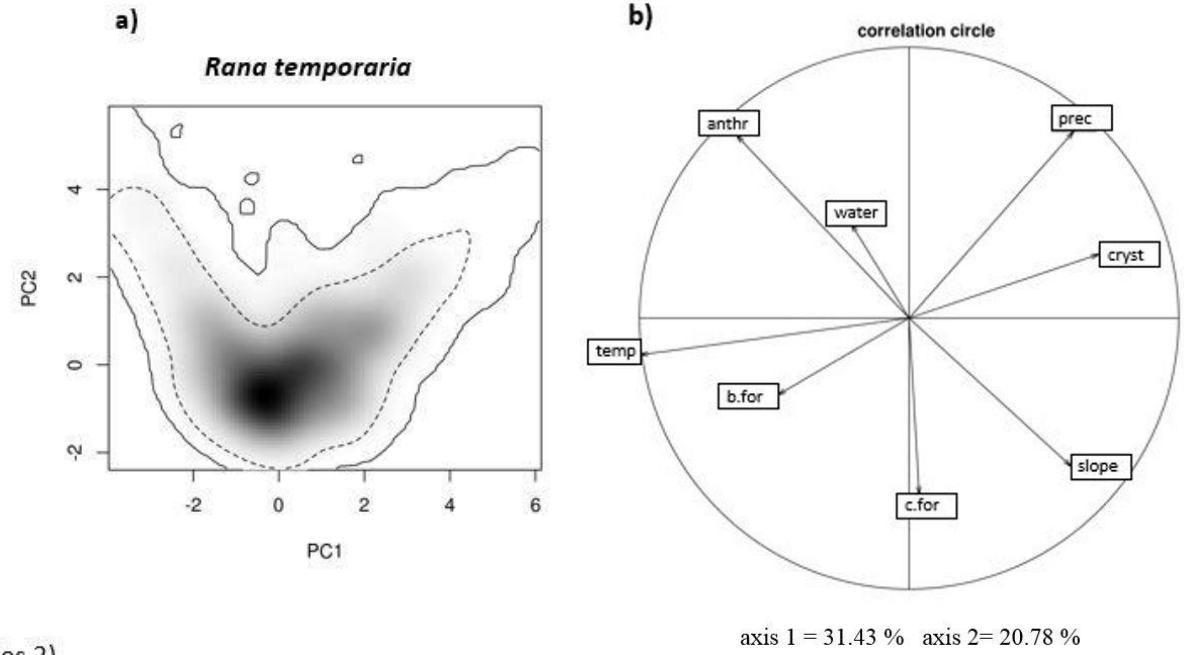
Species diversity data: SR = species richness; BB = *Bufo bufo*; Rt = *Rana temporaria*; Rd = *Rana dalmatina*; Pe = *Pelophylax* synkl. *esculentus*; Ss = *Salamandra salamandra*; Bv = *Bombina variegata*; Ia = *Ichthyosaura alpestris*;

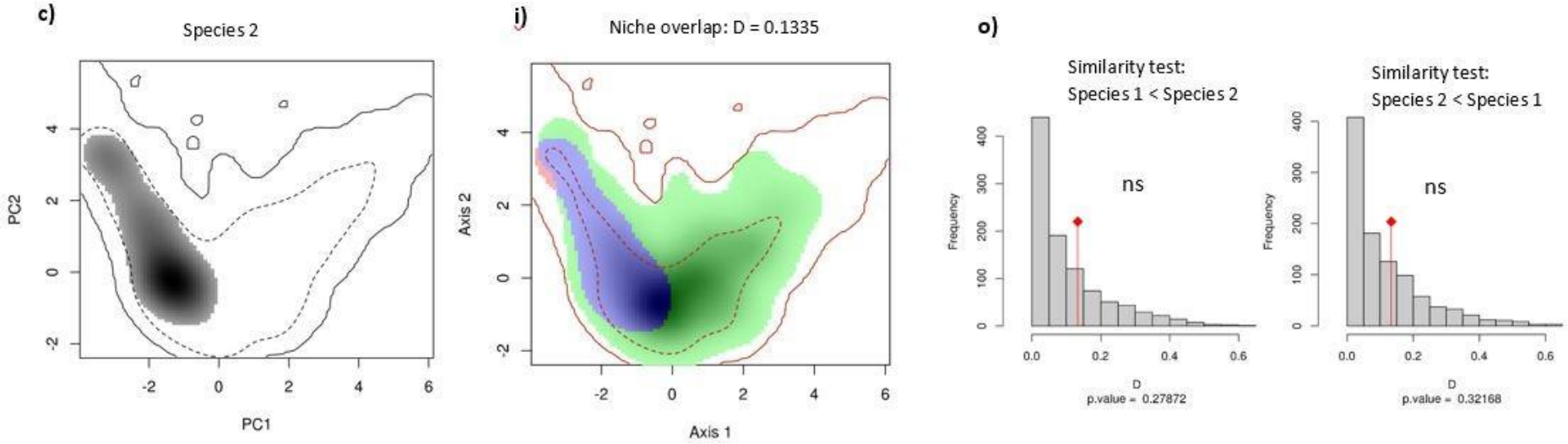
Genetic diversity data, microsatellite data (derived from Chapter 3 of the present thesis): N = number of genotyped samples; AR = rarefacted allelic richness, based on minimum sample size n = 15; He = expected heterozygosity;

Phylogeographic history of *R. temporaria* populations, mtDNA data (derived from Chapter 2 of the present thesis): N lin = number of detected COI lineages; Alp1 = frequency of COI Alpine lineage 1; Alp2 = frequency of COI Alpine lineage 2; Alp4 = frequency of COI Alpine lineage 4; Note: COI lineages are named according to Stefani et al. 2012.

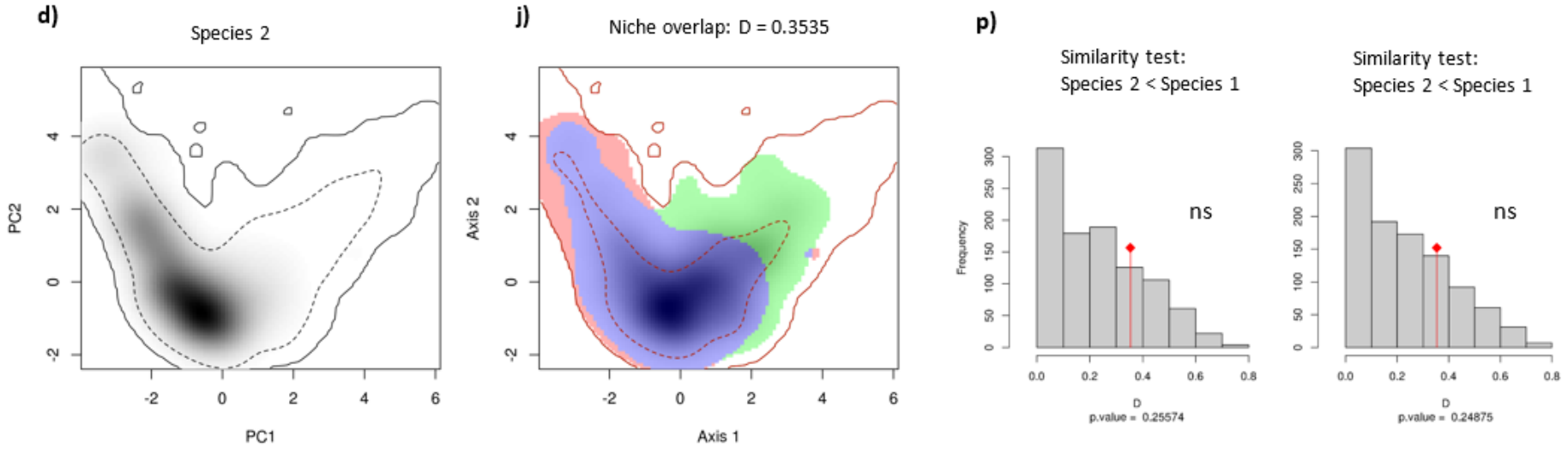
	Amphibian richness and community composition							Genetic diversity (of *R. temporaria* populations)			Phylogeographic history (of *R. temporaria* populations)			
Site	**SR**	***Bb***	***Rt***	***Rd***	***Pe***	***Ss***	***Ia***	***Bv***	**N**	**AR**	**He**	**N lin**	**Alp1**	**Alp2**	**Alp4**
Amp	2	x	x						30	6.287	0.651	1	1	0	0
Bon	2	x	x						24	6.486	0.654	1	1	0	0
Bro	3	x	x				x		21	6.059	0.612	3	0.5	0.3	0.2
CCC	2	x	x						26	5.883	0.625	1	1	0	0
Ech	4	x	x			x	x		24	5.463	0.559	2	0.3	0.7	0
Fia	4	x	x		x		x		31	6.321	0.650	1	1	0	0
Ing	5	x	x		x	x	x		33	4.928	0.533	2	0.8	0.2	0
Lag	4	x	x			x	x		29	6.289	0.603	2	0.6	0.4	0
Lel	6	x	x	x	x	x	x		24	5.567	0.587	3	0.5	0.4	0.1
LRo	2	x	x						15	5.917	0.641	1	1	0	0
MBa	7	x	x	x	x	x	x	x	16	4.826	0.505	2	0.2	0.8	0
Mon	4	x	x		x		x		24	5.583	0.555	2	0.1	0.9	0
MRe	1		x						52	6.685	0.703	1	1	0	0
Mug	5	x	x			x	x	x	28	5.635	0.557	2	0.7	0.3	0
PLa	3	x	x				x		23	6.529	0.637	2	0.8	0	0.2
PMa	3	x	x				x		42	6.463	0.625	2	0.7	0.3	0
PS2	3	x	x				x		22	5.659	0.597	3	0.5	0.1	0.4
PT1	1		x						18	5.545	0.600	1	1	0	0
PTe	3	x	x			x			23	5.471	0.604	2	0.5	0.5	0
PTr	3	x	x				x		20	5.731	0.610	1	1	0	0
Ron	5	x	x		x	x	x		31	5.628	0.625	2	0.9	0.1	0
Ste	2	x	x						15	6.417	0.636	3	0.6	0.1	0.3
Tov	2	x	x						26	5.378	0.612	1	1	0	0
Va1	2	x	x						21	6.191	0.657	1	1	0	0
VD1	2	x	x						40	6.355	0.678	1	1	0	0
VG1	3	x	x			x			42	6.285	0.654	1	1	0	0

Figure S1. Results of the PCA-env analysis, comparing the realized ecological niche of *Rana temporaria* and the other 6 amphibian species; **a)** Niche of *R. temporaria* along the two first axes of the PCA. Grey shading represents the density of occurrences. The solid and dashed lines represent, respectively, 100% and 50% of the available (background) environment; **b)** Contribution of the eight environmental variables on the two axes of the PCA and the percentage of inertia explained; for a legend of the different variables, see Tab. S5**; c-h)** Niches of the other amphibian species; **i-n)** Observed niche overlap D (blue) of *R. temporaria* with the other amphibian species; **o-t)** Histograms showing the observed niche overlap (D) between the two species (bars with a diamond) and simulated niche overlaps (grey bars) on which tests of niche similarity are calculated. The significance of the tests is shown (ns = non-significant; P < 0.05).
See Broennimann *et al.* (2012) for more details.

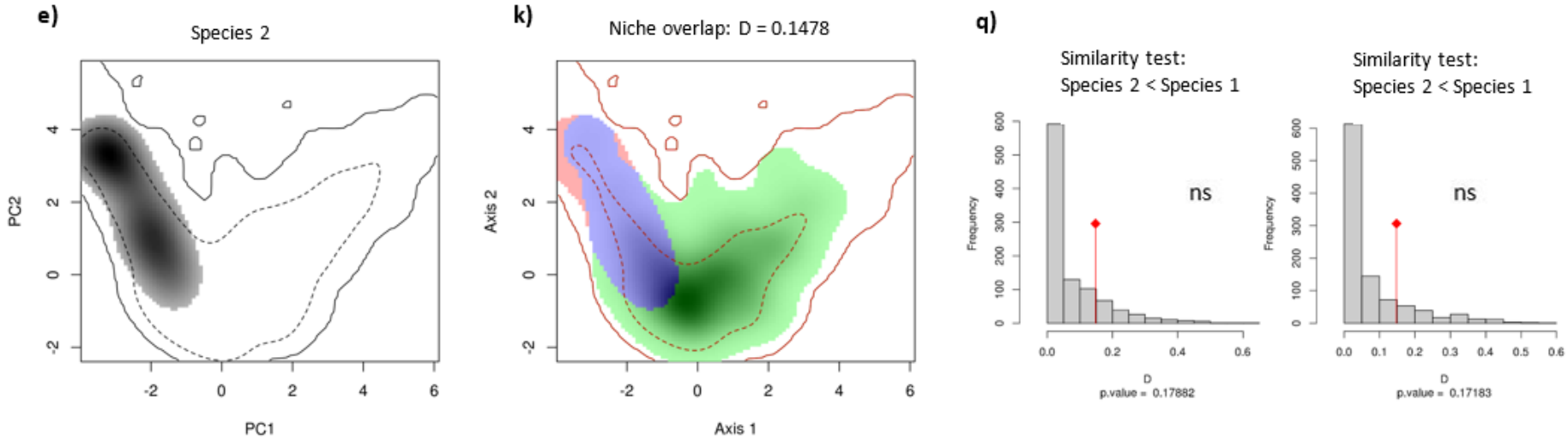




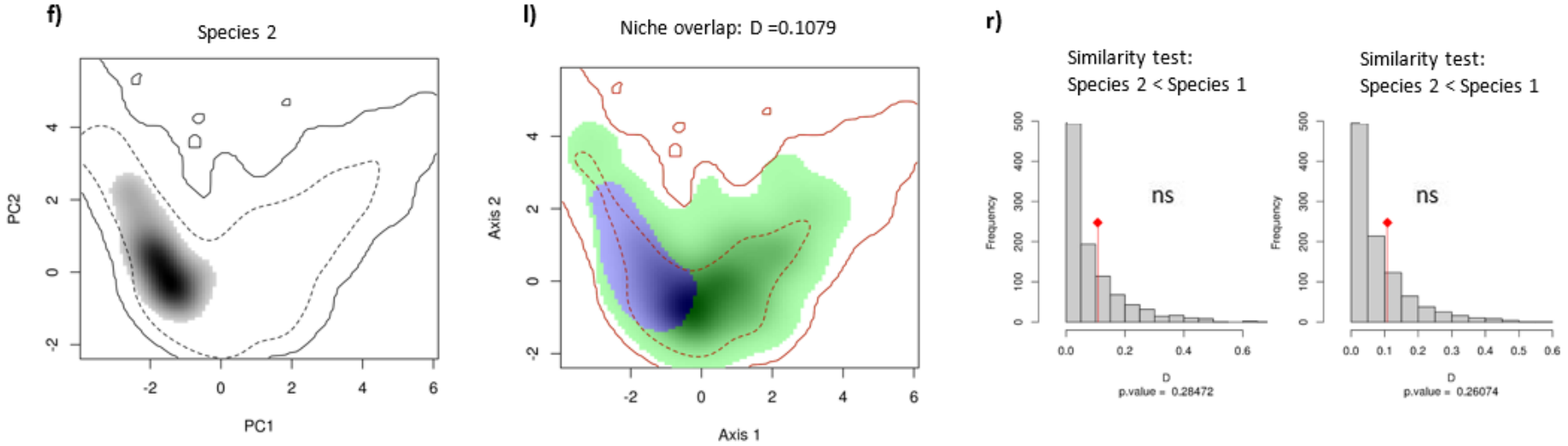
Rana temporaria (Species 1) vs ***Bufo Bufo*** (Species 2)



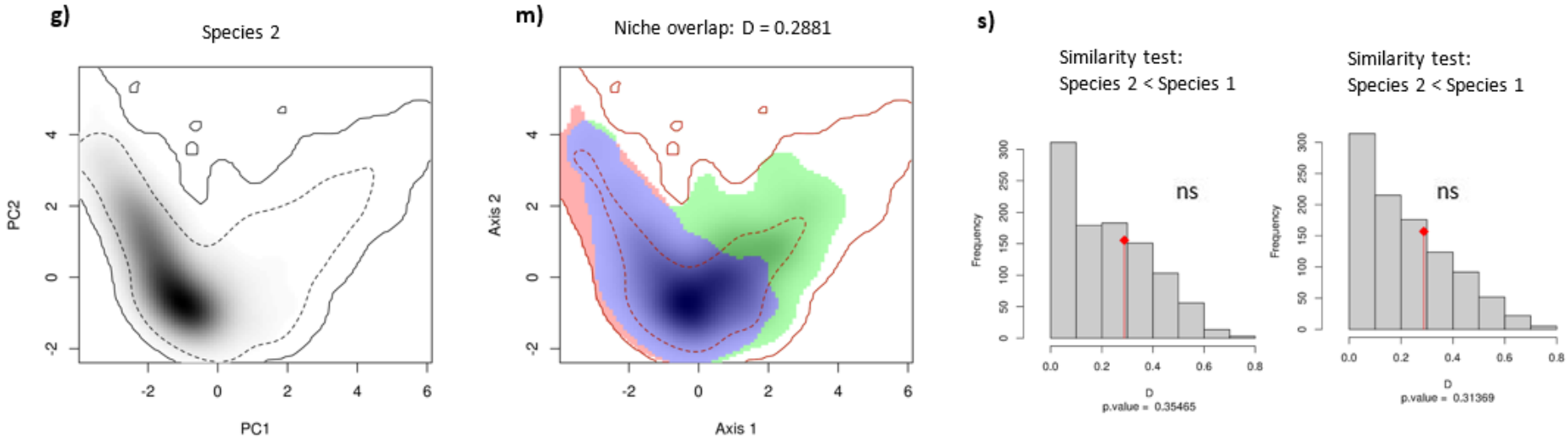
Rana temporaria (Species 1) vs ***Pelophylax*** **synkl.** ***esculentus*** (Species 2)



Rana temporaria (Species 1) vs ***Rana dalmatina*** (Species 2)



Rana temporaria (Species 1) vs ***Salamandra salamandra*** (Species 2)



Rana temporaria (Species 1) vs ***Ichthyosaura alpestris*** (Species 2)

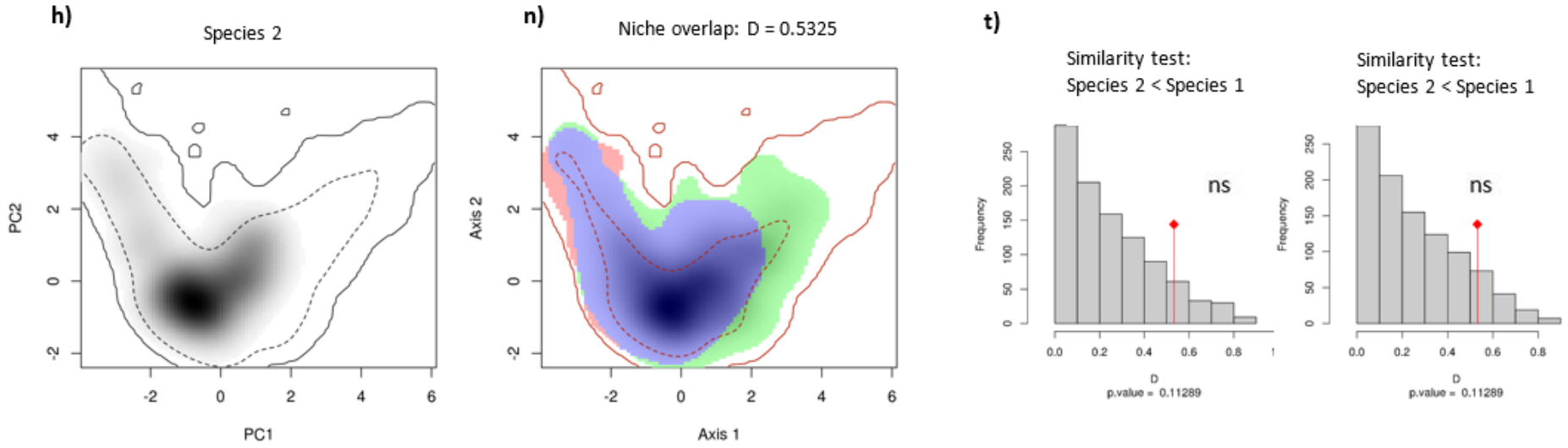


Table S4 Maxent: training AUC, test AUC and AUC standard deviation for the different habitat suitability models (average values for the 10 replicate runs); n = number of occurrences

Model/Species	n	Training AUC	Test AUC	AUC sd
Bufo bufo	454	0.7712	0.7482	0.0373
Bombina variegata	130	0.9097	0.8773	0.0406
Pelophylax synkl. *esculentus*	71	0.9441	0.9166	0.0239
Rana dalmatina	101	0.9365	0.9123	0.0376
Rana temporaria	1287	0.7984	0.7824	0.0275
Salamandra salamandra	260	0.8453	0.8267	0.0327
Ichthyosaura alpestris	231	0.8237	0.7946	0.0473

Table S5 Maxent: Relative percentage contribution of the selected environmental variables on the habitat suitability models of the different amphibian species. Bold represents the top 2 variables.

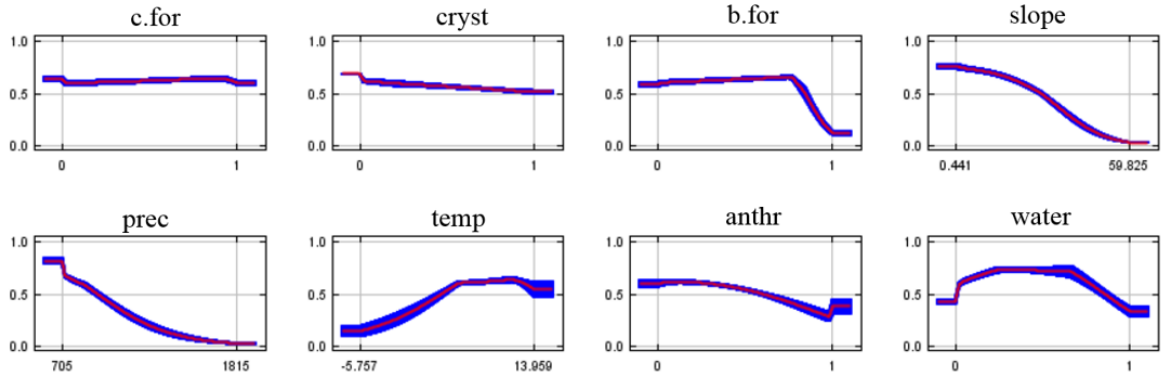
	Rana temporaria	*Bufo bufo*	*Bombina variegata*	*Pelophylax* synkl. *esculentus*	*Rana dalmatina*	*Salamandra salamandra*	*Ichtyosaura alpestris*
variable	**% contr.**	**% contr.**	**% contr.**	**% contr.**	**% contr.**	**% contr.**	**% contr.**
anthr	1.7	7.1	**16.9**	**46.1**	14.8	23.1	10
b.for	2.4	5.2	5.6	0.5	**27.1**	**25**	2.1
c.for	5.5	0.4	3.2	2.3	0.4	2.3	1.8
cryst	11.3	11.5	5.3	5.3	2.4	0.9	**31.7**
prec	7	6.1	2.2	0.9	2.1	7.7	8.2
slope	16.1	**26.2**	8.2	4	10.6	2.6	**24.4**
temp	**29**	15.7	**45.2**	**21.2**	**25.8**	**34.2**	3.3
water	**27.1**	**27.7**	13.4	19.7	16.9	4.2	18.5

Legend of environmental variables:

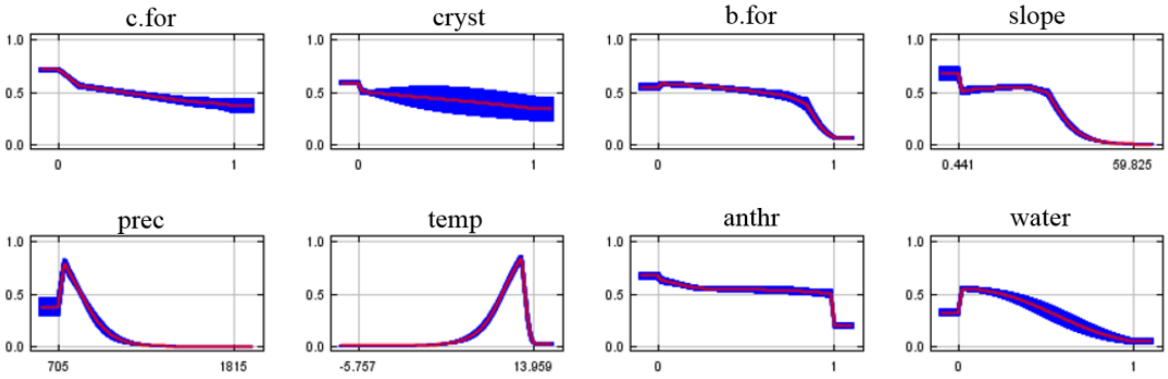
temp = mean annual temperature;
water = water areas (lakes, rivers and wetlands);
slope = slope;
cryst = crystalline rock;
prec = annual precipitation;
c.for = coniferous forest;
b.for = broad-lived and mixed forest;
anthr = anthropized areas (urban and agricultural areas)

Figure S2 Maxent habitat suitability modeling: curves showing, for the different species, how each environmental variable affects the Maxent prediction. The curves show how the logistic prediction changes as each environmental variable is varied, keeping all other environmental variables at their average sample value (red = mean response of the 10 replicate Maxent runs; blue = mean response +/- one standard deviation)

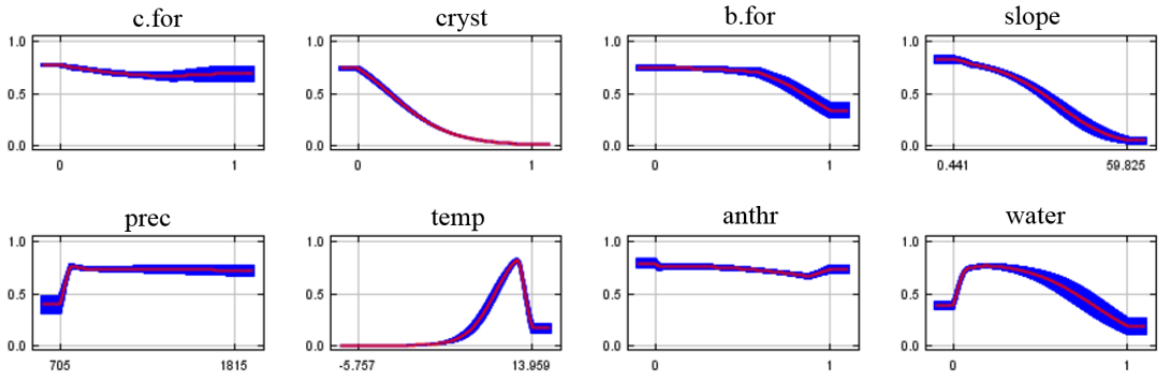
Bufo bufo



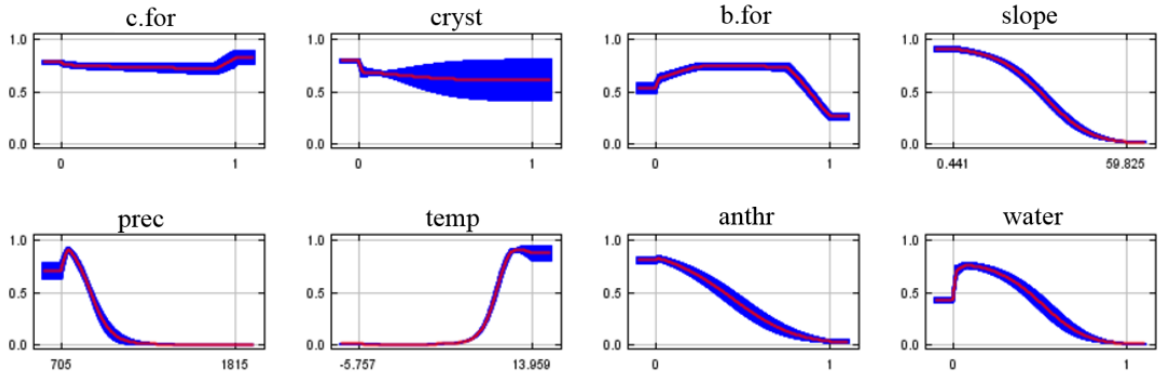
Bombina variegata



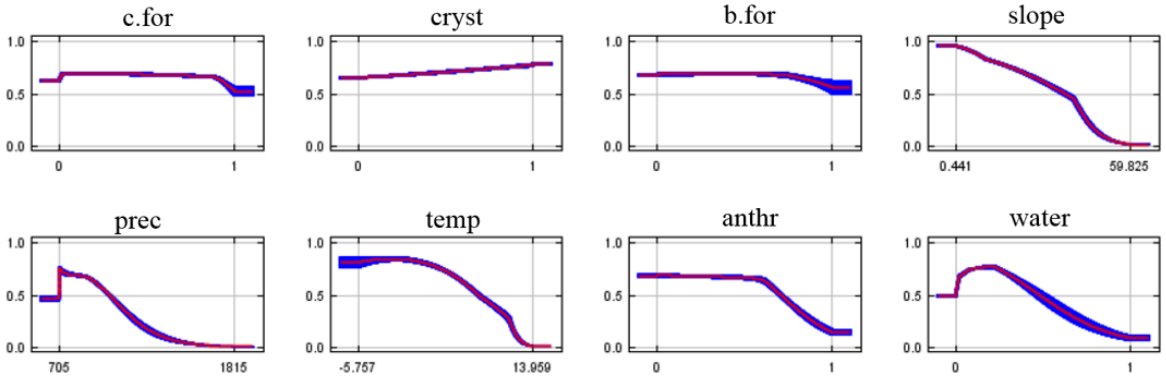
Pelophylax synkl. *esculentus*



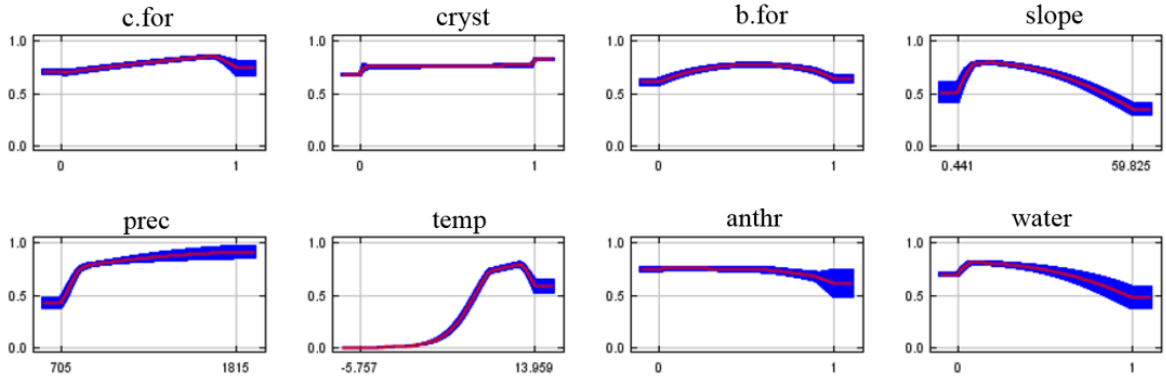
Rana dalmatina



Rana temporaria



Salamandra salamandra



Ichthyosaura alpestris

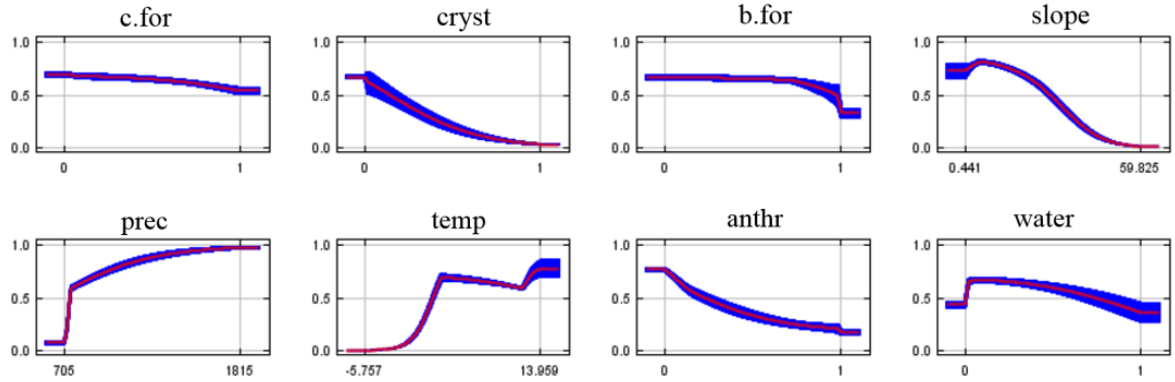
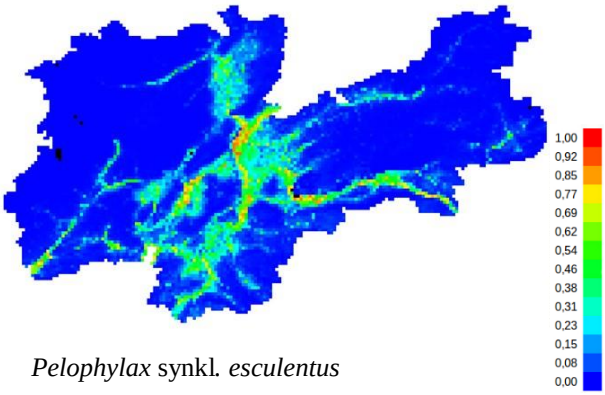
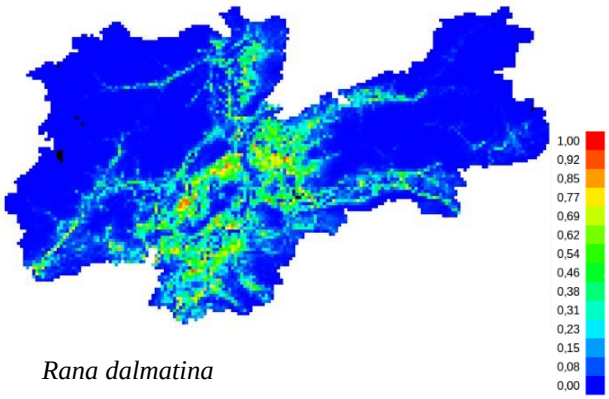


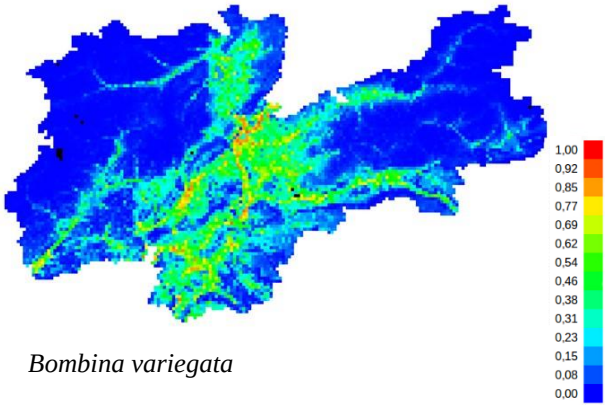
Figure S3 Habitat suitability maps (Maxent) for the different amphibian species



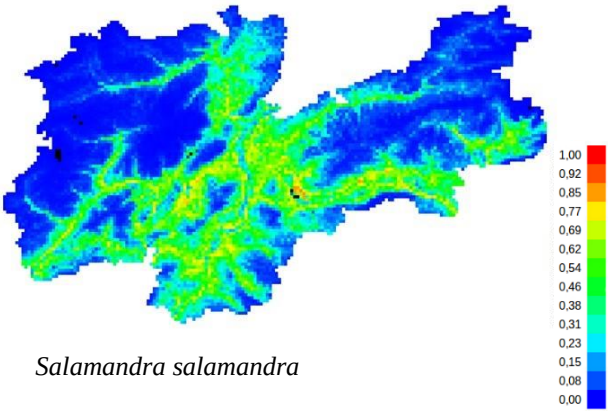
Pelophylax synkl. *esculentus*



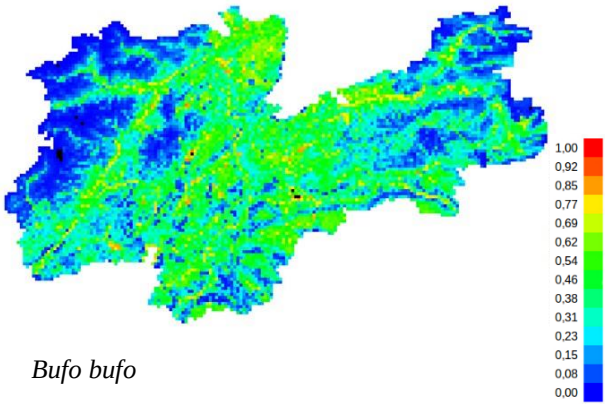
Rana dalmatina



Bombina variegata



Salamandra salamandra



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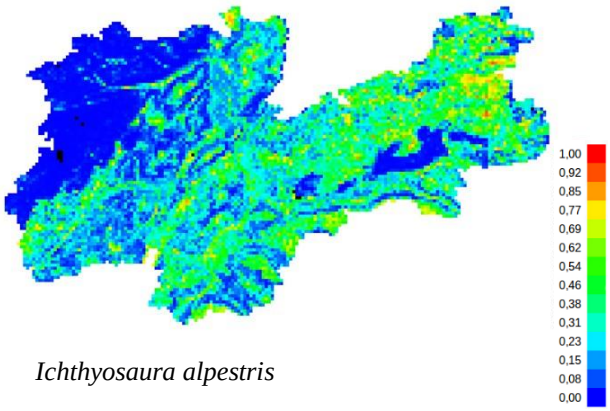

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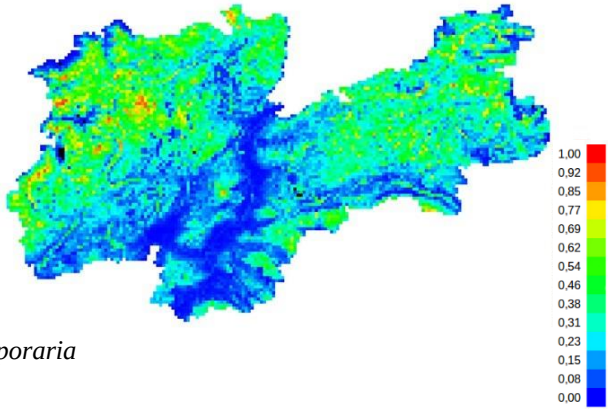

Rana temporaria

Chapter 5. General conclusions

5.1 General discussion

In the ongoing global biodiversity crisis, amphibians are the most endangered group of vertebrate, with increasing reports of population declines and extinctions worldwide (Gardner 2001; Stuart *et al.* 2004). A growing body of evidence is showing that the different levels in which biological diversity may be divided (i.e. genes, species, and ecosystems) are broadly linked, and ecological processes results from the complex interactions between these levels (Hughes *et al.* 2008). As a result, modern conservation biology is increasingly recognizing the need for an integrative approach.

In this project, we adopted such an approach: focusing on a south-eastern Alpine region and choosing a model organism, the common frog (*Rana temporaria*), as target species for the evaluation of genetic diversity, we investigated the evolutionary and ecological processes affecting amphibian biodiversity at different levels and different temporal scales, within a systemic perspective.

In the first study (Chapter 2), by means of a fine scale phylogeographic reconstruction we provided strong support for a "refugia-within-refugia" scenario (sensu Gomez & Lunt, 2007) as most likely explanation for the Pleistocene evolutionary history of *Rana temporaria* in the Alpine area. According to this hypothesis, first proposed for the species by Stefani *et al.* 2012 (in contrast to previous hypotheses; Teacher *et al.* 2009), *Rana temporaria* survived the last glacial period in multiple peripheral refugia on the southern slopes of the Alps, separated by inhospitable intervening regions. Trentino was almost completed covered by the Adige glacier until Last Glacial Maximum (Caldonazzi & Avanzini 2011) and our data clearly indicates the presence of two different refugia in the southern margin of the region (or in the near proximity), and another, more far refugium in the eastern Alps. Starting from these non-glaciated areas, re-colonization occurred following irregular routes, reflecting the complex orography of the region. A contact zone among different evolutionary lineages was highlighted in the eastern part of the region. Interestingly, the Adige river valley was found to be an "asymmetric barrier" relative to re-colonization routes: while one lineage crossed the valley, penetrating in the eastern part of the region from north-west, the other two lineages remained confined to the eastern part. This intriguing pattern remains an open question: potential speculative explanations are listed in Chapter 2, but our data does not allow a conclusive answer. The puzzle could be

completed by means of future investigations, combining a specific sampling design (including "pure" populations for all the three lineages), a multi-gene approach allowing robust demographic inference, and a detailed paleoclimatic reconstruction. Additional support might come from paleogeographic information from different organisms, too.

The second study (Chapter 3), a population and landscape genetic analysis performed using microsatellite data, confirmed the Adige river valley as a barrier also for present connectivity. This time, however, the detected barrier effect was complete and bi-directional (with only one local exception; see Chapter 3 for a detailed discussion). A spatial analysis of intra-population genetic diversity patterns revealed an unexpected pattern, namely an opposite latitudinal trend for genetic diversity levels among the two sub-regions. This discordance is difficult to explain invoking hypothetical ecological differences between the two sub-regions, but seem to well match the phylogeographic patterns highlighted in Chapter 2, suggesting a key role for the different Pleistocene evolutionary history of the species in the two sub-regions. However, reduced (current) connectivity seems to play an additional role for the considerably low genetic diversity of some specific populations, located in isolated, calcareous, Prealpine mountain massifs. Moreover, a significant difference among the two sub-regions emerged from patterns of fine-scale population structure, too.

Overall, considering the different spatial scales, genetic patterns in the common frog seem to be shaped by a combination of historical and present factors. This outcome stresses the importance of a combined approach consisting in the use of different molecular markers characterized by different rates of substitution (e.g. mtDNA and microsatellites), which allow to capture signatures of population processes at different times in evolutionary history (Avise *et al.* 1987; Thomson *et al.* 2010). Without a parallel mtDNA analysis, our interpretation of the recorded patterns of genetic diversity at microsatellite loci could have been misleading. Notably, only microsatellite markers are generally considered in landscape genetic studies (Wang 2010). Although most landscape genetic studies generally focus on small spatial scale (Holderegger & Wagner 2008), our intensive sampling design pointed out complex phylogeographic patterns for the common frog even at very limited spatial scale: this aspect should not be underestimated in studies focusing on small-sized, low-vagility vertebrates.

Lastly, our ultimate study (Chapter 4) consisted in an empirical evaluation of the correlation between species and genetic diversity (SGDC; Vellend 2003). We tested whether species richness in the amphibian communities and genetic diversity in the focal species

(*Rana temporaria*) co-vary in space in the considered alpine region (Trentino), and we found a strong, negative correlation. We demonstrated that the recorded pattern was due to the opposite influence of environmental factors on the two levels of biological diversity, ruling out the potential role of interspecific competition. We therefore highlighted a clear and relevant exception to the general assumption that species diversity and genetic diversity co-vary with a prevalence of positive correlations (Vellend 2005; Vellend & Geber 2005). It is worth noting that other examples of negative or non-significant correlations from empirical data have been reported (e.g. Karlin *et al.* 1984; Marshall & Camp 2006; Taberlet *et al.* 2012; Avolio & Smith 2013; Xu *et al.* 2016).

Our aim here is not to provide universal rules: on the contrary, we believe that the assumption of a general positive correlation is a dangerous oversimplification, which ignores the complexity of the underlying factors and their context-dependent interactions.

5.2 Conservation implications

Rana temporaria is a widespread amphibian and it is not currently considered threatened, although local declines are documented for the species, and it might be affected by range reduction and population fragmentation in the near future due to climate change (Bartolini *et al.* 2014). However, the importance of studies focusing on common species is well recognized in conservation genetics, since fine-scale, detailed studies are usually difficult to implement for rare organisms. Information gained from this study may therefore provide important basis for the conservation and management of more threatened amphibians and vertebrate species. For example, the finding of a genetically homogeneous gene pool at mtDNA in the western part of the region, opposite to the admixture patterns found in the east, together with the separation highlighted with microsatellite markers, clearly indicates the need for different management and conservation strategies for the species in the two sub-regions. Notably, an analogous east-west genetic subdivision has been found for other animal species in the Trentino region (Vernesi *et al.* 2016): further studies will be needed for assessing the generality of this pattern.

Under a conservation perspective, past evolutionary events such as range expansion-contraction due to glacial cycles are rarely considered. However, it has been recently recognized that understanding patterns and processes related to Pleistocene refugia may be of crucial importance for developing a robust conservation strategy in the face of ongoing climate change. Indeed, the study of major paleoclimatic events may help understanding the

genetic and evolutionary consequences of range shifts, extinctions and recolonization processes, identifying potential future climate change refugia and implementing priority actions for the specific management of these areas (Morelli *et al.* 2016).

The detected negative species-genetic diversity correlation represents perhaps the most relevant conservation outcome of this study. Indeed, conservation strategies focus mainly on species diversity, because genetic diversity is more difficult to measure at large scales. Even if the importance of genetic diversity is recognized, species richness is usually taken as a surrogate, assuming a positive correlation. Our study argues against the generality of this positive correlation and its indiscriminate use in conservation biology: in the presence of negative or non-significant correlations, using species diversity as a proxy of genetic diversity might result in a dangerous neglection of true genetic diversity hotspots. This is a matter of great concern, since the importance of genetic diversity for the persistence of species and ecosystems is well recognized. The possibility of negative correlations, moreover, may lead to potential conservation conflicts. A possible solution would be to dedicate some areas towards the conservation of species richness, and others to genetic diversity, considering different forms of management. As an alternative, "trade-off" areas can be identified, although this approach would probably lead to a sub-optimal preservation of both levels of diversity. Again, comparative studies aimed at identifying common patterns among species may be of great help in this respect, even if we cannot deny that critically endangered species, affected by particular threats, need specific conservation actions.

In particular, according to IUCN (2016), the most endangered amphibian taxa in the study region is the golden Alpine salamander (*Salamandra atra* ssp. *aurorae*). This urodele, endemic of a restricted area in Veneto and Trentino (and absent from the wetlands sites included in this study), is considered to be critically endangered by the IUCN red list, because of its very limited geographic distribution and the potential threat of "exploitation of its natural habitat through wood harvesting". In the conservation management of the species, this particular aspect should be carefully considered, trying to avoid mechanical harvesting or at least limiting it to non-breeding period. Besides this specific case, however, forest management and sylvicultural practices are recognized to have an important influence on the survival and fitness of many different amphibian species (see DeMaynadier & Hunter 1995, for a review).

Concerning forest habitats, another important, implicit indication emerging from this study is the primary role highlighted for broad-leaved forests in determining habitat suitability for

some amphibian species (e.g. *Rana dalmatina* and *Salamandra salamandra*). Broad-leaved forests represent only 5% of total forest area in Trentino (APPA 2012): this limited presence is partly due to the typical alpine climate of the region, but also to massive habitat alteration (i.e. destruction of lowland and riparian forests) in low elevation valleys, with particular reference to the Adige river valley. We therefore stress the importance of the conservation and restoration of these critical forest habitats, particularly in view of the fact that these lowland areas harbor the higher amphibian richness in the region (Caldonazzi et al. 2002). In the remaining, degraded small patches of lowland forests, the preservation of breeding habitats, as well as of forest complexity and natural dead wood dynamics (providing suitable microclimates and refugia habitats for amphibians; DeMaynadier & Hunter 1995) should be a conservation priority.

In summary, we think that conservation strategies may largely benefit from an integrate approach, which takes into account the ecological and evolutionary processes affecting and sustaining biodiversity at different levels, i.e. from genes, to species and ecosystems, with the ultimate goal of preserving not only current biodiversity patterns, but also future potential.

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