Fine-scale phylogeography of *Rana temporaria* Linnaeus, 1758 (Anura, Ranidae) in a southern Alpine putative secondary contact zone (Trentino, Italy)

ALEXIS MARCHESINI[1,2], GENTILE FRANCESCO FICETOLA[3,4], LUCA CORNETTI 3, ANDREA BATTISTI[2] and CRISTIANO VERNESI[1]

[1]*Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, 38010, S. Michele all'Adige, Trento, Italy.*

[2]*Department of Agronomy, Food, Natural Resources, Animals, & Environment (DAFNAE), University of Padua, Agripolis, Viale dell'Università 16, 35020 Legnaro (PD), Italy*

3 to be added byLuca
3 Department of Environmental Science and Policy, Università degli Studi di Milano. Via Celoria 26, 20133 Milano, Italy
[4]*Laboratoire d'Ecologie Alpine (LECA), Université Grenoble-Alpes. Grenoble F-38000 Grenoble, France*

The common frog (*Rana temporaria*) has been focus of several broad scale phylogeographic studies, revealing a deep split between eastern and western European populations, most likely induced by the onset of the Pleistocene glaciations. However, 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 refuge in the Italian peninsula and suggested the hypothesis of multiple separated microrefugia ("refugia-within refugia" model), but fine-scale studies required to confirm this hypothesis are lacking. We examined the phylogeographic structure of 54 common frog populations (540 individuals) by means of COI (cytochrome oxydase I) mitochondrial gene, focusing on a south eastern alpine region (Trentino, Italy) with an intensive sampling design. Phylogeographical 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. This study on a widespread species, confirmed the biogeographic peculiarity of the Trentino region with clear conservation implications.

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; Avise, 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 shifting the distribution of many species to suitable areas south of the glaciated regions, the so-called “glacial refugia”, while during the interglacials some species were able to recolonize previously glaciated areas. These repeated contractions-expansions had important genetic consequences, leading to (1) the genetic differentiation of populations isolated in distinct 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; Hewitt, 2004; Petit *et al.*, 2003). Mitochondrial DNA (mtDNA) is often a marker of choice in traditional phylogeographic studies. 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).

Phylogeographic studies are often performed over broad spatial scales, trying to cover the whole range of target species, and this approach has allowed to identify the main glacial refugia of species. However, within the main refugia, a strong genetic structure often existed throughout the Pleistocene, suggesting the existence of "refugia witin refugia". Such refugia within refugia are a major source of present-day genetic diversity,

105 but the identification of fine scale patterns requires high resolution data that are not always
106 available in phylogeographic analyses.
107 Amphibians generally have limited dispersal and often exhibit high fidelity to
108 breeding sites (Beebee, 1996). As a consequence, populations tend to be highly structured
109 genetically and retain strong signals of past evolutionary processes, so amphibians have
110 become popular subjects in many phylogeographic studies (see Zeisset & Beebee, 2008,
111 for a review). The common frog (*Rana temporaria* Linnaeus, 1758) is one of the most
112 widespread and abundant amphibians in Europe (Gasc, 1997) and has the greatest genetic
113 variability of all western Palearctic brown frogs (Veith, Kosuch & Vences, 2003; Vences
114 *et al.*, 2013), therefore it is a perfect model organism for examining phylogeographic
115 processes.
116 Large-scale phylogeographic studies (Palo *et al.*, 2004; Teacher, Garner & Nichols,
117 2009), based on mtDNA cytochrome b gene (cyt b), identified two main lineages for *Rana*
118 *temporaria* in the Palearctic region, with an Eastern lineage mainly distributed in Eastern
119 Europe and Scandinavia (but documented also for the northern Alpine border), and a
120 Western lineage in France, Germany, Iberian Peninsula, and the British Isles. The split
121 between the two major lineages was dated at approximately 700 000 BP, roughly
122 corresponding to the onset of the Middle Pleistocene glaciations (Palo *et al.,* 2004). The
123 Iberian Peninsula has been proposed as the main refugium for the western lineage (Teacher
124 *et al.* 2009), with a potential secondary refugium in Ireland. Conversely, the eastern
125 lineage was supposed to originate from a single refugium in Italy or the Balkans.
126 Nevertheless, Stefani *et al*. (2012), based on a genetic survey covering the whole Italian
127 distribution of the species, proposed an alternative phylogeographic scenario. These
128 authors detected only the western cyt b lineages in Italy but, using the cytochrome oxidase
129 I (COI) gene, they found high genetic diversity in the Italian populations, with five

different COI lineages: four in the Alps and one in the Apennines. Therefore, these authors proposed the Italian Peninsula as an important glacial refugium for the western lineage during the last phase of Pleistocene, and the observed pattern of diversity of Italian populations was interpreted as evidence for a "refugia-within-refugia" (Gómez & Lunt, 2007). Under this scenario, the Italian peninsula hosted a system of multiple separate sub-refugia, located on the southern slopes of the Alps and Apennines, where the species survived the last glacial-interglacial cycles in fragmented populations (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 (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 different putative glacial refugia for the species. Although common frog specimens from this area were not included in previous studies, a broad-scale survey on surrounding mountain massifs suggested the presence of a contact zone among different mtDNA lineages (Stefani *et al.*, 2012).

Specifically, we addressed the following questions: (1) do our local-data conform with 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? (3) What are the routes and modes of postglacial recolonization in the study region?

Under a conservation perspective, the study of Pleistocene climatic oscillations and their influence on genetic diversity patterns is of crucial importance in the face of ongoing climate change, providing a basis for understanding the evolutionary consequences of predicted range shifts, identifying hotspots of "evolutionary potential" as well as potential future climate change refugia (Sgro, Lowe & Hoffmann, 2011; Morelli *et al.*, 2016). While the effects of Quaternary glaciations have been widely investigated for many organisms over large spatial scales, they are still poorly understood at regional and local scales, which correspond to the scales at which conservation planning is actually performed.

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).

SAMPLE COLLECTION

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% above 1000 m a.s.l). The Adige river valley (Fig. 1) 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 2009-2012, the selected areas were screened for common frog spawn during the breeding season. To minimize the probability of collecting full-sibs, we collected one fertilized egg from each clutch, or tadpoles coming from separate ponds. 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 *et al.*, 2005; Johansson, Primmer & Merilä, 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 they represent the southern margin of *Rana temporaria* distribution range in the considered part of the Alps.

Overall we collected 1522 individuals from 90 different sampling 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 (Figure 1 and Table S1 in Supporting Information).

DNA EXTRACTION, POLYMERASE CHAIN REACTION (PCR) 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 individuals, a fragment of 569 base pairs (bp) of mtDNA cytochrome C oxidase subunit I region (COI) 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 DIVERSITY, PHYLOGEOGRAPHICAL AND DEMOGRAPHIC ANALYSIS

Sequences obtained were collapsed into haplotypes by using DnaSP v5 (Librado & Rozas, 2009). DnaSP v5 was also used to calculate the 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 Development Core Team, 2006).

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.

As suggested for intraspecific gene genealogies (Posada & Crandall, 2001), we analyzed the phylogenetic relationships between sequences by means of haplotype networks. Statistical parsimony networks were generated with the software TCS 1.21 (Clement, Posada & Crandall, 2000), using the 95% limit for a parsimonious connection. First, we constructed a COI haplotype network, combining our DNA sequences with all the available haplotypes of *Rana temporaria* from 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 sequences, 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, Schneider & Excoffier, 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 10 000 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 ARLEQUIN, using the best-fit grouping pattern suggested by SAMOVA. The HKY model (Hasegawa, Kishino & Yano,

1985) was identified using jModelTest 2.1.6 (Darriba *et al.*, 2012) as the best-fit model of nucleotide substitution, based on the Akaike Information Criterion (AIC). As the HKY model is not implemented in ARLEQUIN, the closely related Tamura-Nei model (TrN; Tamura & Nei, 1993) was selected for AMOVA. The statistical significance of the variance components was computed by 10 000 permutations.

The location of major genetic discontinuities was also assessed using the software BARRIER 2.2 (Manni, Guerard & Heyer, 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 the highest to the lowest ''rank". We continued adding barriers until no more barriers showed a significant PhiST value (Manni *et al.* 2004).

Finally, the demographic history of the detected COI lineages was investigated using two different approaches: (a) neutrality tests; (b) mismatch distribution analysis. First, the population history of each lineage was inferred by testing departure of neutrality using Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989) statistics in ARLEQUIN 3.5 with 10 000 simulations. Second, we analysed patterns of mismatch distribution computed using the software DnaSP v5. Mismatch distribution is the frequency distribution of the observed number of differences between pairs of haplotypes. A unimodal Poisson-like distribution is indicative of populations that have experienced a recent demographic expansion (Rogers &

278 Harpending, 1992; Slatkin & Hudson, 1991) or a range expansion with high levels of gene
279 flow between neighboring demes (Ray *et al.*, 2003; Excoffier, 2004). In contrast, a
280 multimodal distribution generally indicates that populations are at demographic
281 equilibrium, reflecting the highly stochastic shape of gene trees (Rogers & Harpending,
282 1992). Demographic analyses were conducted using all Alpine accessions of *Rana*
283 *temporaria* COI lineages.
284
285 **RESULTS**
286
287 GENETIC DIVERSITY AND PHYLOGEOGRAPHICAL ANALYSIS
288 We found a total of 12 COI gene haplotypes (569 bp long), that differed at 19 polymorphic
289 sites (19 parsimony-informative sites). Six of these were previously unreported (Table 1).
290 Newly discovered COI haplotypes were deposited in GenBank database under Accession
291 **No. X–Y**.
292 Phylogenetic reconstruction including haplotypes available from public repositories
293 (Figure S2 in Supporting Information) led to the assignment of the detected haplotypes to
294 three of the four COI lineages (= haplogroups) known for the Alps (Stefani *et al.*, 2012).
295 Specifically, nine haplotypes were found for Alpine lineage I (hereafter Alp1), two
296 haplotypes for AlpinRae lineage IV (hereafter Alp4) and on haplotype for Alpine lineage II
297 (hereafter Alp2). A phylogenetic network for the haplotypes found in the study region,
298 with node size proportional to their frequencies, is reported in Figure 2.
299 Alp1 was ubiquitous in the study region, while Alp2 and Alp4 were detected only in the
300 eastern part of the region. In particular, Alp2 was predominant in south-eastern populations
301 (Venetian Prealps), while lineage 4 was present only in 10 sites, located in the north-

302 eastern corner of the region, always in admixture with other lineages. Overall, complex
303 spatial patterns of admixture among the three lineages were detected in the eastern part of
304 the region (hereafter East Trentino), while the western part (hereafter West Trentino), was
305 characterized by the presence of a single COI lineage, Alp1 (Figure 3).

306 Overall, the haplotype with the highest frequency was CA2, belonging to Alp1
307 (overall frequency = 0.526; see Table 1). CA2 was distributed across the whole study
308 region and it was present in all sites, except for one (RM1). The second most frequent
309 haplotype was VC6 (lineage Alp1; overall frequency = 0.246), although being present only
310 in Eastern Trentino. Within this sub-region, the haplotype VC6 was present in all the sites
311 but one (PLa), and showed frequency ≥ 0.5 in 14/28 sites. Nine of them (64%) were
312 located in the southern half of the region. All the other haplotypes were present with global
313 frequency < 0.1. The haplotype TN1 was present only in one site (Tre), located in the
314 southernmost part of West Trentino.

315 The most diverse lineage, Alp1, displayed a "star-like" shaped topology in the
316 haplotype network, with the most abundant central haplotype surrounded by several less
317 abundant haplotypes, a pattern that is generally interpreted as an evidence of past
318 population-wide demographic expansion (Rogers & Harpending, 1992; Bandelt *et al.*,
319 1995).

320 The western portion of the study area showed a larger number of haplotypes than the
321 eastern portion, despite the presence of only one COI lineage. Considering the haplotypes
322 belonging to the Alp1 lineage, seven of them were exclusive of Western Trentino. The
323 spatial distribution of the different haplotypes showed a pattern of geographical clustering,
324 although with frequent irregularities, particularly at local scale (Figure S1 in Supporting
325 Information). Haplotype occurrences for all populations are reported in Table 2.

326 Different populations exhibited different levels of intra-population genetic diversity
327 (see Table 2), sometimes even at short geographic distance. No correlation was found
328 between latitude and standard genetic diversity measures. However, when considering the
329 two separate subsets (Western and Eastern Trentino), a moderate significant correlation
330 was detected in both cases, but with opposite sign. The number of haplotypes (n) decreased
331 from south to north in West Trentino ($r = -0.42$, $p < 0.05$), while in East Trentino an
332 opposite trend was highlighted, with genetic diversity increasing with latitude ($r = 0.59$, p
333 < 0.05). Other measures of genetic diversity (e.g. haplotype diversity, h; nucleotide
334 diversity, π) yielded very patterns (data not shown). A correlation between longitude and h
335 was detected only in East Trentino ($r = 0.42$, $p < 0.05$).

336 Pairwise PhiST values (Table S3, Supporting Information) highlighted an overall
337 high level of genetic differentiation among populations, with 686/1431 comparisons
338 (47.9 %) yielding significant values ($p < 0.05$, after adjustment for multiple comparisons
339 using False Discovery Rate method; Benjamini & Hochberg, 1995). This result is
340 remarkable, considering the fine spatial scale of our study, and the fact that we employed a
341 single mtDNA gene. Significant PhiST values were frequently found even for populations
342 separate by less than 10 km, particularly in East Trentino (e.g. PR1-PS1, PR1-PS2, PR2-
343 PS1, PR2-PS2, Ech-DDB, Ech-Ing, Mon-DDB, Mon-Ing, Ste-MBa, Ste-Bed).

344 Spatial analysis of molecular variance (SAMOVA) indicated $K = 3$ (3 groups) as
345 the most likely population structure, when FCT was maximized ($FCT = 0.494$) and the
346 increment of FCT was the largest ($\Delta FCT = 0.0018$) (Table S2 in Supporting Information).
347 Group 1 included all populations with a prevalence of the lineage Alp1 (frequency of
348 Alp1 > 0.5); group 2 included all populations with a prevalence of the lineage Alp2
349 (frequency of Alp 2 > 0.5); group 3 included only 2 populations, characterized by
350 admixture of 3 lineages and lineage Alp 4 present at high frequencies (≥ 0.4). However, it

351 is worth noting that: (1) all the other tested grouping schemes (K) yielded similar
352 proportions of explained variance (FCT values were relatively constant among the
353 different K; (2) in all the tested K, the proportion of genetic variability found among
354 populations (F_{ST}) was higher than the proportion of genetic variability found among groups
355 (F_{CT}).

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

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

366 For the detection of major genetic discontinuities (software BARRIER), scenarios
367 imposing from 1 to 8 barriers were investigated, until the identified discontinuities were
368 corresponding to statistically significant PhiST values (Figure 4). The analysis firstly
369 indicated the isolation of single populations fixed for single haplotypes (e.g. barrier a,
370 isolating RMa) or small groups of populations (e.g. barrier b). Then, the imposition of the
371 third barrier (barrier c) resulted in the separation of populations from the Venetian Prealps.
372 Adding more barriers, more general patterns started to appear. Major separations resulted
373 from the addition of different adjacent barriers, e.g. barrier h + b, separating the whole
374 north-eastern part of Trentino from the rest of the region. As a final output, eight barriers
375 lead to the almost complete separation of the western and eastern side of the Adige valley

(Figure 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.

Tajima's D neutrality test yielded negative values for lineages Alp1 and Alp2, although marginally significant only for Alp1 ($p = 0.061$), and positive non-significant value for Alp4. Notably, lineage Alp1 also showed a highly negative (-7.5951) and significant value for Fu's Fs ($p < 0.05$), suggesting demographic expansion (Table S4 in Supporting Information).

The mismatch distribution for lineage Alp1 showed a clear unimodal shape (Figure S4 in Supporting Information), with a peak at 0 mutational steps, as expected in the case of a very recent population expansion (Rogers & Harpending, 1992). The peak corresponds to the comparisons between individuals that share the same allele, the most common CA2 (further details on mismatch distribution and relative goodness-of-fit tests are discussed in Supporting Information, Table S4 and Appendix S1). Mismatch distribution for Alp2 and Alp4 did not show any signature of expansion.

DISCUSSION

"REFUGIA-WITHIN-REFUGIA" IN THE SOUTHERN ALPS?

Our fine-scale intensive sampling design revealed the complex evolutionary history of *Rana temporaria* in the considered southern alpine region (Figure 5), providing strong support for the hypothesis of a "refugia-within-refugia" model for common frogs in the Italian Alps. According to this scenario, first proposed by Stefani *et al.* (2012), and in contrast to previous hypotheses (see Teacher *et al.*, 2009), *Rana temporaria* survived the

401 last glacial period in multiple peripheral refugia on the southern slopes of the Alps and
402 Prealps, separated by inhospitable intervening regions.

403 The global levels of genetic diversity observed for the species in the Trentino
404 region are considerably high. We found 12 different haplotypes, a striking number
405 considering the small spatial scale of our study given that Stefani *et. al* (2012) observed 18
406 haplotypes across the whole Italian Alps. Such high levels of genetic diversity are typical
407 of areas located in the proximity of glacial refugia (Hewitt, 1996; Taberlet *et al.*, 1998;
408 Widmer & Lexer, 2001; Provan & Bennett, 2008). The detection of three different COI
409 lineages, the strong association between genetic variation and geography and the presence
410 of a contact zone in the eastern part of the region suggest a history of allopatric divergence
411 in different refugia, followed by secondary contacts and population admixture. SAMOVA
412 provided support for these three main groups, but also identified alternative grouping
413 schemes explaining a similar proportion of variance, suggesting that the inferred
414 population structure might be better interpreted as spatial clines of admixture among the
415 three lineages.

416 The spatial distribution of lineages and haplotypes indicates that Alp1 colonized
417 East Trentino from the west. Indeed, Alp1 was the only lineage found in the western part
418 of the region, where it exhibits high levels of diversity, while in the east it is present with
419 two haplotypes only, and always admixed with other lineages. The negative correlation
420 between genetic diversity and latitude detected in West Trentino suggests a north-south
421 orientation of the postglacial recolonization process for Alp1 in this half of the region. We
422 can therefore propose that Alp1 originated in a peripheral Pleistocene refugium probably
423 located in the south-western mountains of the region, or in the immediate southern
424 Lombardian Prealps, from where it spread toward the north and toward the east under

425 favorable climatic conditions. Alp2 is instead the dominant haplogroups in the Venetian
426 Prealps, at the southern margin of the region, suggesting this area as potential refugium.
427 Further support for the proposed locations of the refugia for Alp1 and Alp2
428 lineages stems from fossil records: fossils remains of *Rana temporaria* were found in
429 Pleistocene paleontological localities in the North-Western Lombardian Prealps (Bona,
430 Laurenti & Delfino, 2009) and in Lessinia, in the Venetian Prealps (Delfino, 2002),
431 providing evidence that the species survived the Pleistocene glacial cycles in these areas
432 lying outside the current distribution of the species (Bartolini *et al.*, 2014). The frequency
433 of Alp4 depicts a penetration line from north-east to the middle of the region. Its presence
434 is marginal in the study region and does not allow speculations on its geographic origin,
435 however data from Stefani et al. (2012) seem to indicate the eastern margin of the Alps as
436 its potential refugial area (see Figure S3a in Supporting Information). The relatively strong
437 increase in genetic diversity with latitude, detected in East Trentino, and opposite to the
438 western trend, can be explained by the admixture of three different COI lineages occurring
439 in the northern part of this sub-region, which is a common feature of contact zones (Petit *et*
440 *al.*, 2003). The moderate correlation among number of haplotypes (n) and longitude,
441 detected only for East Trentino, seems to be also driven by the presence of three lineages
442 particularly in the north-eastern part of this sub-region.
443 Interestingly, both Alp2 and Alp4, did not penetrate in West Trentino. The location
444 of major genetic discontinuities, detected with the Monmonier algorithm (BARRIER),
445 provided further details on the potential colonization routes. Western and Eastern Trentino
446 appeared to be completely separated except for a strict corridor in the central part of the
447 region. This area matches with the Valsugana valley, a west-east oriented valley that could
448 have been used as a corridor by Alp1 for its eastward expansion. The hypothesis of one
449 single penetration corridor is supported by the rapid loss of genetic diversity that this

lineage seems to have experienced moving eastward. 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, Ziegenhagen & Petit, 2006).

Nevertheless, the observed high levels of genetic differentiation (PhiST values), 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 the three different lineages met. This could be due to the complex orography of the study region, characterized by different mountain massifs and deep valleys. 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). Allele surfing can occur more often in small, rapidly growing populations under limited dispersal (Klopfstein *et al.* 2006), and this may be the case of our study species. The two proposed explanations are not mutually exclusive.

DO THE LINEAGES ORIGINATED IN DIFFERENT SUB-REFUGIA HARBOR DIFFERENT LEVELS OF GENETIC DIVERSITY?

Another major outcome of this study is the remarkable difference in overall genetic diversity levels between Alp1 and Alp2 lineages, with 9 haplotypes in Alp1 and only 1 found for Alp2. Stefani *et al.* (2012), in their survey covering the whole Alpine chain, found only 2 haplotypes for this lineage. Given the large number of sampled sites, the strong difference in genetic diversity cannot be due to sampling bias, and might reflect different conditions experienced by the two lineages in their respective glacial refugia.

475 Alp1 might have persisted in a large refugium with widely connected populations and an
476 overall high effective population size, while Alp2 might have been restricted to a smaller,
477 less favorable area, therefore experiencing a strong loss of genetic diversity due to drift or
478 bottlenecks. The different current spatial distributions of the two lineages, with Alp1 being
479 widespread in a large sector of central and eastern Alps, and Alp2 limited to a small
480 portion of eastern Alps and Prealps (Figure S3a), seem to corroborate this hypothesis. The
481 "star-like" shaped topology of Alp1 supports the idea of a larger and more suitable refuge
482 for Alp1 (compared to other lineages), as this pattern is interpreted as an evidence of past
483 population-wide demographic expansion (Rogers & Harpending, 1992; Bandelt *et al.*,
484 1995). However, we cannot exclude the possibility of a sudden spatial expansion during
485 the recolonization process (Appendix S1): different models of population growth may in
486 some cases lead to similar gene tree patterns (Slatkin & Hudson, 1991; Ray *et al*., 2003);
487 moreover, the two potential explanations are here not mutually exclusive. Nevertheless, the
488 fact that demographic expansion was detected for Alp1 only suggests that conditions
489 experienced in different refugial areas might had played an important role.
490
491 ROUTES AND MODES OF POSTGLACIAL RECOLONIZATION
492 The main genetic discontinuity detected in our study region, corresponding to the
493 low elevation Adige river valley, has a strong paleoclimatic foundation. Broad valleys are
494 major genetic barriers for common frogs also outside the study area (Stefani *et al.* 2012),
495 and this barrier effect may be explained by the fact that, during the interglacials, broad
496 Alpine valleys were occupied for slong periods by slowly retreating glaciers, preventing
497 gene flow between the opposite sides. During the last Alpine Last Glacial Maximum
498 (ALGM), about 25 000 years ago, the whole Trentino region was indeed covered by the
499 Adige glacier, approximately 1600-2000 m thick (Caldonazzi & Avanzini, 2011). In

500 contrast, Prealpine areas were only partially covered by glaciers (Bassetti & Borsato,
501 2005). The ice sheet started to retreat 17 000–11 500 years ago, and in the final stage of the
502 retreat, complete ice melt led to massive flooding in the central part of the region
503 (Angelucci, 2013). Meanwhile, forests started to cover both sides of the region. It is likely
504 that, during glacier retreat, both surrounding forests and the swampy central valley
505 provided suitable habitat for the common frog, but the central valley become unsuitable
506 with further temperature increases, re-establishing their role as barrier to gene flow.
507 However, the Pleistocene history of the common frog revealed a more complicated
508 scenario than a simple east-west separation: lp2 and Alp4 remained confined to the eastern
509 part of the region, but Alp1 crossed the Adige river valley, colonizing East Trentino. Three
510 not-mutually exclusive hypotheses might explain this pattern: (1) the recolonization by the
511 different COI lineages occurred in different times; (2) the three different putative refugia
512 were located at different distances from the central valley: under this scenario, the glacial
513 refuge for Alp1 should have been located closer to the central part of the region, so that
514 this lineage reached the valley when it was not a barrier, while the other lineages arrived
515 later; (3) recolonization from different refugia took place at different recolonization rates.
516 Testing these hypotheses would require future investigations, combining a specific
517 sampling design (including "pure" populations for all the three lineages), a multi-gene
518 approach allowing robust demographic inference, and a detailed paleoclimatic
519 reconstruction. Nevertheless, the supposed location of different refugia supports hypothesis
520 2, as Alp4 was was detected in East Trentino only in few sites, with frequency rapidly
521 decreasing toward the center, with a pattern resembling the ending tail of a penetration
522 line. On the other habnd, ; (b) evidence supporting hypothesis 3 for explaining the failure
523 of Alp2 may come from its low genetic diversity. Indeed, supposing low effective
524 population size and/or density in its corresponding refugium for explaining the low levels

525 of genetic diversity, we could assume that the same factor negatively affected dispersal
526 rates and connectivity, and, ultimately, recolonization potential. Considering its limited
527 geographic distribution across the Alps, it must be noted that this lineage doesn't seem to
528 have spread toward the east, neither (Stefani *et al.*, 2012; Figure S3a).
529
530 CONCLUSIONS
531 Amphibians are considered the most endangered group of vertebrates (Wake, 1991;
532 Houlahan *et al.*, 2000; Gardner, 2001; Stuart *et al.*, 2004; IUCN, 2016). Genetic diversity
533 is required for populations to adapt to a changing environment (Booy *et al.*, 2000; Reusch
534 *et al.*, 2005; Höglund, 2009), and genetic monitoring should therefore be considered a
535 fundamental aspect in the study of amphibian declines, together with a better
536 understanding of the underlying evolutionary processes (Allentoft & O'Brien, 2010;
537 Blaustein & Bancroft, 2007).
538 *Rana temporaria* is a widespread amphibian and it is not currently considered
539 threatened, although local declines are documented for the species (IUCN, 2016), and it
540 might be affected by range reduction and population fragmentation in the near future due
541 to climate change (Henle *et al.*, 2008; Bartolini *et al.*, 2014). However, the study of
542 widespread species can help unravelling the fine-scale legacy of past climatic oscillations,
543 helping to detect historical hotspots of genetic diversity and to identify management units
544 of relevant evolutionary significance. For example, the finding of a genetically
545 homogeneous gene pool at mtDNA in the western part of the region, opposite to the
546 admixture patterns found in the east, suggests the need for different management strategies
547 for the species in the two sub-regions. Information gained from this study may therefore
548 serve as a term of comparison with other more rare and threatened amphibians, for which

549 detailed studies are usually more difficult to implement, in order to identify common
550 patterns or to highlight relevant evolutionary differences among organisms.

551 Combining mtDNA and microsatellites, Vernesi *et al.* (2016) found strong genetic
552 differentiation among populations from the eastern side of the Adige valley for several
553 vertebrate species (roe deer, red deer, mountain hare and, only for mtDNA, chamois). A
554 similar east-west genetic differentiation along this line was also detected for the mid-
555 altitude butterfly *Erebia euryale* (Haubrich & Schmitt, 2007) and for different alpine plant
556 species (e.g. Schönswetter *et al.*, 2002; Albach, Schönswette & Tribsch, 2006). At the
557 species diversity level, the so-called "Brenner-line", which include the Adige valley up to
558 the Brenner pass, was proposed as a barrier for plant species distributions in the 19th
559 century (Kerner, 1870). An analogous discontinuity, corresponding to the Adige river
560 valley was also recognized for cave-dwelling species (Ruffo, 1950; Ruffo, 1958; Vailati,
561 1975; Latella, Verdari & Gobbi, 2012). Our findings provide further evidence for this
562 biogeographic peculiarity of the Trentino region, which is so far not recognized in an
563 organic theoretical framework, nor in conservation planning.

564 Under a conservation perspective, past evolutionary events such as range
565 expansion-contraction due to glacial cycles are rarely considered. However, understanding
566 patterns and processes related to Pleistocene refugia may be of crucial importance for
567 developing a robust conservation strategy in the face of ongoing climate change. Indeed,
568 the study of major paleoclimatic events may help understanding the genetic and
569 evolutionary consequences of range shifts, extinctions and recolonization processes,
570 identifying potential future climate change refugia and implementing priority actions for
571 management (Morelli *et al.*, 2016). Moreover, a recent meta-analysis showed that the
572 conservation status of European amphibians is negatively correlated with distance from
573 refugia, thus the phylogeographic status of populations (i.e., refugial vs. post-glacial

574 colonization) should be considered in conservation assessments (Dufresnes & Perrin,
575 2015).

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

588

589 **ACKNOWLEDGMENTS**

590

591 We thank Barbara Crestanello and Matteo Girardi for their contributions to field sampling
592 and lab activities. Map of sampling sites was generated with the help of Markus Neteler
593 and Duccio Rocchini (FEM, GIS and Remote Sensing Unit). We thank Andrea Gandolfi
594 for precious suggestions and help in figure editing. Paolo Pedrini and Michele Menegon
595 (Museo delle Scienze di Trento, Sezione Zoologia dei Vertebrati), Michele Caldonazzi and
596 Sandro Zanghellini (ALBATROS S.R.L.) provided useful information on amphibian
597 distribution in the study area. This research was partially funded by Autonomous Province
598 of Trento (Italy) as part of the ACE-SAP project (University and Scientific Research

599 Service regulation number 23, 12 June 2008) and by FIRST (FEM International Research
600 School of Trentino).
601
602
603 **References**
604
605 Albach, D.C., Schoenswetter, P. & Tribsch, A. (2006). Comparative phylogeography of the
606 Veronica alpine complex in Europe and North America. *Molecular Ecology*, 15, 3269–
607 3286.
608 Angelucci, D. (2013). *La valle dell'Adige: genesi e modificazione di una grande valle*
609 *alpina come interazione tra dinamiche naturali e fattori antropici*. In: Il fiume, le terre,
610 l'immaginario. L'Adige come fenomeno storiografico complesso. Osiride, Rovereto, pp.
611 9-43.
612 Avise, J.C. (2000). *Phylogeography: the history and formation of species*. Harvard
613 University Press.
614 Avise, J.C. (2009). Phylogeography: retrospect and prospect. *Journal of biogeography*, 36,
615 3–15.
616 Avise, J.C. (2010). Perspective: conservation genetics enters the genomics era.
617 *Conservation Genetics*, 11, 665–669.
618 Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., *et al.* (1987).
619 Intraspecific phylogeography: the mitochondrial DNA bridge between population
620 genetics and systematics. *Annual review of ecology and systematics*, 489–522.
621 Bandelt, H.-J., Forster, P., Sykes, B.C. & Richards, M.B. (1995). Mitochondrial portraits of
622 human populations using median networks. *Genetics*, 141, 743–753.

623 Bartolini, S., Cioppi, E., Rook, L. & Delfino, M. (2014). Late Pleistocene fossils and the
624 future distribution of Rana temporaria (Amphibia, Anura) along the Apennine Peninsula
625 (Italy). *Zoological Studies*, 53, 1.
626 Bassetti, M. & Borsato, A. (2005). Evoluzione geomorfologica della Bassa Valle
627 dell'Adige dall'Ultimo Massimo Glaciale: sintesi delle conoscenze e riferimenti ad aree
628 limitrofe. *Studi Trentini di Scienze Naturali Acta Geol.*, 82, 31–42.
629 Beebee, T. (1996). *Ecology and conservation of amphibians*. Springer Science & Business
630 Media.
631 Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and
632 powerful approach to multiple testing. *Journal of the royal statistical society. Series B*
633 *(Methodological)*, 289–300.
634 Bialozyt, R., Ziegenhagen, B. & Petit, R. (2006). Contrasting effects of long distance seed
635 dispersal on genetic diversity during range expansion. *Journal of evolutionary biology*,
636 19, 12–20.
637 Bona, F., Laurenti, B. & Delfino, M. (2009). Climatic fluctuations during the last glacial in
638 the North-Western lombardian prealps: the upper Pleistocene faunal assemblages of the
639 Caverna Generosa (Como, Italy). *Rivista Italiana di Paleontologia e Stratigrafia*
640 *(Research In Paleontology and Stratigraphy)*, 115.
641 Bonin, A., Taberlet, P., Miaud, C. & Pompanon, F. (2006). Explorative genome scan to
642 detect candidate loci for adaptation along a gradient of altitude in the common frog (Rana
643 temporaria). *Molecular Biology and Evolution*, 23, 773–783.
644 Brede, E. & Beebee, T. (2004). Contrasting population structures in two sympatric
645 anurans: implications for species conservation. *Heredity*, 92, 110–117.

646 Brede, E.G. & Beebee, T.J. (2006). Consistently different levels of genetic variation across
647 the European ranges of two anurans, Bufo bufo and Rana temporaria. *The Herpetological*
648 *Journal*, 16, 265–271.
649 Buskirk, J. (2012). Permeability of the landscape matrix between amphibian breeding sites.
650 *Ecology and evolution*, 2, 3160–3167.
651 Canestrelli, D., Bisconti, R., Sacco, F. & Nascetti, G. (2014). What triggers the rising of an
652 intraspecific biodiversity hotspot? Hints from the agile frog. *Scientific Reports*, 4, 5042.
653 Caldonazzi, M., & Avanzini, M. (2011). *Storia geologica del Trentino*. Albatros, Trento.
654 Clement, M., Posada, D. & Crandall, K.A. (2000). TCS: a computer program to estimate
655 gene genealogies. *Molecular ecology*, 9, 1657–1659.
656 Darriba, D., Taboada, G.L., Doallo, R. & Posada, D. 2012. jModelTest 2: more models,
657 new heuristicsand parallel computing. Nature Methods 9(8), 772.
658 Delfino, M. (2002) *Erpetofaune italiane del Neogene e del Quaternario*. Università degli
659 Studi di Modena e Reggio Emilia. Ph.D. Dissertation.
660 Dufresnes, C. & Perrin, N. (2015). Effect of biogeographic history on population
661 vulnerability in European amphibians. *Conservation Biology*, 29, 1235–1241.
662 Dupanloup, I., Schneider, S. & Excoffier, L. (2002). A simulated annealing approach to
663 define the genetic structure of populations. *Molecular Ecology*, 11, 2571–2581.
664 Excoffier, L. & Lischer, H.E. (2010). Arlequin suite ver 3.5: a new series of programs to
665 perform population genetics analyses under Linux and Windows. *Molecular ecology*
666 *resources*, 10, 564–567.
667 Excoffier, L. & Ray, N. (2008). Surfing during population expansions promotes genetic
668 revolutions and structuration. *Trends in ecology & evolution*, 23, 347–351.

669 Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992). Analysis of molecular variance
670 inferred from metric distances among DNA haplotypes: application to human
671 mitochondrial DNA restriction data. *Genetics*, 131, 479–491.
672 Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for
673 amplification of mitochondrial cytochrome c oxidase Subunit 1 from diverse metazoan
674 invertebrates. *Molecular Marine Biology and Biotechnology.*
675 Gasc, J.-P., Cabela, A., Crnobrnja-Isailovic, D., Dolmen, K., Grossenbacher, K., Haffner,
676 P., *et al.* (1997). Atlas of amphibians and reptiles in Europe.
677 Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes
678 on identification. *Herpetologica*, 16, 183–190.
679 Gugerli, F., Sperisen, C., Büchler, U., Magni, F., Geburek, T., Jeandroz, S., *et al.* (2001).
680 Haplotype variation in a mitochondrial tandem repeat of Norway spruce (Picea abies)
681 populations suggests a serious founder effect during postglacial re-colonization of the
682 western Alps. *Molecular Ecology*, 10, 1255–1263.
683 Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and
684 analysis program for Windows 95/98/NT. In: *Nucleic acids symposium series*. pp. 95–98.
685 Hampe, A. & Petit, R.J. (2005). Conserving biodiversity under climate change: the rear
686 edge matters. *Ecology letters*, 8, 461–467.
687 Hasegawa, M., K. Kishino, and T. Yano (1985). Dating the human-ape splitting by a
688 molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, 22, 160–174.
689 Haubrich, K. & Schmitt, T. (2007). Cryptic differentiation in alpine-endemic, high-altitude
690 butterflies reveals down-slope glacial refugia. *Molecular Ecology*, 16, 3643–3658.
691 Hewitt, G.M. (1996). Some genetic consequences of ice ages, and their role in divergence
692 and speciation. Biol. J. Linn. Soc. 58, 247–276.
693 Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. *Nature*, 405, 907–913.

694 Hewitt, G. (2004). Genetic consequences of climatic oscillations in the Quaternary.
695 *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 359,
696 183–195.
697 Hickerson, M., Carstens, B., Cavender-Bares, J., Crandall, K., Graham, C., Johnson, J., *et*
698 *al.* (2010). Phylogeography's past, present, and future: 10 years after. *Molecular*
699 *Phylogenetics and Evolution*, 54, 291–301.
700 Johansson, F., Veldhoen, N., Lind, M.I. & Helbing, C.C. (2013). Phenotypic plasticity in
701 the hepatic transcriptome of the European common frog (Rana temporaria): the interplay
702 between environmental induction and geographical lineage on developmental response.
703 *Molecular ecology*, 22, 5608–5623.
704 Johansson, M., Primmer, C.R. & Merilae, J. (2007). Does habitat fragmentation reduce
705 fitness and adaptability? A case study of the common frog (Rana temporaria). *Molecular*
706 *Ecology*, 16, 2693–2700.
707 Johansson, M., Primmer, C.R., Sahlsten, J. & Merilä, J. (2005). The influence of landscape
708 structure on occurrence, abundance and genetic diversity of the common frog, Rana
709 temporaria. *Global Change Biology*, 11, 1664–1679.
710 Kerner, A. (1870). Die natürlichen Floren im Gelände der Deutschen Alpen. Jena:
711 Fromann.
712 Klopfstein, S., Currat, M. & Excoffier, L. (2006). The fate of mutations surfing on the
713 wave of a range expansion. *Molecular biology and evolution*, 23, 482–490.
714 Librado, P. & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA
715 polymorphism data. *Bioinformatics*, 25, 1451–1452.
716 Manni, F., Guerard, E. & Heyer, E. (2004). Geographic patterns of (genetic, morphologic,
717 linguistic) variation: how barriers can be detected by using Monmonier's algorithm.
718 *Human biology*, 76, 173–190.

719 Morelli, T.L., Daly, C., Dobrowski, S.Z., Dulen, D.M., Ebersole, J.L., Jackson, S.T., *et al.*
720 (2016). Managing climate change refugia for climate adaptation. *PLoS One*, 11,
721 e0159909.
722 Moritz, C., Langham, G., Kearney, M., Krockenberger, A., VanDerWal, J. & Williams, S.
723 (2012). Integrating phylogeography and physiology reveals divergence of thermal traits
724 between central and peripheral lineages of tropical rainforest lizards. *Philosophical*
725 *Transactions of the Royal Society B: Biological Sciences*, 367, 1680–1687.
726 Palo, J.U., Schmeller, D.S., Laurila, A., Primmer, C.R., Kuzmin, S.L. & Merilä, J. (2004).
727 High degree of population subdivision in a widespread amphibian. *Mol. Ecol.*, 13, 2631–
728 2644.
729 Petit, R.J., Aguinagalde, I., de Beaulieu, J.-L., Bittkau, C., Brewer, S., Cheddadi, R., *et al.*
730 (2003). Glacial refugia: hotspots but not melting pots of genetic diversity. *Science*, 300,
731 1563–1565.
732 Provan, J., & Bennett, K. D. (2008). Phylogeographic insights into cryptic glacial refugia.
733 Trends in ecology & evolution, 23(10), 564-571.
734 Rogers, A.R. & Harpending, H. (1992). Population growth makes waves in the distribution
735 of pairwise genetic differences. *Molecular biology and evolution*, 9, 552–569.
736 Rowe, G., Beebee, T. & Burke, T. (1998). Phylogeography of the natterjack toad Bufo
737 calamita in Britain: genetic differentiation of native and translocated populations.
738 *Molecular Ecology*, 7, 751–760.
739 Ruffo, S. (1950). Descrizione di due nuovi Catopidi cavernicoli del Veronese ed
740 osservazioni sul genere Neobathyscia Müll. *Mem. Mus. Civ. St. Nat. Verona*, 2, 125–133.
741 Ruffo, S. (1958). *Speleofaune regionali e biogeografia italiana. Le caratteristiche della*
742 *fauna cavernicola pugliese in rapporto alla paleageografia della regione adriatica*.
743 Actes II Congr. lnt. Spél. Bari-Lecce-Salerno.

744 Schmeller, D.S., Palo, J.U. & Merilä, J. (2008). A contact zone between two distinct Rana
745 temporaria lineages in northern Germany. *Alytes*, 25, 93–98.
746 Schönswetter, P., Tribsch, A., Barfuss, M. & Niklfeld, H. (2002). Several Pleistocene
747 refugia detected in the high alpine plant Phyteuma globulariifolium Sternb. & Hoppe
748 (Campanulaceae) in the European Alps. *Molecular Ecology*, 11, 2637–2647.
749 Sgro, C.M., Lowe, A.J. & Hoffmann, A.A. (2011). Building evolutionary resilience for
750 conserving biodiversity under climate change. *Evolutionary Applications*, 4, 326–337
751 Slatkin, M. & Hudson, R.R. (1991). Pairwise comparisons of mitochondrial DNA
752 sequences in stable and exponentially growing populations. *Genetics*, 129, 555–562.
753 Stapley, J., Reger, J., Feulner, P.G., Smadja, C., Galindo, J., Ekblom, R., *et al.* (2010).
754 Adaptation genomics: the next generation. *Trends in ecology & evolution*, 25, 705–712.
755 Stefani, F., Gentilli, A., Sacchi, R., Razzetti, E., Pellitteri-Rosa, D., Pupin, F., *et al.* (2012).
756 Refugia within refugia as a key to disentangle the genetic pattern of a highly variable
757 species: the case of Rana temporaria Linnaeus, 1758 (Anura, Ranidae). *Mol. Phylogenet.*
758 *Evol.*, 65, 718–726.
759 Stevens, V.M., Verkenne, C., Vandewoestijne, S., Wesselingh, R.A. & Baguette, M.
760 (2006). Gene flow and functional connectivity in the natterjack toad. *Molecular Ecology*,
761 15, 2333–2344.
762 Taberlet, P., Fumagalli, L., Wust-Saucy, A. G., & Cosson, J. F. (1998). Comparative
763 phylogeography and postglacial colonization routes in Europe. Molecular Ecology, 7(4),
764 453-464.
765 Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the
766 control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*
767 10:512–526

768 Teacher, A.G.F., Garner, T.W.J. & Nichols, R.A. (2009). European phylogeography of the
769 common frog (Rana temporaria): routes of postglacial colonization into the British Isles,
770 and evidence for an Irish glacial refugium. *Heredity (Edinb)*, 102, 490–496.
771 Templeton, A.R., Crandall, K.A. & Sing, C.F. (1992). A cladistic analysis of phenotypic
772 associations with haplotypes inferred from restriction endonuclease mapping and DNA
773 sequence data. III. Cladogram estimation. *Genetics*, 132, 619–633.
774 Teske, P.R., Papadopoulos, I., Newman, B.K., Dworschak, P.C., McQuaid, C.D. & Barker,
775 N.P. (2008). Oceanic dispersal barriers, adaptation and larval retention: an
776 interdisciplinary assessment of potential factors maintaining a phylogeographic break
777 between sister lineages of an African prawn. *BMC Evolutionary Biology*, 8, 1.
778 Veith, M., Kosuch, J. & Vences, M. (2003). Climatic oscillations triggered post-Messinian
779 speciation of Western Palearctic brown frogs (Amphibia, Ranidae). *Mol. Phylogenet.*
780 *Evol.*, 26, 310–327.
781 Vences, M., Hauswaldt, J.S., Steinfartz, S., Rupp, O., Goesmann, A., Künzel, S., *et al.*
782 (2013). Radically different phylogeographies and patterns of genetic variation in two
783 European brown frogs, genus Rana. *Mol. Phylogenet. Evol.*, 68, 657–670.
784 Vernesi, C., Hoban, S.M., Pecchioli, E., Crestanello, B., Bertorelle, G., Rosà, R., *et al.*
785 (2016). Ecology, environment and evolutionary history influence genetic structure in five
786 mammal species from the Italian Alps. *Biological Journal of the Linnean Society*, 117,
787 428–446.
788 Widmer, A., & Lexer, C. (2001). Glacial refugia: sanctuaries for allelic richness, but not
789 for gene diversity. Trends in Ecology & Evolution, 16(6), 267-269.
790 Zeisset, I. & Beebee, T. (2008). Amphibian phylogeography: a model for understanding
791 historical aspects of species distributions. *Heredity*, 101, 109–119.
792

Table 1. Overall haplotype frequencies and distributions among sampling sites (N° sites; % sites) and sub-regions (E TN = East Trentino; W TN = West Trentino). Newly discovered haplotypes are highlighted in bold.

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

Table 2. Frequency of COI lineages, number of lineages (nl), 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), and detected haplotypes (n° of occurrences in brackets). Sites are numbered according to map in Figure 1; geographical coordinates are reported in Supporting Information, Table S1.

N°	site	Alp1	Alp2	Alp4	nl	n	h	π	k	s	haplotypes
1	Amp	1	0	0	1	2	0.533	0.0009	0.533	1	CA2(4); TN2(6)
2	Bed	0.1	0.8	0.1	3	3	0.378	0.0054	3.067	13	CA2(1); VC6(8); PR4(1)
3	Bon	1	0	0	1	4	0.711	0.0015	0.867	3	CA2(5); TN2(3); TN3(1); DE10(1)
4	Bro	0.5	0.3	0.2	3	4	0.778	0.0098	5.556	14	CA2(4); TN4(1); VC6(3); PR4(2)
5	Cad	1	0	0	1	3	0.600	0.0012	0.667	2	CA2(6); TN5(3); MT5(1)
6	Can	0.4	0.6	0	2	2	0.533	0.0047	2.667	5	CA2(4); VC6(6)
7	CCC	1	0	0	1	3	0.511	0.0010	0.556	2	CA2(7); TN5(1); MT5(2)
8	Ce1	0.5	0.2	0.3	3	3	0.689	0.0104	5.911	13	CA2(5); VC6(2); PR4(3)
9	Ce2	0.8	0.2	0	2	2	0.356	0.0031	1.778	5	CA2(8); VC6(2)
10	DDB	0.9	0.1	0	2	2	0.200	0.0018	1	5	CA2(9); VC6(1)
11	Ech	0.3	0.7	0	2	2	0.467	0.0041	2.333	5	CA2(3); VC6(7)
12	Fia	1	0	0	1	4	0.800	0.0020	1.133	3	CA2(3); TN2(1); TN3(3); TN5(3)
13	Ing	0.8	0.2	0	2	2	0.356	0.0031	1.778	5	CA2(8); VC6(2)
14	Lag	0.6	0.4	0	2	2	0.533	0.0047	2.667	5	CA2(6); VC6(4)
15	LCa	1	0	0	1	1	0	0	0	0	CA2(1)
16	Lel	0.5	0.4	0.1	3	3	0.644	0.0083	4.711	15	CA2(5); VC6(4); SA1(1)
17	LMe	1	0	0	1	3	0.600	0.0012	0.667	2	CA2(6); TN5(3); MT5(1)
18	LPo	0.3	0.7	0	2	2	0.467	0.0041	2.333	5	CA2(3); VC6(7)
19	LSG	1	0	0	1	2	0.467	0.0008	0.467	1	CA2(3); TN5(7)
20	Mon	0.1	0.9	0	2	2	0.200	0.0018	1	5	CA2(1); VC6(9)
21	MP1	0.3	0.7	0	2	2	0.467	0.0041	2.333	5	CA2(3); VC6(7)
22	MP2	0.1	0.9	0	2	2	0.200	0.0018	1	5	CA2(2); VC6(8)
23	MRe	1	0	0	1	3	0.378	0.0007	0.4	2	CA2(8); TN3(1); DE10(1)
24	Mug	0.7	0.3	0	2	2	0.467	0.0041	2.333	5	CA2(7); VC6(3)
25	PLa	0.8	0	0.2	2	2	0.356	0.0075	4.267	12	CA2(8); SA1(2)
26	PLC	0.3	0.7	0	2	2	0.467	0.0041	2.333	5	CA2(3); VC6(7)
27	PMa	0.7	0.3	0	2	2	0.467	0.0041	2.333	5	CA2(7); VC6(3)
28	Pos	0.1	0.9	0	2	2	0.200	0.0018	1	5	CA2(1); VC6(9)
29	PR1	0.2	0.7	0.1	3	3	0.511	0.0065	3.711	13	CA2(2); VC6(7); PR4(1)
30	PR2	0.4	0.6	0	2	2	0.533	0.0047	2.667	5	CA2(4); VC6(6)
31	PS2	0.5	0.1	0.4	3	3	0.644	0.0105	5.978	13	CA2(5); VC6(1); PR4(4)
32	PT1	1	0	0	1	2	0.200	0.0003	0.2	1	CA2(9); TN5(1)
33	PT2	1	0	0	1	2	0.200	0.0003	0.2	1	CA2(9); TN6(1)
34	PTe	0.5	0.5	0	2	2	0.556	0.0049	2.778	5	CA2(5); VC6(5)
35	PTr	1	0	0	1	3	0.511	0.0010	0.556	2	CA2(7); TN5(1); TN6(2)
36	RM1	1	0	0	1	1	0	0	0	0	TN5(1)
37	Ron	0.9	0.1	0	2	2	0.200	0.0017	1	5	CA2(9); VC6(1)
38	Tov	1	0	0	1	3	0.733	0.0017	1	2	CA2(3); TN5(3); TN6(4)
39	Va1	1	0	0	1	4	0.778	0.0018	1.022	3	CA2(4); TN2(1); TN3(2); MT5(3)
40	VD1	1	0	0	1	3	0.511	0.0010	0.556	2	CA2(7); TN5(1); DE10(2)
41	VD2	1	0	0	1	4	0.644	0.0013	0.756	3	CA2(6); TN2(1); DE10(2); MT5(1)
42	VG1	1	0	0	1	2	0.467	0.0008	0.467	1	CA2(7); TN5(3)
43	VG3	1	0	0	1	3	0.689	0.0014	0.822	2	CA2(5); TN3(3); TN5(2)
44	ViT	1	0	0	1	1	0	0	0	0	CA2(1)
45	VN2	1	0	0	1	2	0.533	0.0009	0.533	1	CA2(6); TN5(4)
46	VP1	1	0	0	1	1	0	0	0	0	CA2(1)
47	VP2	1	0	0	1	1	0	0	0	0	CA2(1)
48	Mar	0.3	0.7	0	2	2	0.467	0.0041	2.333	5	CA2(3); VC6(7)
49	MBa	0.2	0.8	0	2	2	0.356	0.0031	1.778	5	CA2(2); VC6(8)
50	PS1	0.3	0.2	0.5	3	3	0.689	0.0113	6.444	13	CA2(3); VC6(2); PR4(5)
51	So2	0.6	0.2	0.2	3	4	0.800	0.0097	5.511	14	CA2(2); TN4(4); VC6(2); PR4(2)
52	Ste	0.6	0.1	0.3	3	3	0.600	0.0111	6.333	15	CA2(6); VC6(1); SA1(3)
53	Tre	1	0	0	1	4	0.711	0.0018	1.022	3	CA2(5); TN1(1); TN2(3); TN3(1)
54	LRo	1	0	0	1	2	0.533	0.0009	0.533	1	CA2(6); TN3(4)

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

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			

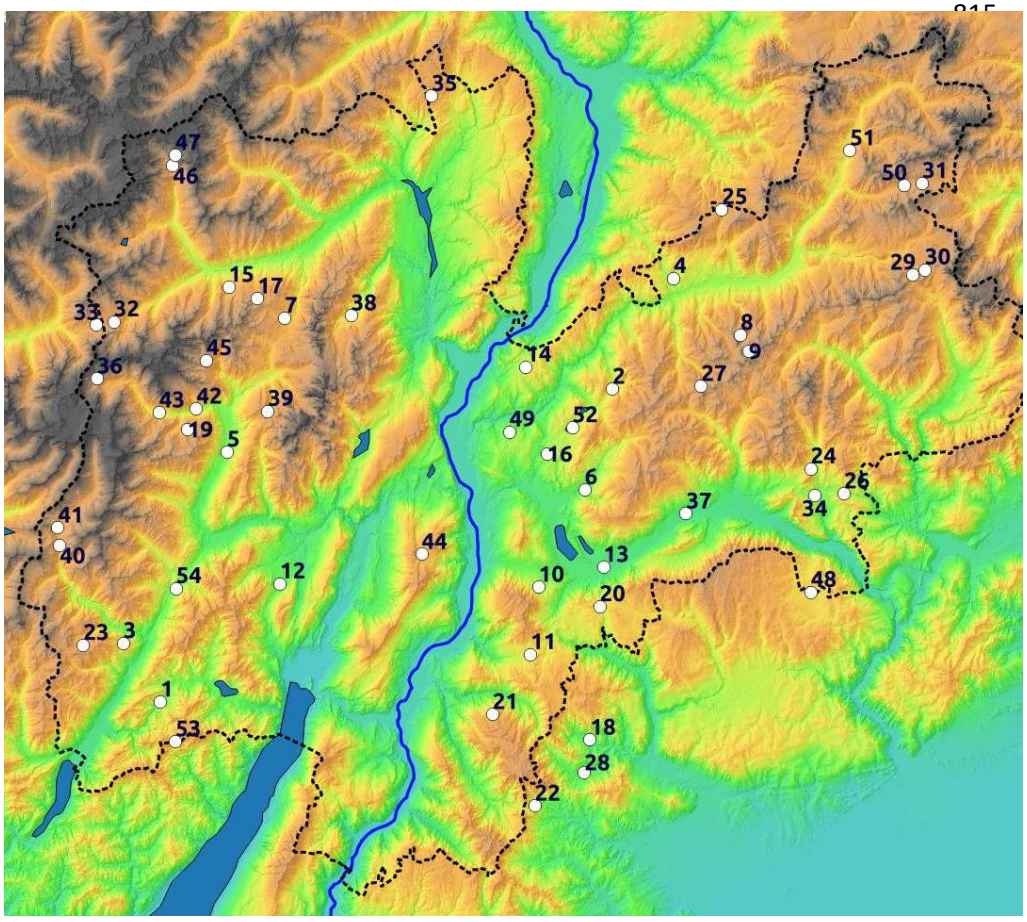
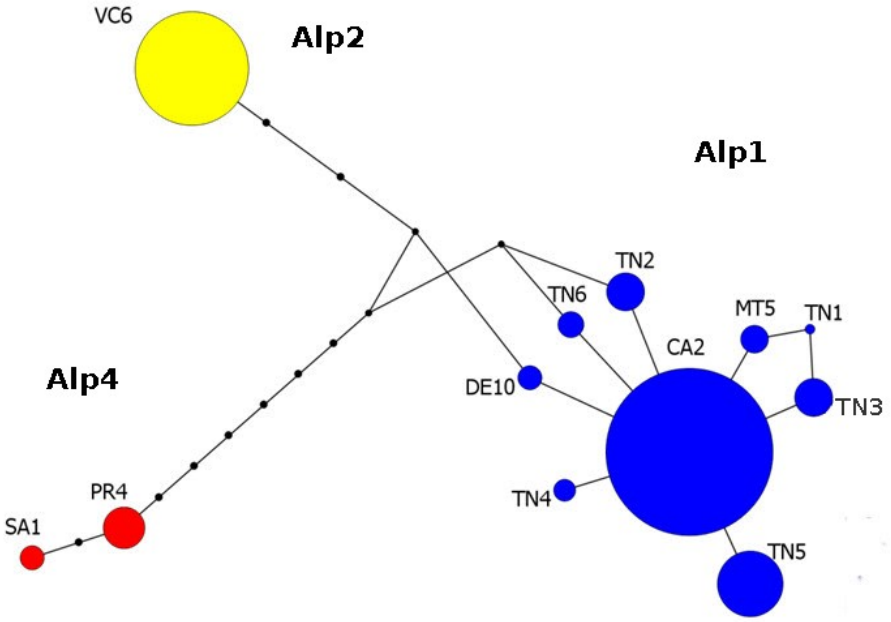


Figure 1. Sampling sites of *Rana temporaria* in the Trentino region. Labels, site names and coordinates are listed in Table S1 (Supporting Information). The blue line in the middle of the region represents the Adige river. Sites are numbered according to Table 2.



834

835

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

840

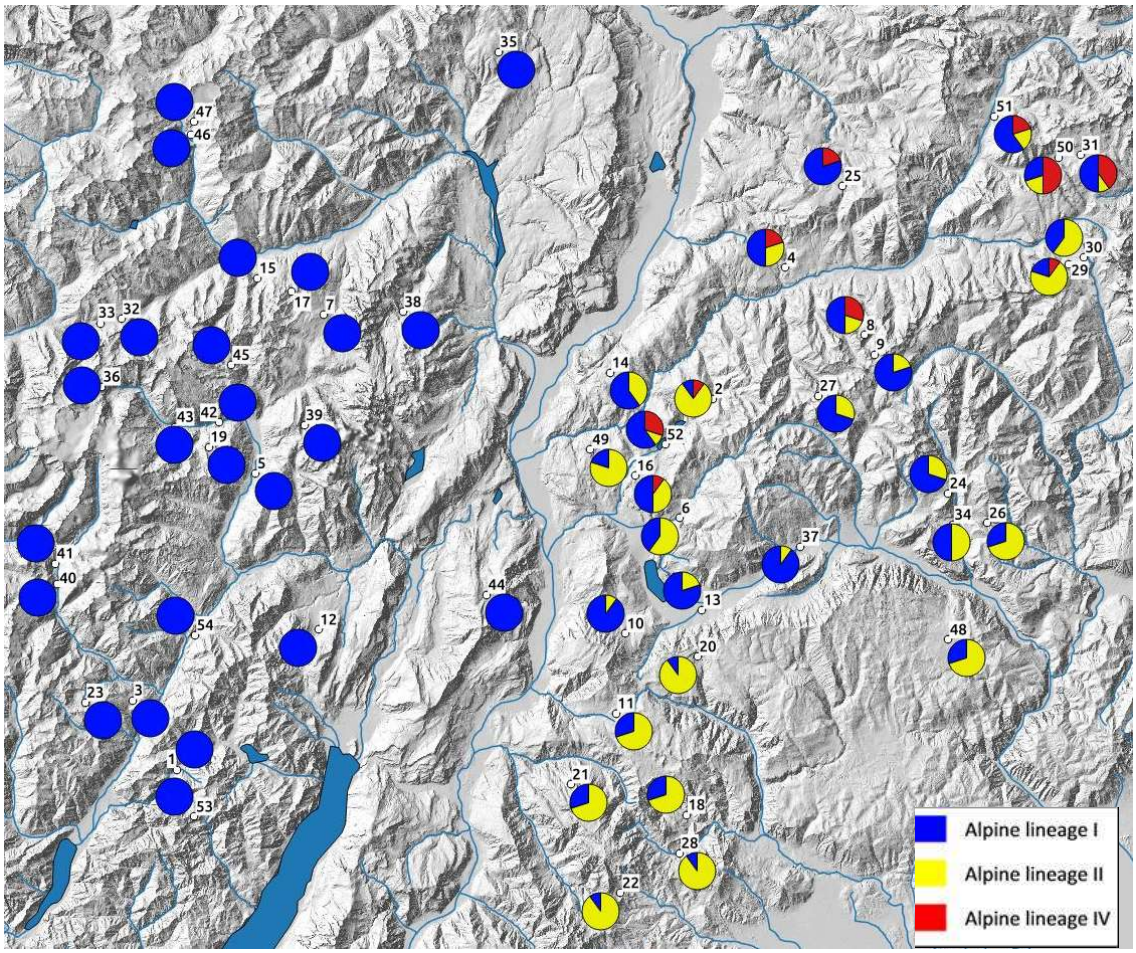


Figure 3. Spatial distribution of COI lineages in the different populations. Different colors correspond to the different lineages. Sites are numbered according to Table S1 (Supporting Information).

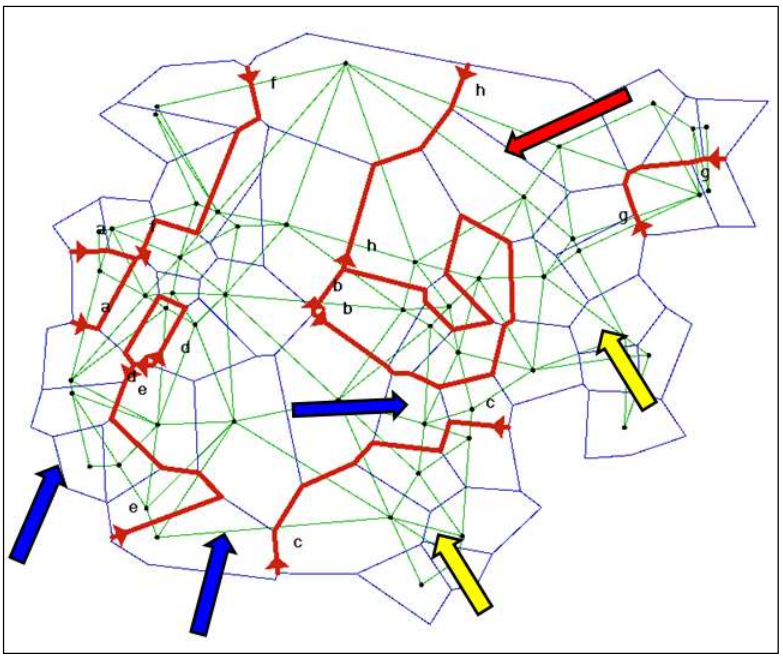


Figure 4. Output of BARRIER analysis, showing the spatial location of major genetic discontinuities. Sample points (populations) are represented by black dots, blue lines correspond to Voronoï tessellation and green lines to Delaunay triangulation. The inferred barriers are depicted with red lines and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

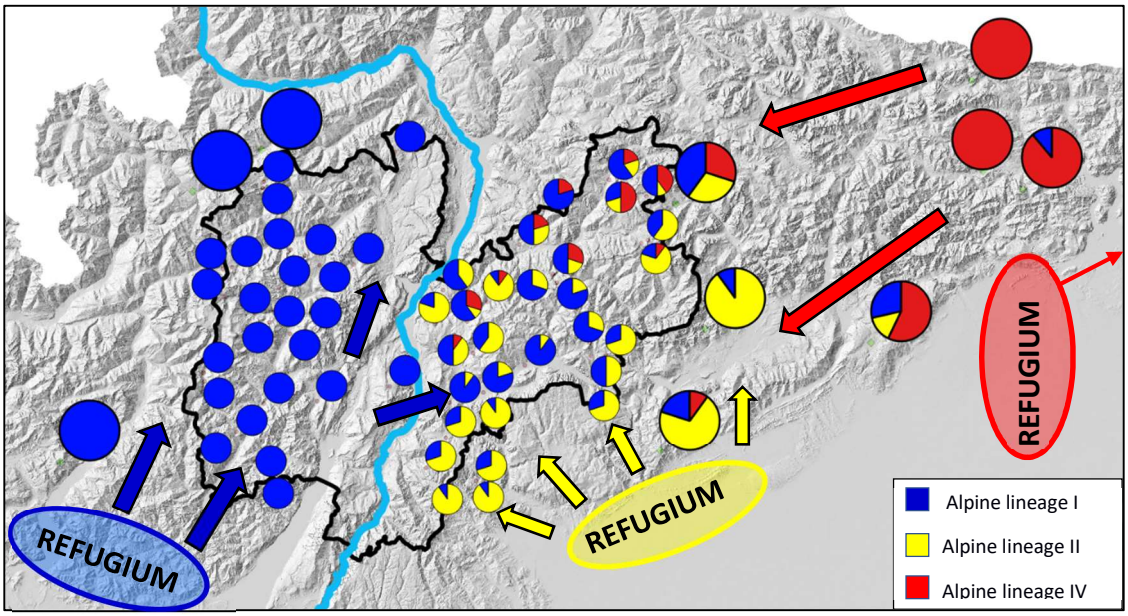


Figure 5. Tentative phylogeographic reconstruction for *Rana temporaria* in the Trentino region. The map shows the different sampling sites, colored according to the frequency of detected COI lineages, together with the approximate location of corresponding glacial refugia and the proposed recolonization 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).

DEMOGRAPHIC ANALYSIS