Ancient, but not recent, population declines have had a genetic impact on alpine yellow-bellied toad populations, suggesting potential for complete recovery

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Abstract

Reduction in population size and local extinctions have been reported for the yellow-bellied toad, *Bombina variegata,* but the genetic impact of this is not yet known. In this study, we genotyped 200 individuals, using mtDNA cytochrome b and 11 nuclear microsatellites. We investigated fine-scale population structure and tested for genetic signatures of historical and recent population decline, using several statistical approaches, including likelihood methods and Approximated Bayesian Computation. Five major genetically divergent groups were found, largely corresponding to geography but with a clear exception of high genetic isolation in a highly touristic area. The effective sizes in the last few generations, as estimated from the random association among markers, never exceeded few dozen of individuals. Our most important result is that several analyses converge in suggesting that genetic variation was shaped in all groups by a 7- to 45-fold demographic decline, which occurred between a few hundred and few thousand years ago. Remarkably, only weak evidence supports recent genetic impact related to human activities. We believe that the alpine *B. variegata* populations should be monitored and protected to stop their recent decline and to prevent local extinctions, with highest priority given to genetically isolated populations. Nonetheless, current genetic variation pattern, being mostly shaped in earlier times, suggests that complete recovery can be achieved. In general, our study is an example of how the potential for recovery should be inferred even under the co-occurrence of population decline, low genetic variation, and genetic bottleneck signals.

Key words: *Bombina variegata,* bottleneck, effective population size, microsatellites, demography

Introduction

Amphibians are among the most threatened vertebrates. Many species from all continents are experiencing a demographic decline (Houlahan et al. 2000), due to multiple causes (Allentoft and O'Brien 2010). Important anthropogenic activities include land use, leading to habitat loss and fragmentation, pollution and indirectly the increase of UV-B irradiation (Weyrauch and Grubb 2006). Also, climate change and global warming affect the distribution of amphibians, influence breeding phenology or lead to pathogen outbreaks (Corn 2005; Rohr et al. 2008). Amphibians appear to be particularly sensitive to all these processes, making them good biological indicators of environmental quality (Blaustein and Wake 1990).

The causes and consequences of amphibian decline, and the utility of this taxon as a biological indicator, cannot be generalized at a global scale. Many factors interact, and their impact likely differs according to geographic areas and focal species (Beebee and Griffiths 2005). Studies at regional scale, where the major factors of habitat disturbance can be identified, the demographic dynamic of a species and its genetic impact can be reconstructed, and the possible causal influences can be inferred, are therefore crucial for understanding, and mitigating, amphibian declines. In this context, the Alpine environment is of particular interest and concern.

The Alpine environment is heavily affected by environmental change, including climate change and land use (Cannone et al. 2008; Vanham et al. 2009; Keiler et al. 2010; Huggel et al. 2010). In particular, temperatures in the European Alps increased in the last century twice as much as the global average increase (Brunetti et al. 2009). Consequences of climate warming, such as the upwards shift of the tree-line (Leonelli et al. 2011), or the change in population genetic structure, have been already demonstrated or predicted in many Alpine plants species (Jay et al. 2012; Moradi et al. 2012). However, few case studies of recent demographic and genetic change are documented in animals (though see for example the decreasing litter size in the marmot, Tafani et al. 2013). Here we analyzed the genetic variation in an amphibian species sampled in the Italian Alps. Our main goal is to estimate the genetic impact, if any, of its recent demographic decline.

In studying influences on genetic patterns, it is always important to consider not only recent but also ancient events in a species' history. For example, many species have undergone range fluctuations, colonization events, or demographic collapse due climatic/ habitat changes that occurred thousand of years ago. Examples include well-documented northward tree migration after the last glaciation (10,000 to 20,000 years ago), mid-Holocene hemlock decline, and elephant population contraction due to drying in tropical Africa 4000 years ago (Bhiry and Filion 1996; Hewitt 2000; Okello et al. 2008; Lee-Yaw et al. 2008). Such events have left genetic and genomic signatures, and may be the dominant drivers of modern genetic patterns in some species. In other species, it may be shown that the drivers are more recent, as in Iberian lynx (Casas-Marce et al. 2013). Determining whether and how ancient and recent environmental change has influenced population genetic patterns is a key unanswered evolutionary (Andrew et al. 2013) and ecological (Sutherland et al. 2013) question, and whether a species is newly rare or was always rare will also determine the relevant management interventions to be applied (Sgro et al. 2011).

Recent statistical genetic methods offer the potential to give a more complete understanding of demographic and genetic histories (Andrew et al. 2013), especially when a direct comparison between genetic variation in modern and museum samples (e.g. Rubidge et al. 2012) is not possible. Specifically, improvements in likelihood and simulation-based methods allow comparison of alternative demographic models (e.g. stability, decline) and estimation of parameters regarding historical and contemporary population sizes and timing of major events. These methods are a major improvement on widely-used but simplistic tests of population equilibrium. Use of multiple complementary analyses, and comparing results among them, should help unravel recent and ancient historical fluctuations in effective population size.

The yellow-bellied toad, *Bombina variegata,* is mainly distributed across central western Europe, from Spain to the Carpathian Mountains (Sillero et al. 2014). Breeding sites are usually ephemeral, and include small puddles in meadows and river loops and occasionally farm ponds or water-filled wheel ruts (Gollmann et al. 1998; Di Cerbo and Ferri,2000, Sillero et al. 2014). Although the species is globally considered of Least Concern by the IUCN (IUCN 2014), extinctions or

99 demographic reductions have been reported in the last decades across the distributional range. In
100 particular, severe declines are documented in Romania, the Netherlands, and Italy (Goverse et al.
101 2007; Barbieri et al. 2004; Covaciu et al. 2010). Only one population is now described in
102 Luxembourg, and the species is probably extinct in Belgium and highly fragmented in France
103 (Kuzmin et al. 2009). Urbanization and consequent loss of suitable habitat (e.g. abandonment of
104 pastures, heavy use of unpaved forestry roads and drainage of natural breeding sites) are considered as
105 the major factors reducing the population sizes and increasing the fragmentation in this species.
106 Additionally, chytridiomycosis has been also suggested to be an important cause of population decline
107 at least in the sister species *Bombina pachypus* (Stagni et al. 2004). As in many amphibians in natural
108 conditions, *B. variegata* has small effective population size (Beebee and Griffiths 2005) and low
109 dispersal ability (Smith and Green 2005; Hartel 2008), making the genetic and non-genetic risks
110 associated to small numbers of highly isolated individuals even higher.
111 In Italy, *B. variegata* was common in the last century (De Betta 1857; Giacomelli 1887;
112 Vandoni 1914), but it is significantly declining in many areas (Stagni et al. 2004). Anthropization of
113 natural habitats, pollution and use of pesticides led to a population decrease in the last decades
114 (Barbieri et al. 2004), fragmentation and local extinctions (Di Cerbo and Ferri 2000). A recent study
115 used simulations, under various models of climate change, environmental alteration and solar
116 irradiation, to predict that the yellow-bellied toad in Italy might lose between 13% and 75% of its
117 suitable natural habitat in the next 50 years (D'Amen et al. 2011).
118 Here we study the pattern of genetic variation at the mitochondrial cytochrome b gene and at
119 11 microsatellite markers in a restricted area in the Italian Alps, where recent extinctions and
120 population declines have been confirmed (Caldonazzi et al. 2002). We typed 200 individuals from 9
121 sites to address the following main questions: Does the genetic pattern show evidence of demographic
122 decline and fragmentation, and, if so, can we directly infer that recent human-related factors are
123 responsible for the genetic pattern? We address this question using a set of complementary statistical
124 methods suitable to estimate the effective population sizes and their temporal dynamic, the population
125 structure, the individual genomic compositions and the pattern of isolation by distance, and to

probabilistically compare alternative demographic models. Our results have specific implications for the conservation of *Bombina variegata*, and provide general guidelines for avoiding over-estimation of extinction risks when genetic data are analysed.

Materials and methods

Samples collection and DNA extraction

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

Genetic typing

We initially sequenced a fragment (471 bp) of the mitochondrial DNA (mtDNA) cytochrome b gene to verify the haplotypic affiliation of the samples, with respect to the known maternal phylogeographic pattern in Europe. We used the primer pairs L14850 and H 15410 according to Tanaka et al. (1994).

PCR amplifications were conducted in 20 µl (containing 1ul of template DNA, 2 µl of 10X buffer, 0.1 µM of each pair of primers, 1 unit of Hot Master Taq polymerase and ultra pure water) under the following conditions: 10 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 45 sec at 52°C, 60 sec at 65°C, and a final extension step for 10 min at 65°C. Sequences were edited using Finch TV 1.4.0 (an open source application developed by Geospiza Research Team, http://www.geospiza.com/Products/finchtv.shtml), assembled with Sequencer v.4.7 and aligned using ClustalX (Thompson et al. 1997) using default parameters.

The genetic variation level and structure at the local scale were then investigated typing 11 autosomal microsatellites (Supp. Table 1) previously isolated in *Bombina variegata* or *Bombina bombina* (Nürnberger et al. 2003; Stuckas and Tiedemann, 2006; Hauswaldt et al. 2007). PCR amplifications were conducted in four different multiplex reactions in a final volume of 20 ul containing: 1µl of template DNA, 2 µl of 10X buffer, 0.05 µM of each pair of primers, 1 unit of Hot Master Taq polymerase (Applied) and ultra-pure water. The amplification protocol consisted of an initial denaturation step at 94°C for 10 minutes, followed by 30 cycles of the series: 94°C for 30 seconds, annealing temperature (Ta: 53°C for Bv11 and Bv32; Ta: 56°C for 1A, 10F and F22; Ta: 45°C for B13 and 8A; Ta: 52°C for 5F, 9H, 12F and B14) for 30 seconds, 65°C for 45 seconds; then, a final extension step at 65°C for 10 minutes. PCR labeled products were run on a four capillary system ABI 3130 Genetic Analyzer (Applied Biosystem) and scored with an internal lane standard (LIZ) using GeneMapper software.

Statistical analysis

Mitochondrial DNA

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

180

181 *Microsatellites*

182 Microsatellites were tested for the presence of null alleles, allele drop-out and scoring errors using
183 MicroChecker (Van Oosterhout et al. 2004). We used GENEPOP 3.4 (Raymond and Rousset 1995) to
184 test for deviations from Hardy–Weinberg equilibrium for each locus and globally. We also tested
185 genotypic Linkage Disequilibrium (*LD*) for each pair of loci. To evaluate overall genetic variation,
186 expected and observed heterozigosity (H_e and H_o) and number of alleles (N_a) within each population
187 were calculated using Arlequin v3.5 (Excoffier and Lischer 2010); FSTAT software (Goudet 1995)
188 was used to calculate allelic richness (A_r). In addition, pairwise F_{st} values between populations and
189 their significance were computed with Arlequin v3.5 and the corresponding triangular matrix of
190 distances was visualized using Principal Coordinates analysis (PCoA) implemented in GenAlex v6.5
191 (Peakall and Smouse 2012). Pairwise distances were also computed using two indices of genetic
192 differentiation that, differently from F_{st}, do not depend on the level of variation within populations:
193 G'_{st} (Hedrick 2005), and Jost's *D* (Jost 2008).

194

195 *Bayesian clustering analyses*

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

205

206

207 *Genetic vs. geographic distances*

208 The correlation between genetic similarity and geographic distance was evaluated at both individual
209 and population levels. At the individual level, we computed with the software SPAGeDi (Hardy et al.
210 2002) the kinship coefficient estimator derived by Loiselle et al. (1995) for all pairs of individuals.
211 These coefficients were then pooled in classes with similar number of comparisons, corresponding to
212 different geographic distances. At the population level, we used a Mantel test to analyse the
213 relationship between the linearized F_{st} based distance ($F_{st}/(1-F_{st})$) and the logarithm of the linear
214 geographic distance. The statistical significance was evaluated using a permutation test as
215 implemented in GenAlex v6.5 (Peakall and Smouse 2012).

216

217 *Recent effective population size*

218 Two methods were used to estimate the recent effective population size (N_e) of each population: LDNe
219 (Waples and Do 2008; Do et al. 2014) and ONeSAMP (Tallmon et al. 2008). LDNe is based on the
220 linkage disequilibrium among unlinked loci created by random drift and the estimated N_e reflects the
221 population size in the last few generations (Hare et al. 2011). As suggested by the authors (Waples and
222 Do 2008), we excluded the alleles with frequencies smaller than 0.02 to avoid bias related to rare
223 alleles. ONeSAMP implements an Approximate Bayesian Computation analysis (Beaumont et al.
224 2002; Bertorelle et al. 2010). Eight summary statistics are used by ONeSAMP to compare observed
225 and simulated data sets; the inclusion of linkage disequilibrium among these statistics makes this
226 method particularly sensitive to recent population sizes (Skrbinsek et al. 2012). The lower and upper
227 limits of the uniform prior distribution of N_e were set to 2 and 5000, respectively.

228

229 *Demographic dynamic*

230 We analysed the demographic dynamic of each population using five approaches: 1) the M-ratio test
231 (Garza and Williamson 2001); 2) the heterozygosity excess test implemented in the software
232 BOTTLENECK 1.2.02 (Piry et al. 1999), 3) a Bayesian analysis based on the coalescent framework
233 and able to estimate the posterior distributions of the parameters of a contraction/expansion

demographic model, as implemented in the software MSVAR v1.3 (Beaumont 1999, 2004); 4) a likelihood analysis based on the coalescent framework and specifically designed to infer population size contractions and simultaneously the parameters of the mutation model for microsatellites (as implemented in the software MIGRAINE, Leblois et al. 2014); 5) a model comparison based on the Approximate Bayesian Computation approach (Beaumont et al. 2002; Bertorelle et al. 2010), as implemented in the software in DIYABC v 1.0.4.46b (Cornuet et al. 2008, 2010). The first two approaches are simple statistical tests of the null hypothesis of demographic stability, while the last three approaches are model-based, and produce parameter estimates and/or model probabilities using most or all the information provided by the data.

Each method has different statistical properties, which depend on the number of markers, the specific feature of the bottleneck (e.g. age, initial population size, intensity, recovery or not) and possible violations of the model they assume (e.g., migration events among populations). Therefore, none can be considered superior to the others in all conditions (e.g. Swatdipong et al. 2010; Chikhi et al. 2010; Peery et al. 2012; Hoban et al. 2013a). We briefly describe these methods, and we will return to their properties in the discussion.

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

The heterozygosity excess test is based on the comparison between heterozygosity and number of alleles, which is predicted to deviate from the expectation after a bottleneck because the former decreases more slowly than the latter. Statistical significance (one tail) is computed using the

Wilcoxon's signed ranked test to compare observed and expected heterozygosities (Cornuet and Luikart 1996), where expected values are computed by simulations assuming again a two phase mutation model, a variance among multiple steps equal to 12 (corresponding to δ_g = 3.1, see Peery et al. 2012) and p_s = 0.22.

The method implemented in MSVAR assumes that an ancestral population with effective size N_1, increased or decreased (linearly or exponentially) to its current size N_0, starting T generations ago. The estimation algorithm is based on Markov Chain Monte Carlo simulations, and the simple Single-step Mutation Model (SMM) is assumed. Simulations were run for $4x10^8$ iterations; convergence and posterior distributions of the parameters were evaluated with Tracer v1.5 (Rambaut et al. 2014), after discarding the first 10% of the chains (burn-in). For each population, three independent runs were performed assuming an exponential demographic change. The possible effect of this choice was tested assuming a linear change in an additional run of the program. Priors means for the ancestral and current population sizes were set equal to a log-10 transformed value of 3 (1000 individuals), with a standard deviation equal to 1. The prior distributions are log-normal, and this setting allows the testing of population sizes from few tens to hundreds of thousands of individuals. Three different prior distributions of the time since the demographic change were tested, with means equal to 2, 3, and 4, respectively (corresponding to 100, 1000, and 10000 years) and standard deviations equal to 1. The prior distribution of the average mutation rate across loci was set to $1.27x10^{-3}$ per generation. This value corresponds to the direct measure of the microsatellite mutation rate available for amphibians, as estimated from 7,906 allele transfers from parents to offspring in the tiger salamander (Bulut et al. 2009). All the other prior settings in the hierarchical model implemented in MSVAR are reported in Supp. Table 2 and follow standard choices used in other studies (e.g. Storz et al. 2002; Goossens et al. 2006).

Considering that MSVAR provides little information on the mutation rate (Girod et al. 2011), but this rate is necessary to convert the scaled parameters $\theta_0=4N_0\mu$, $\theta_1=4N_1\mu$, and $t=T/2N_0$ into the natural parameters N_0, N_1, and T, we estimated the natural parameters in two different ways: a) using

286 the posterior distribution of the mutation rate as estimated by MSVAR; and b) using the posterior
287 distribution of the scaled parameters and subsequently generating the distributions of the natural
288 parameters N_0, N_1, and T using either the "amphibian specific" rate = 1.27×10^{-3} or the commonly used
289 rate of 5.0×10^{-4} per generation (Garza and Williamson 2001; Storz et al. 2002). Time estimates are
290 transformed in years assuming a generation time of 3 years (Szymura 1998; Gollmann and Gollmann
291 2002). Estimates were made on each population separately, and also after pooling populations that are
292 not significantly differentiated.

293 The same scaled parameters estimated by MSVAR were also estimated with MIGRAINE, a
294 computer package that implement a coalescent method based on importance sampling of gene
295 genealogies (Leblois et al. 2014). Under this method, microsatellites are allowed to mutate under the
296 generalized stepwise mutation model (GSM), which is more realistic for this type of markers and
297 reduces the risk of false positive in bottleneck testing (Peery et al. 2012). Scaled parameters were then
298 converted to natural parameters assuming the "amphibian specific" mutation rate (see above).

299 Lastly, the demographic dynamic was analysed comparing three alternative scenarios with the
300 ABC (Approximate Bayesian Computation) approach as implemented in DIYABC (Cornuet et al.
301 2010): constant effective population size, ancient bottleneck and recent bottleneck. The models
302 assuming ancient or recent reductions were simulated to mimic the demographic effects possibly
303 related to the post-glacial founding of the Alps populations and the human-mediated processes
304 affecting amphibians in the last century, respectively. Hereafter, we call these models *Con* (constant
305 population size), *AnD* (ancient post-glacial decline), and *ReD* (recent, human-related, decline). Ten
306 different settings and prior distributions were tested for each population to check the robustness of the
307 results, for a total of 90 analyses (Supp. Table 3). The prior distribution for the mutation rate was set
308 to either uniform from 1×10^{-5} to 5×10^{-3}, or gamma shaped with shape parameter equal to 3.2, mean
309 equal to 1.27×10^{-3}, and range from 0.01 to 0.0001, thus covering a wide range of plausible values
310 estimated empirically for microsatellites in different species.
311
312

Results

Mitochondrial sequences

Three polymorphic sites, and an average pairwise divergence of 0.043% among individuals, were found in the 420 bp alignment of the cytb gene. Four different haplotypes were detected, three of which had never been observed before in this species. The maximum likelihood (ML) phylogenetic tree (Supp. Fig. 1) indicates that the samples we analyzed belong to the previously described "Balkano-Western" clade of the nominal form, *Bombina variegata variegata* (Hofman et al. 2007).

Microsatellite markers

All 200 samples from 9 populations were successfully genotyped at all 11 amplified loci. MicroChecker results did not suggest any significant presence of null alleles, scoring errors or allelic drop-out. Systematic deviation from Hardy-Weinberg and linkage equilibrium can be excluded: only 5 out of 99 (11 loci x 9 populations) Hardy-Weinberg tests were significant with $P<0.05$, and only 2 out of 55 locus pairs showed significant genotypic linkage with $P<0.05$, after controlling for false discovery rate (FDR) for multiple testing following Benjamini and Hochberg (1995).

All loci were polymorphic and the number of alleles per locus ranged from 2 for F22 to 11 for Bv32 (Supp. Table 1). Genetic variation was relatively low in all populations (Table 1). Heterozygosity values were around 0.50, with lower values in SPI (H_e = 0.41) and NAG (H_e = 0.34). The allelic richness per locus was between 3 and 4 for most populations, again with SPI and NAG showing the lowest values (2.5 and 2.4, respectively).

Population differentiation

Significant genetic differentiation (after following FDR correction) was found in 34 out 36 pairwise F_{st} comparisons. The only exceptions are the comparisons between two pairs of geographically adjacent populations (ZAM vs. MEZ and PRA vs. POZ). F_{st} values (see Supp. Table 4) ranged between 0.05 and 0.15 in most cases, with higher values (up to 0.32) when the NAG site was involved. The matrix of distances is graphically visualized in Fig. 2 using the PCoA. Pairwise distances

computed with G'_{st} and Jost's D were linearly and highly correlated to F_{st} (R^2 equal to 0.99 and 0.98, respectively), with regression coefficients very close to 1 (1.35 and 0.92, respectively), and intercepts very close to 0 (0.016 and 0.012, respectively). The PcoA plot based on these two additional measures of differentiation (not shown) are virtually identical to that reported in Fig. 2.

Bayesian clustering analyses

The inspection of the likelihood plot for different *K* values (Supp. Fig. 2a), and the plot based on the rate of change of the likelihoods (Supp. Fig. 2b), suggests that the two most relevant partition of the data are those with 2 and with 5 inferred groups. For K=2, in fact, we observe the highest rate of likelihood change, and for K=5, the likelihood plot reaches a plateau and a peak in the rate of likelihood change is also observed. We present therefore these results.

For *K*=2, the inferred groups are predominant in central/northern and southern locations (Supp. Fig. 3), respectively. All the individuals in 4 populations can be entirely or almost entirely assigned to the central/northern (PRA, POZ and SPI) or the southern (NAG) groups. Individuals in the other 5 populations show admixed composition with very similar fractions of the two inferred groups within the same locality, suggesting shared ancestry rather than recent admixture (e.g. Jarvis et al. 2012)

For *K*=5, the groups inferred by STRUCTURE roughly correspond to the groups graphically identified by the PCoA plot (Fig. 2): from South to North, we can easily identify MBA+LOP, NAG, SPI, ZAM+MEZ, VER+POZ+PRA. In the southern area, NAG is genetically distinct from MBA and LOP, but with a clear portion of shared ancestry with these neighboring localities. Some individuals in NAG also appear as recent hybrids, with ancestors both in NAG and in MBA or LOP. SPI appears as a genetic isolate in the central portion of the sampled area. In the North, two major groups can be identified: one including the two western samples located along the major Adige valley (ZAM and MEZ), and the other grouping the eastern samples at higher altitude along the Avisio side valley (VER, POZ, and PRA). Interestingly, all the individuals in VER, the sampling locality along the side

366 Avisio valley that is closer to the main Adige valley, show large affinity with the southern localities of
367 MBA and LOP.
368
369 ***Genetic vs. geographic distances***
370 The relationship between linearized F_{st} and the logarithm of geographic distance is positive, weak (R^2
371 = 0.07, Supp. Fig. 4), and statistically significant (Mantel test, P= 0.04). Estimated kinship coefficients
372 are relatively high (1/16, as among first cousins) when individuals from localities separated by 5
373 kilometers or less are compared, and very low otherwise (Supp. Fig. 5).
374
375 ***Recent effective population sizes***
376 Point estimates of recent effective population sizes are low or very low (Table 1). The maximum value
377 is around 170 individuals for the Loppio population using the LDNe method, but for the same
378 population the estimated size is less than 30 when the ONeSAMP method is applied. All the other
379 values range approximately between 10 and 50, with LDNe producing in most cases larger estimates
380 than ONeSAMP. The confidence intervals have large upper limits in most LDNe estimates, but the
381 posterior distributions of Ne produced by ONeSAMP have very small probabilities for N_e>50.
382
383 ***Demographic dynamic***
384 All the populations have M-ratio values (see Table 2) below the 0.68 threshold usually taken as
385 evidence for a bottleneck (Garza and Williamson 2001). When M-ratios are tested controlling for false
386 positives (Benjamini and Hochberg 1995), significant support of the bottleneck (P<0.05) is found in
387 all populations, the only exception being ZAM and MEZ when the largest values of θ = 5 is assumed.
388 The heterozygosity excess test indicates that heterozygosities are higher than predicted from the
389 number of alleles, as expected after a bottleneck, but this difference is significant only for SPI.
390 The posterior distributions of ancestral and current population sizes, as estimated directly by
391 MSVAR in each population, have very limited overlap, and, although rather large confidence intervals
392 were found, support a demographic decline in all populations (Fig. 3, Supp. Table 5a). Different

populations show similar distributions, but considering the point estimates we note that the ratio between ancestral and current median sizes varies approximately between 7 and 45. NAG, MBA, LOP, and SPI show the most extreme reduction (>25 fold), and a less extreme decline is estimated for the other populations (<15 fold). Ancient sizes distributions have peaks at around 1000-2000 individuals, and current sizes estimates vary between 35 to 150 animals in different populations. Only small differences from this pattern are observed assuming either an exponential or a linear decline (Supp. Table 5a).

The best supported value for the time when the decline started varies in different populations between 250 and 1500 years before present (BP) when the exponential decline was assumed (see Fig. 4a, Supp. Table 5a) and between 500 and 3000 years BP when the linear decline was assumed. Given the evident overlap between prior and posterior distributions, we checked the influence of the former on the latter by performing additional tests with different priors. The posterior distributions support a decline starting point between few hundred and few thousand years even when the prior mean was decreased or increased by a factor of 10 (see Fig. 4b and 4c).

All these general results of MSVAR are consistent across runs and when scaled, instead of natural, parameters are estimated, or when samples from pairs of populations not genetically differentiated were pooled to increase the sample size (Supp. Table 5a and 5b). On the contrary, population sizes and decline ages estimates are approximate doubled if the "generic" mutation rate is used instead of the "amphibian specific" rate is used to convert scaled into natural parameters (Supp. Table 5c). Credible intervals and median values of the posterior distributions estimated in different MSVAR analyses are all reported in Supp. Tables 5.

When population size and the time since the population started to decline are estimated with the method implemented in MIGRAINE, thus allowing for multiple steps in the mutation process, the general conclusions reflect those produced by MSVAR, with some differences in the parameter estimates (Supp. Table 6). Modern but especially ancestral sizes are larger, varying among different populations between 50 and 400, and 5000 and 15000, respectively, and thus increasing the estimated

intensity of the decline. The beginning of the decline, on the contrary, is almost the same as estimated by MSVAR, ranging between 600 and 3500 years.

The results of the ABC analysis are clearly affected by the priors setting, but, overall, the evidence against demographic stability (*Con* model) is strong and the model *AnD* (ancient post-glacial decline) appears the most plausible to explain the pattern of genetic variation (Fig. 5, Supp. Table 3). However, it is also important to note that in 9 out of 90 analyses performed under different priors setting (10 for each population), the posterior probability of *ReD* (recent decline, human related) was higher than the probability of *AnD*. This situation occurred only for three populations, MEZ (once), NAG (5 times) and SPI (3 times), and can be visualized by the overlap of probability ranges for *AnD* and *ReD* reported in Fig. 5. The results of the complete set of ABC analyses are reported in Supp. Table 2.

Discussion

This study was motivated by the recent demographic decline and habitat change and fragmentation observed in multiple Alpine population of the yellow-bellied toad (Caldonazzi et al. 2002). Considering the heightened susceptibility of this mountain environment to ongoing increase of temperatures, our main goal was to understand whether or not recent demographic and habitat change had already produced negative genetic effect in terms of variation levels, within and between populations, and inbreeding. After a preliminary phylogeographic analysis based on cytochrome b sequences, we addressed this main question typing 11 nuclear microsatellites in 200 individuals from 9 populations. In general, the data supported a genetic bottleneck occurring in the past, and we dedicated particular attention in the statistical analysis to estimate the timing of this event. We clearly show that ancient reductions of genetic variation likely occurred in this species, but a recent and recorded demographic decline, possibly associated to human activities, did not leave a significant genetic signature. Therefore the conservation situation may be optimistic, due to possibly ancient

445 purging effects and adaptation occurring in many generations at low density, and also the lack (as yet)
446 of human impacts.
447
448 ***Phylogeographic affiliation***
449 The phylogenetic analysis of mitochondrial sequences showed that all samples included in this study
450 fall in the Balkan-Western clade (Supp. Fig. 1). The level of variation in our sample was very low,
451 with 89% of the individuals sharing the same mtDNA sequence, and only 4 haplotypes in total. This
452 result agrees with previous studies suggesting a severe reduction of variation in the Western areas of
453 the distributions (Hofman et al. 2007; Fijarczyk et al. 2011), and support the hypothesis that the
454 populations characterized by this clade originated in a Balkan refugium and expanded northwestward
455 after the last glaciation, losing genetic variation during the colonization process. Further statistical
456 analyses at this marker were not possible, as only thee polymorphic sites were observed.
457
458 ***Nuclear variation and estimates of contemporary Ne and kinship***
459 Microsatellite markers confirmed that genetic variation levels are very low in at least two Alpine
460 populations (NAG and SPI), and in general lower than observed in comparable populations of this
461 species or the sister species *B. bombina*. When samples sizes are adjusted to be equal by resampling,
462 and only the loci shared among studies are considered, the average number of alleles was about 27%
463 and 40% lower in the Alps than in a *B. variegata* and *B. bombina* population in Northern Germany,
464 respectively (Hauswaldt et al. 2007). The number of alleles and the heterozygosities are similar to the
465 values found in endangered frog or toad species (e.g. Morgan et al. 2008; Beauclerc et al. 2010; Wang
466 2012; Igawa et al. 2013). If compared to large collections of heterozygosity values observed in both
467 endangered and non-endangered species (e.g. Frankham et al. 2010; Hughes 2010), the average level
468 of genetic variation in *B. variegata* populations should be probably considered "medium-low". For
469 example, the average value of heterozygosity observed in the Alpine toad populations, 0.47,
470 correspond to the 10^{th} and 30^{th} percentile in two lists of 221 non-endangered and 73 endangered or
471 vulnerable bird species, respectively (Hughes 2010).

Low genetic variation in modern samples may be produced by different demographic scenarios, including low and constant census size, recent bottlenecks, and even in recently large and expanding populations when mutations have not the time to accumulate yet. As a first step, our analyses excluded the last scenario (expansion), specifically our estimation of effective contemporary population size using the linkage disequilibrium pattern among physically unlinked markers, and the ABC-based OneSAMP method. Most populations showed values smaller than 50 individuals, and some of them values smaller than 20. These values are lower than those estimated in other ranid species (Wilkinson et al. 2007; Phillipsen et al. 2011), and similar to the estimates obtained in endangered anuran species (Ficetola et al. 2010; Wang 2012). We conclude therefore that these yellow-bellied toad populations have today relatively low genetic variation and evolutionary potential, with high risk of local extinction due to demographic stochasticity, considering the very limited number of breeders. Inbreeding within populations or between individuals sampled at very short geographic distances is probably unavoidable in this condition, as suggested by the kinship coefficients estimated in this study, but the negative effects in terms of individual fitness is not easily predictable (see below) and should be directly evaluated.

Genetic structure

Gene flow, which could counteract the loss of variation and inbreeding in small populations, is unlikely to occur in a fragmented landscape and especially in species with reduced movement capabilities such as frogs (e.g., Dolgener et al. 2012; Igawa et al. 2013). In particular, most of the sample sites in our study are separated by highly urbanized areas, and a previous mark-recapture field study in *B. variegata* showed that travel distances covered each year by adults or subadults rarely exceed 500 meters (Hartel 2008). As expected, a clear evidence of genetic substructure was found, and only two pairs of populations, separated by less than 7 kilometers, were not genetically differentiated between them. Genetic distances were substantial, which was observed using both classical Wright's F_{st} and more recently developed metrics for multi-allelic markers, Jost's D and Hedrick's G'_{st}. This

result is not unexpected and it suggests that when genetic variation is medium to low, Wright's F_{st} is suitable for any type of marker.

Five major genetic groups were identified, with two of them corresponding to two single and highly divergent populations (NAG and SPI), and the others associated to geographically homogenous areas. Genetic data also showed that kinship levels are high only at very short distances. Overall, these results indicate that gene flow among local small populations is limited, and rapidly decreases as the geographic distance increases.

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

Newly rare or always rare?

Small local population size can occur at demographic equilibrium, i.e. a natural and stable condition reached in patchy habitats by species with limited dispersal, or can result from recent demographic decline. Field studies in *B. variegate* suggest recent demographic decline, and genetic evidence suggest small effective sizes and fragmentation. But can we directly infer that recent decline is the cause of the low genetic variation? In other words, given that field studies indicate that the demographic equilibrium has been recently perturbed, can we also conclude that the genetic variation pattern has also been recently perturbed? Answering this question is clearly relevant in terms of conservation actions, since the dual threat, demographic and genetic, should be considered a much higher concern, as a likely step further in the extinction vortex (Lankau and Strauss 2011). If, on the contrary, the recent demographic decline is not the cause of the current genetic pattern, milder measures of protection could be sufficient to favor demographic re-expansion, and prevent the

524 beginning of genetic erosion. Therefore we dedicated a large effort to estimate the genetic impact of
525 the recent demographic decline.

526 Five approaches were used. Two of them, the M-ratio and the heterozygosity excess tests, are
527 classical statistical tests that test whether simple properties of the observed data are compatible with
528 what is expected under the null hypothesis of demographic stability. The genetic data appear mostly
529 incompatible with demographic stability. In almost all the analyses and populations, the M-ratio
530 strongly supports a demographic bottleneck. Considering that the power of this test is reasonably high
531 when a bottleneck occurs in an isolated population between few (Peery et al. 2012, Hoban et al.
532 2013a) and few hundreds (Garza and Willamson 2001, Swatdipong et al. 2010) generations, it might
533 be inferred that our data are compatible with a recent decline. However, it has been shown by
534 simulation that even relatively low migration rates (m = 0.001) can extend the time frame of the
535 bottleneck signal based on the M-ratio to several thousand of generations (Swatdipong et al. 2010). A
536 significant excess of heterozygosity compared to value expected from the number of alleles was
537 observed only in three populations (one after the multiple test correction). Considering that the
538 heterozygosity excess is a transitory event rarely extending more than 50 or approximately 0.5 to 4 *Ne*
539 generations (Cornuet and Luikart 1996; Henry 2009; Peery et al. 2012), and in general a shorter time
540 compared to the gap in the allelic size distribution contributing to the M-ratio (Spear et al. 2006;
541 Hundertmark and Van Daele 2009; Marshall et al. 2009), these results can be considered as a
542 statistically weak and unconvincing evidence of recent decline, with stronger support on ancient
543 declines.

544 The above inference based on the temporal power window of the M-ratio (older bottlenecks)
545 and the heterozygosity excess test (younger ones) is speculative, though not uncommon in the
546 literature (e.g. Spear et al. 2006; Lumibao and McLachlan 2014). More robust and direct evidence on
547 the age and the intensity of the bottleneck can be obtained, as shown by simulation and empirical
548 studies (Girod et al. 2011, Peery et al. 2012) when the demography is modeled and the whole
549 information contained in the data is used (rather than one summary statistic). Here we used the model-
550 based methods implemented in MSVAR, MIGRAINE, and DIYABC; all these methods clearly

551 support the hypothesis of a non-recent demographic decline in all the populations. In particular,
552 MSVAR and MIGRAINE indicated that the demographic decline most likely started not later than
553 approximately 250 year ago, and not earlier than approximately 3500 years ago. In other words, these
554 estimates point to a decline predating the currently documented human-induced changes, and
555 postdating the most recent complete deglaciation and climate stability reached about 10,000 years ago
556 (Cusinato and Bassetti 2007), when several plant and animal species had probably re-colonized the
557 Alpine area of our study. These two values, 250 and 3500 years before present, correspond to the
558 range of median values estimated in different populations, but considering that the support intervals
559 are rather large and also that the main genetic impact in all these close Alpine populations is probably
560 related to a shared demographic dynamic, we can prudently take them as an estimate of the temporal
561 boundaries of a population size change. We prefer here not to speculate on which historical or
562 environmental factor may have caused this decline, since the time interval is quite large and it may
563 even reflect an average between the ages of two or more independent declines (Sharma et al. 2012).
564 The clear inference is that the genetic impact of the population decline recently observed in field
565 studies, if any, is limited and surpassed by the impact of a much older decline. A study in salamanders
566 in North America also suggested a decline some thousands of years ago (Jordan et al. 2008).
567 Population sizes dropped by at least one order of magnitude. Some differences can be detected
568 among populations, but the support intervals of the estimates are large: the only safe conclusion
569 appears that the decline was more intense in the most southern populations of Nago, Monte Baldo,
570 Loppio, and Spiz. Only in these population, in fact, the estimated ratio between ancient and current
571 size was at least as large, or larger than 25. Finally, when three explicit models were compared using
572 the ABC approach, the highest posterior probabilities always favored, with the exception of a few
573 analyses in Nago and Spiz, an ancient demographic decline, i.e. a decline occurring at least 200 years
574 ago but probably in more ancient times. The fact that similar results were found in all the populations,
575 especially regarding the time and strength of decline, suggests a range-wide rather than localized
576 influence.
577

Caveats to our demographic dynamic inference

Statistical testing and parameter estimation imply of course assumptions that, when violated, may produce biased results. Direction and magnitude of the bias are difficult to predict in different conditions and for different approaches, but some general notes regarding the robustness of our results can be drawn. 1) Population structure may produce false bottleneck signals in MSVAR (Chikhi et al. 2010) and probably in all coalescent-based methods (e.g. Wakeley 1999; Heller et al. 2013). Following the empirical suggestions by Chikhi et al. 2010 and Heller et al. 2013, we repeated the MSVAR analysis in two data sets created sampling either 3 or 10 individuals per population. This approach is likely to reduce the power to detect bottleneck occurring only in some populations, but can be used to exclude that population structure is the only responsible of the bottleneck inference. The ratio between the estimated ancient and modern population sizes was very close to 5 in both analyses, suggesting that a real decline occurred in the Alpine populations we considered. 2) Rare alleles may go undetected in small samples, thus producing gaps in the allelic size distribution and false signals of bottlenecks in the M ratio. This effect is probably small in our case, since the M ratio remains small and significant when genetically similar samples (MEZ+ZAM and PRA+POZ) are pooled, or when the whole data set is jointly analyzed. 3) When only few individuals contribute to the next generation, and the vast majority does not, i.e. when the variance in reproductive success is high, false signals of bottleneck may emerge in stable populations (Hoban et al. 2013b). Direct measure of the variance in reproductive success are not available for *B. variegata*, but we know that the vast majority of females reproduce and the number of eggs per clutch is relatively small (Barandun et al. 1997), and also that the likely mating system in this species probably allows many males to fertilize eggs (Lorchner 1969; Sanderson et al. 1992; Vines 2003). It seems therefore unlikely that large variance in reproductive success is the cause of our results. 4) Wrong mutation models and rates, and a wrong generation time, obviously introduce bias in inference and estimates. Most of the methods applied here used a microsatellite-specific multistep mutation model, a mutation rate based on the only direct estimate known for an amphibian (1.27×10^{-3}), and a generation time estimated for *B. variegata* (3 years). Estimates of population sizes and times since the beginning of the inferred decline increase by

605 applying the slightly slower "generic" rate commonly used for this type of markers in non-amphibian
606 species (5.0 x 10^{-4}), or by increasing the generation time to the value of 5 years estimated in some
607 populations of the related species *B. bombina* (Vines et al. 2003; Fog et al. 2011). Nevertheless, the
608 major conclusions of this study, i.e. that the recent population decline is not the main responsible for
609 the observed genetic variation pattern, remains robust, and this is true even increasing the mutation
610 rate to very large and uncommon values of 2 to 4 x10^{-3} per generation (Peery et al. 2012). 5)
611 Bottleneck detection and estimation is based on the assumption that only one demographic event
612 occurred in the past. This is an oversimplification of the real history of a population, but it is unclear
613 how multiple events (e.g. sequential bottlenecks) can affect these analyses (Goossens et al. 2006;
614 Okello et al. 2008; Sharma et al. 2012). More simulation studies (Hoban et al. 2012) are required to
615 better understand the behavior of these methods under complex demographic scenarios including
616 "multi-events" models, necessarily requiring several parameters. Highly informative genomic data sets
617 could be used in such situations.
618
619 *4.6 Conservation issues and actions*
620 Protecting *B. variegata* populations where demographic reductions have been documented, and
621 possibly favoring a demographic increase, is important to prevent further genetic variation erosion. As
622 shown also in a recent simulation study, much of the variation can be preserved if quick action is
623 implemented (Hoban et al. 2014). However, since the current level of genetic variation in most of the
624 populations we analyzed is not extremely low, and the genetic impact of the recent decline, if any,
625 appears limited, some optimism regarding the possibility of a complete recovery without risks of
626 negative genetic consequences is justified. Our results do not clearly indicate a specific environmental
627 situation where, in general, conservation efforts should be focused, and even some recent studies
628 based on non-genetic data suggest that this question has not a simple answer. Hartel and von Wehrden
629 (2013), in fact, found that traditional farming practices produce a large number of suitable ponds and
630 should be preserved, but Scheele et al. (2014) observed that the pasture ponds, compared to those in
631 forest, tend to host individuals in worse body conditions. However, our study does show that highest

priority might be given to the populations of Spiz and Nago, since they showed lowest values of diversity, clear evidence of extreme contraction of effective population size, and some (weak) evidence of the genetic impact of a recent decline. Spiz is one of the two highly isolated populations in our study located at high altitude, where the negative effects of global warming may additionally increase the risk of local extinction. In fact, if early breeding is commonly associated with increasing temperature (Blaustein et al. 2001), the increased probability of late frosts can have fatal consequences on early-bred spawn (Henle et al. 2008). Nago is located in a high tourism area, where anthropogenic disturbance may be impactful. Interestingly, some signature of the recent introduction of individuals from other areas has been found in Nago, and it would be useful to determine if this migration (likely due to human releases) could have inadvertently, but positively, reduced the risk of inbreeding in this highly homogenous and genetically isolated population.

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Data Accessibility

Mitochondrial sequences are available in GenBank with accession numbers KP784451 to KP784453. Microsatellite data are deposited in Dryad: http://XXXXXX.

Author Contributions

LC and CV conceived and designed the study, collected the samples, and genetically typed the individuals. LC, GB and AB planned and performed the statistical analyses and interpreted them. GB wrote the manuscript, with the assistance of SH and LC. All authors examined data, discussed results, contributed to manuscript revision and approved the final draft.

Table 1. Genetic diversity and effective population size estimates of 9 populations of *B. variegata.* Sampling localities, including the corresponding acronym, number of samples collected (*N*), number of alleles (N_a), allelic richness (A_r), observed (H_o) and expected (H_e) heterozygosity, and estimates of effective population size by linkage-disequilibrium method (N_e (LDNe)) and by Bayesian method (N_e (OneSamp)). N_a, A_r, H_o and H_e are mean among loci. Intervals in brackets are 95% confidence intervals (LDNe) and 95% credible limits for the posterior distribution (OneSamp).

Population	**Label**	**N**	**N_a**	**A_r**	**H_o**	**H_e**	**N_e (LDNe)**	**N_e (OneSamp)**
Zambana	ZAM	29	4.2	3.7	0.58	0.54	51.9 (23.9 - 552.1)	26.2 (20.3 - 49.9)
Mezzolombardo	MEZ	10	3.4	3.4	0.52	0.49	31.1 (6.9 - inf)	12.2 (9.4 - 19.4)
Nago	NAG	23	2.5	2.4	0.36	0.34	6.5 (2.0 - 25.7)	17.8 (13.4 - 30.0)
Monte Baldo	MBA	25	3.5	3.2	0.47	0.51	61.5 (22.7 - inf)	23.1 (17.1 - 39.6)
Prà	PRA	17	3.6	3.2	0.49	0.48	10.0 (3.2 - 32.3)	17.6 (14.1 - 27.7)
Pozzolago	POZ	25	4.0	3.5	0.53	0.51	55.7 (17.1 - inf)	25.5 (19.7 - 41.2)
Verla	VER	24	3.9	3.3	0.54	0.48	50.8 (17.4 - inf)	19.1 (15.3 - 27.6)
Loppio	LOP	32	3.6	3.1	0.50	0.50	166.6 (29.1 - inf)	26.6 (20.5 - 41.1)
Spiz	SPI	15	2.5	2.5	0.42	0.41	65.8 (12.7 - inf)	13.1 (10.9 - 18.6)
Mean			3.47	3.14	0.49	0.47	55.5	20.1

Table 2. Tests of demographic bottleneck. The heterozygosity excess is tested using the Wilcoxon approach implemented in the software BOTTLENECK. Significant P values (α=0.05) for the M-ratio and the heterozygosity excess tests, after controlling (separately for each test) for multiple testing, are underlined. Three different values of theta ($\theta = 4N_e\mu$, the population-mutation parameter) were used for the M-ratio test.

Population	Label	N	M-ratio	P value (M-ratio)			Heterozygosity excess (p-value)
				θ=1	θ=2	θ=5	
Zambana	ZAM	29	0.63	<u>0.010</u>	<u>0.029</u>	0.062	0.052
Mezzolombardo	MEZ	10	0.60	<u>0.003</u>	<u>0.011</u>	0.072	0.216
Nago	NAG	23	0.48	<u>0.001</u>	<u>0.001</u>	<u>0.001</u>	0.326
Monte Baldo	MBA	25	0.51	<u>0.001</u>	<u>0.001</u>	<u>0.001</u>	0.042
Prà	PRA	17	0.53	<u>0.001</u>	<u>0.001</u>	<u>0.001</u>	0.080
Pozzolago	POZ	25	0.61	<u>0.002</u>	<u>0.003</u>	<u>0.008</u>	0.350
Verla	VER	24	0.61	<u>0.001</u>	<u>0.003</u>	<u>0.010</u>	0.382
Loppio	LOP	32	0.53	<u>0.001</u>	<u>0.001</u>	<u>0.001</u>	0.042
Spiz	SPI	15	0.60	<u>0.005</u>	<u>0.016</u>	<u>0.008</u>	<u>0.002</u>

Legend to Figures

Fig. 1. Map of the nine sampling sites (indicated by red dots) in the Alpine region of Trentino Alto-Adige. Major lakes are shaded. The population codes used throughout the papers are reported in brackets.

Fig. 2. Principal Coordinate Analysis of pairwise F_{st} among populations and plots of proportion of ancestry of each sampled individual for five genetic clusters inferred using STRUCTURE.

Fig. 3. Posterior distribution of the effective population sizes (in log 10 units) for each population obtained with MSVAR assuming an exponential demographic change. Dashed lines represent current *Ne*, while dotted lines represent pre-bottleneck *Ne*. The solid line is the prior distribution for both current and ancient population sizes.

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

Fig. 5. Graphical representation of the posterior probabilities of three different demographic scenarios tested with the ABC approach. For each population, the black bars are proportional to the range of posterior probabilities obtained under 10 different priors settings. *Con* = constant population size; *ReD* = recent decline, associate to human activities; *AnD* = ancient decline, associated to the post-Glacial colonization of the Alps. Details of the prior distributions, and model probabilities in each analysis, are reported in Supp. Table 2.