

# Spatial prioritization of amphibian intraspecific genetic diversity: The need of accounting for palaeoenvironmental legacies

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## ABSTRACT

Biodiversity is a multifaceted concept, and conservation actions must consider all levels of biodiversity. Spatial prioritization of conservation efforts usually targets species diversity, while consideration of intraspecific genetic diversity is frequently hampered by the lack of data. Here, we combined genetic data (mtDNA) from multiple amphibian species and measures of environmental stability to i) identify the overall drivers of present-day intraspecific genetic diversity (nucleotide diversity and phylogenetic originality); ii) define priority areas for the conservation of genetic diversity, and iii) assess the surrogacy value of species diversity for genetic diversity in Italy and nearby areas. We tested for potential environmental predictors of genetic diversity and originality by fitting spatially-explicit Bayesian mixed models, and used species-specific predictions to generate spatial prioritizations of intraspecific genetic diversity. Present-day phylogenetic originality was positively correlated with climate and habitat stability since the Last Glacial Maximum (*pseudo-R*<sup>2</sup>: 0.61), while the same set of predictors had limited explanatory power for nucleotide diversity (*pseudo-R*<sup>2</sup>: 0.15). The spatial pattern of originality remarkably matched previously identified refugial areas for plants, as well as the distribution of some micro-endemic species. Prioritizations targeting phylogenetic originality showed that high conservation performance might be achieved with relatively low effort. However, the surrogacy between genetic and species-diversity was limited, implying that larger efforts would be needed to preserve genetic diversity while taking species diversity into account. Our study provides an example of how spatially-explicit approaches allow prioritization of conservation efforts accounting for both species and intraspecific genetic diversity, thus improving the long-term conservation of biodiversity.

## 1. Introduction

Past climate changes strongly affected the distribution of most living organisms and are recognized as a major driver of evolutionary processes (Hewitt, 2000; Dawson et al., 2011). Current distribution patterns of many terrestrial species are largely a legacy of dramatic climate fluctuations that occurred during the late Pleistocene (Hewitt, 2004; Hofreiter and Stewart, 2009; Hewitt, 2011). Depending on their dispersal abilities and to the patchiness of suitable habitats, species persisted in climatically stable areas (i.e. refugia) or tracked the geographical displacement of their habitats. These spatial dynamics were associated with local demographic processes, such as colonization,

extinction and population contraction/expansion. The demographic fluctuations and the strong selective forces experienced during these range contractions and shifts had both stochastic and selective effects on genetic diversity, and conditioned the ability of the species to persist, adapt and evolve in response to global environmental changes (Steffen et al., 2015).

The worldwide accelerating decline in biodiversity compels the scientific community, administrators and stakeholders towards the identification and protection of those areas most critical for the preservation of species and genetic resources (Convention on Biological Diversity, strategic goal C; <http://www.cbd.int/sp/targets>). Conservation efforts often target species diversity, with species' distribution being the

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fundamental piece of information for conservation planning. While species-based prioritizations, at least for some regions, can be a good surrogate for functional and phylogenetic diversity (i.e. have a high surrogacy value; Rapacciolo et al., 2019) some mismatches exist (e.g. Devictor et al., 2010; Mazel et al., 2017). Additionally, while conservation plans targeting phylogenetic diversity aim at preserving evolutionary histories, intraspecific genetic diversity is pivotal for the adaptive potential of species, in terms of their ability to deal with current and future selective pressures, persist and evolve in response to environmental changes (Steffen et al., 2015; Theodoridis et al., 2020; Hanson et al., 2020), and can help maintaining their role for the functioning of communities and ecosystems (Carvalho et al., 2017; Andreello et al., 2022).

Traditionally, most of phylogeographic studies focused on the idea that, during cold-arid periods of the Pleistocene (ice ages), many species survived at the southern edges of their current distribution (glacial refugia: e.g. Hewitt, 1996; Taberlet et al., 1998). Rapid expansion from refugia following post-glacial climate warming shaped phylogeographic patterns characterized by intraspecific diversity declining away from refugia, as a consequence of sequential founder events (Hewitt, 1996; Hewitt, 2000; Petit et al., 2003). Later research expanded the refugium concept by pointing out the existence of both multiple and cryptic (high-latitude or high-elevation) refugia, as well as the individualistic responses of species to these changes, showing that diversity patterns are often at odds with predictions of simple colonization models (Stewart and Lister, 2001; Schönswetter et al., 2005; Gómez and Lunt, 2007; Provan and Bennett, 2008; Médail and Diadema, 2009). Assessing the responses of individual species to Pleistocene climate shifts remains a fundamental step to understand the drivers of their current distribution and gauge their adaptive potential to future changes (e.g., Marta et al., 2016). However, approaches based on a single species or sets of ecologically similar species with congruent distributions may fail to identify ecological and evolutionary factors determining the overall patterns of genetic diversity occurring nowadays (Gratton et al., 2017a). Several authors pointed at habitat stability and topographic heterogeneity as main drivers of species persistence (Graham et al., 2006; Svenning and Skov, 2007). Stable areas have been shown to harbour higher genetic diversity (e.g. Carnaval et al., 2009) and endemism (e.g. Jansson, 2003; Sandel et al., 2011), to represent the core of the distribution for dispersal-limited alpine species (Marta et al., 2016; Marta et al., 2019), and stability has been shown to predict global spatial patterns of genetic diversity in mammals, amphibians and molluscs (Theodoridis et al., 2020; de Kort et al., 2021). Furthermore, topographic heterogeneity could have favoured local persistence of populations, by supporting a larger number of potential niches and allowing to cope with environmental fluctuations through short-distance (altitudinal) movements (Thuiller et al., 2006; Sandel et al., 2011; Ficetola et al., 2013). This, in turn, would have resulted in relatively stable effective population sizes (i.e. limited founder effect), enabling the maintenance of higher genetic diversity (Svenning and Skov, 2007).

The spatially discrete nature of field sampling in most phylogeographic data do not allow a continuous mapping of intraspecific genetic diversity. To overcome this limitation, several authors made use of complex interpolation techniques (e.g. Carvalho et al., 2017), based on the decay of similarity with distance. However, predictive models accounting for the combined effects of past environmental changes and purely spatial processes in shaping intraspecific diversity might allow identifying the determinants of diversity hotspots, and, thus, improve prioritization of conservation targets.

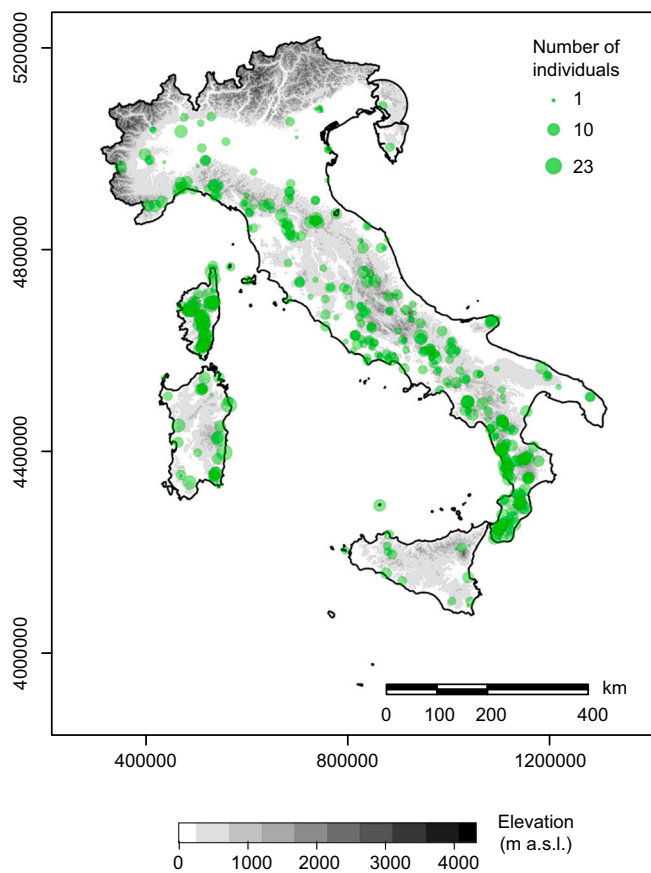
Amphibians are a highly threatened group, characterized by reduced dispersal abilities and a large number of phylogeographic data, which often allowed the identification of clear spatial genetic structures. These features make them excellent candidates for robust, genetic-based conservation planning (e.g. Carvalho et al., 2017). In this study, we use DNA sequence data from 16 species of amphibians distributed across Italy and nearby areas (Corsica and Istria) to i) evaluate whether environmental

stability coherently explains present-day patterns of intraspecific genetic diversity across multiple species; ii) identify priority areas for conservation of intraspecific genetic diversity and evaluate the effectiveness of the current network of protected areas, and iii) assess whether species diversity, which is more easily measurable than genetic diversity, can be a good surrogate for intraspecific genetic diversity (Devictor et al., 2010; Rapacciolo et al., 2019). To do so, we summarized the spatial pattern of intraspecific genetic diversity of each species (i.e. phylogeographic structure) by two genetic indices (nucleotide diversity and mean per-population phylogenetic originality) and fitted spatially-explicit mixed models to determine the effect of the palaeoclimatic and palaeoenvironmental predictors on these measures of the phylogeographic structure of species, while controlling for the effects of anthropic pressure. We expected that present-day genetic diversity would be i) negatively related to climate anomalies since the Last Glacial Maximum (LGM); ii) positively related to habitat stability since the LGM and iii) positively related to topographic heterogeneity. Species-specific spatial predictions of genetic diversity allowed mapping conservation priorities for intraspecific genetic diversity. We expect medium to large differences between genetic-based and either species-based prioritization, or protected areas network, given that species richness rarely is a perfect surrogate of genetic diversity, and that genetic diversity is rarely accounted for during the designation of protected areas. Our work enabled to identify areas that might complement the current network of protected areas to ensure the long-term persistence of intraspecific genetic diversity.

## 2. Methods

### 2.1. Model organisms and genetic data

We focused our analyses on Italy and nearby areas [Corsica (France), part of Slovenia and Istria (Croatia); Fig. 1], which represent a biogeographically coherent region (Rueda et al., 2010; Vilhena and Antonelli, 2015; Ficetola et al., 2018) investigated in a large number of studies on amphibian biogeography (Lanza et al., 2007). According to the IUCN Red List maps (<http://maps.iucnredlist.org>; last accessed on 04/07/2022), 45 native amphibian species are present within the study area: 21 urodeles (8 genera in 3 families) and 24 anurans (8 genera in 7 families). For each species, we thoroughly searched the literature to gather as many mtDNA sequences as possible and carefully reviewed available information on the geographic origin of sequenced specimens. Given the limited number of species present within the study area, we searched GenBank for the whole set of species, and reviewed all the published sources reported as reference for GenBank accessions. We selected the model organisms according to three criteria: i) presence of at least one mitochondrial haplogroup endemic to the study area (identified either by taxonomy - e.g., *Ichthyosaura alpestris apuana* - or by the original studies; Supplementary Table 1); ii) availability of >15 individuals sequenced at the same mtDNA marker (>450 bp) from >5 sampling sites and belonging to endemic haplogroups; iii) individuals unequivocally georeferenced (i.e. possibility of finding a match between the information from GenBank and the manuscript, to reproduce the spatial population structure and correctly assign sequences to sampling sites). Focusing only on haplogroups endemic to the region of interest minimizes the probability that the genetic diversity patterns observed in the study reflect processes that have occurred or occur outside the study area and, therefore, cannot be captured by our analyses (see, e.g., Buckley, 2009; Buhay et al., 2009). For example, if the Julian Alps (at the extreme northeast of Italy), received mtDNA from a Balkan refuge, this would increase the local genetic diversity because of the presence of a nearby stable area not included in the study area. Sequences were downloaded from GenBank (last accessed on 13/04/2023) and multiple sequence alignment was performed in ClustalW (Larkin et al., 2007) to obtain, for each species, a final dataset of aligned and georeferenced mtDNA sequences. Preliminary bibliographic searches showed that,



**Fig. 1.** Study area and sampling sites. Dots are proportional to the number of individuals sampled in a given site for a given species (ranging between 1 and 23); site locations and per-site number of individuals refer to the original studies, referenced in Supplementary Table 1. Base map: digital elevation model based on the data from the Shuttle Radar Topography Mission (SRTM). Map projection: UTM zone 32 N (EPSG: 32632).

conversely to mtDNA, nuclear DNA data (e.g. microsatellite DNA, genome-wide SNPs) are available for a much smaller number of species and often only covered small portions of species' ranges. This is probably due to mtDNA having been – thanks to the ready PCR amplification from low-quality samples and high per-bp information content – the marker of choice for broad-scale phylogeographic study up to at least early 2010s, while most studies based on nuclear DNA data focused on landscape genetics/genomics and targeted recent evolutionary changes (Wang, 2010; Blair, 2023).

## 2.2. Genetic indices

For each species, we calculated two measures of genetic diversity, namely nucleotide diversity ( $\pi$ ), and mean per-population phylogenetic originality, each of them capturing alternative outcomes of demographic persistence in the face of environmental changes. High nucleotide diversity represents higher effective (female) population size (in a Wright-Fisher haploid population with constant effective size  $N_e$  and mutation rate  $\mu$ ,  $\pi = N_e\mu$ ; Nei, 1987), while originality is determined by divergence among populations by genetic drift (Pavoine et al., 2005). Phylogenetic originality is considered as synonymous with several other phylogenetic metrics, such as 'evolutionary distinctiveness', 'evolutionary isolation' or 'phylogenetic rarity', and represents one of the best-suited metrics for measuring evolutionary rarity (Kondratyeva et al., 2019 and reference therein). Combining nucleotide diversity and phylogenetic originality might thus allow for a comprehensive and adequate evaluation of conservation priorities for both genetic diversity

and differentiation (Nielsen et al., 2023).

We described the geographical structures of genetic diversity using Voronoi tessellation, so to obtain "compact" polygons (spatial clusters of grid cells) with straight sides that can be used to identify the sampling sites (or grid cells, for environmental variables) contained within each of them. Voronoi diagram was built using the *deldir* R package (Turner, 2021); to identify the set of coordinates to be triangulated, we performed a k-means clustering (number of clusters = 150) of all grid cell centroids from a 1 km resolution raster of the study area. The resulting tessellation, composed by 150 cells, was clipped using the boundaries of the study area (coordinate reference system: WGS 1984 UTM Zone 32 N - EPSG: 32632); after clipping with the boundaries of the study area, cell areas ranged between 27.0 and 3119.7 km<sup>2</sup> (cf. Supplementary Fig. 1; 10 %–90 % quantiles = 1618–2744 km<sup>2</sup>). Polygons with small cell size were the ones located at the boundaries of the study area or along the coastlines (see Supplementary Fig. 1 for a spatial representation of the obtained Voronoi cells). If, for a given species, multiple sampling sites were located in the same cell, we calculated genetic indices for the pooled sets. The selection of the number of clusters was somehow subjective and different numbers of clusters result in different grain size for the spatial analyses, possibly affecting model estimates. We thus tested the robustness of results to the selection of a different number of clusters (50 and 450).

Nucleotide diversity measures the average DNA sequence divergence in a set of aligned sequences (Nei, 1987), and was calculated in the *pegas* (Paradis, 2010) R package. The phylogenetic originality of each set of sequences in a cell is the average QE-based (QE = quadratic entropy) originality of the sequences composing the unit itself (which, in turn, measures the average rarity of all the features belonging to a given haplotype; Pavoine et al., 2005). Specifically, to calculate phylogenetic originality we first built for each species an UPGMA dendrogram using the unique sequence set (i.e., with no duplication), and then calculated per-haplotype QE-based originality in the *ade4* (Dray and Dufour, 2007) R package. Per-cell per-species average originality was finally calculated as the average originality of the full set of sequences from a given cell. Genetic indices were calculated for all the cells with at least ten individuals for the same species, and for species occupying at least two cells (Goodall-Copestake et al., 2012).

## 2.3. Environmental parameters

We considered several variables describing environmental change between LGM and present: climate anomalies, temporal habitat (vegetation) stability and topographic heterogeneity. Additionally, we included human footprint to account for human-mediated effects on genetic diversity (Miraldo et al., 2016).

Climate anomalies measure change in the temperature and precipitation regimes between the LGM and present (i.e. the temporal climate gradient). Large anomalies are thought to have negative effects on the local persistence of single species, and hence on the overall biodiversity (Jansson, 2003). Current mean annual temperature and annual precipitation data were obtained at the 30 arcsec resolution from the CHELSA dataset (Karger et al., 2017), and reprojected to UTM zone 32 N (EPSG:32632) with 1 km resolution. Estimates of LGM temperature and precipitations were obtained from the whole set of general circulation models (GCMs) available on CHELSA (7 GCMs downscaled at 30 arcsec resolution: NCAR-CCSM4, MRI-CGCM3, MPI-ESM-P, MIROC-ESM, CESS-FGOALS-g2, IPSL-CM5A-LR, CNRM-CM5), and reprojected to UTM zone 32 N (EPSG:32632) with 1 km resolution. To reduce single model uncertainties, we built multi-model ensembles by averaging the projections from the seven GCMs for both LGM temperature and precipitation (Loarie et al., 2009; Sandel et al., 2011). Finally, climate anomalies were calculated as the ratio between the current and LGM temperature (in °K) or precipitation (in mm) from the ensembles. We used ratios instead of differences to obtain measures of change relative to its current value (i.e., change expressed as a proportion of the current

temperature or precipitation in a given cell).

Temporal habitat stability provides a measure of the time elapsed since each map cell acquired its current value (i.e. forested or not), under the assumption that the longer this time, the longer a given species should have been able to continuously occupy the cell, and hence, the higher the genetic diversity. To calculate temporal habitat stability, we relied on a millennial-scale (binary) model of suitability for temperate and warm-temperate forests at high resolution (Marta et al., 2013). We aggregated data from Marta and colleagues at 1 km resolution and identified the millennium in which each cell acquired its present-day habitat (forested or not). Consequently, the index ranged between 23 (completely stable during the period of interest - i.e. from the LGM to present days) and 1 (habitat conditions changed during the last millennium).

Topographic heterogeneity measures the spatial variability in topography and is usually expressed as the standard deviation in elevation within a given reference area. Large heterogeneity is usually thought to be positively related with biodiversity, as it potentially supports a larger number of potential niches and allows coping with environmental fluctuations through short-distance (altitudinal) movements (Thuiller et al., 2006; Ficetola et al., 2013). The index was calculated as the standard deviation in elevation within each Voronoi cell; elevation data were retrieved from the Shuttle Radar topographic mission (SRTM, 90 m resolution). For the sake of mapping model predictions, we also generated a map at 5 km resolution where each cell represents the topographic heterogeneity within a 25 km buffer (the radius needed to obtain areas comparable with the mean Voronoi cell size - 2124 km<sup>2</sup>).

Finally, to control for the effects of current human disturbance on ecosystems, we retrieved human footprint information for the year 2009 (Venter et al., 2016), which combines data on land use and transformation, population density, human access, and presence of infrastructures. Climate anomalies, temporal stability and human footprint were aggregated at the Voronoi cell level using average values for each variable. Maps of the environmental variables used are shown in Supplementary Fig. 2.

#### 2.4. Modelling genetic diversity

We used Bayesian Generalized Linear Mixed Models (GLMMs) to determine the effect of the palaeoclimatic, palaeoenvironmental and anthropic predictors on our measures of the phylogeographic structure of species, while taking into account species identity and the spatial structure of the dependent variables. For each species, cells with genetic data for <10 individuals were excluded from the analyses; all species with <2 remaining cells were also discarded. As nucleotide diversity and phylogenetic originality (the two dependent variables) are bounded on the closed set [0,1], we first removed fixed zeros and ones by taking  $y' = \frac{y \times (N-1) + 0.5}{N}$ , where  $N$  is the number of sequences for each species within a cell (Smithson and Verkuilen, 2006). This transformation has substantially no effect on estimated coefficients but allows fitting models with beta error structure and a logit link function ( $\ln \frac{p}{1-p}$  would return Infinity with both  $p = 0$  and  $p = 1$ ). All the independent variables were scaled to zero mean and unit variance before modelling to improve convergence of analyses and make coefficients easily comparable. Collinearity between pairs of predictors was weak to moderate ( $0.01 \leq |\text{Pearson's } r| \leq 0.50$ ), suggesting the lack of multicollinearity issues. We used Cleveland dotplots to detect outliers in the independent variables (Zuur et al., 2010); two outliers were identified for topographic heterogeneity and the corresponding samples were excluded from the analysis. Mixed models included species identity as random intercept, reflecting the assumption that each species may have a different baseline value for the dependent variables, but the effect of predictors on the dependent variable is expected to be the same across all species.

Spatial autocorrelation may occur when geographically close observations have similar values for a given variable. This is often the case

with genetic indices, as genetic similarity is expected to decrease with increasing distance (Gratton et al., 2017b). Spatial non-independence of residuals can result in biased estimates of the model parameters (Dormann et al., 2007). In order to account for spatial non-independence, we introduced a spatial random field assuming a Matérn covariance function (Lindgren et al., 2011), with penalized complexity priors on range ( $\rho_0$ : 100 km with  $P(\rho < \rho_0)$ : 0.1) and standard deviation ( $\sigma_0$ : 0.1 with  $P(\sigma > \sigma_0)$ : 0.1) of the random field. The Matérn covariance model was solved using stochastic partial differential equations (SPDEs); SPDEs were evaluated at the vertices of an irregular lattice (mesh) with non-intersecting triangular cells (Lindgren et al., 2011). The inner boundary of the mesh corresponded to the current boundary of the study area; to represent dispersal corridors potentially available in the past, the outer boundary was set to the LGM coastline. Maximum edge length was set to 40 km for the inner area of the mesh, and to 80 km for the outer one, so as to obtain a finer resolution within the study area. The resulting mesh was composed by 939 vertices and 1609 triangles. For each of the dependent variables, we mapped the linear predictions using the median coefficient estimates for the fixed effects and the spatial random field, considering both the overall trend of diversity for the two indices and the cumulative diversity obtained summing species-specific predictions, after clipping predictions with the species range. Each dependent variable was brought back on the original response scale using inverse logit.

To evaluate the fit of mixed models, we calculated the amount of variation of dependent variables explained by the fitted models as a measure of *pseudo-R*<sup>2</sup> using the *rsq* R package (Zhang, 2022). The per-cell number of individuals was highly variable across species. To assess the potential effect that differences in sample size may have on the correct estimation of the indices (Goodall-Copestake et al., 2012), and hence on the robustness of the analyses, we performed 100 random samplings of 10 individuals from each sampling unit (species × cell). Genetic indices were then calculated in each replicate and models re-run for each dependent variable. The estimates of models including all the observations were finally compared with the ones obtained from the resampled datasets. To assess the sensitivity of model outputs to the spatial scale of analysis, two additional runs of the models were performed using the 50- and 450-cells clustering. Additionally, as the processes generating patterns of genetic diversity might differ between strictly insular and mainland species, we repeated the analyses removing insular endemics (i.e., *E. montanus*, *E. platycephalus*, *D. montalentii*, *H. sarda* and *S. corsica*). We fitted Bayesian GLMMs using integrated nested Laplace approximation, as implemented in the *INLA* and *spdep* R packages (Rue et al., 2009; Lindgren and Rue, 2015). *INLA* allows reliably approximating posterior marginals in models with complex spatial structures, while considerably reducing computational load and solving convergence issues (Rue et al., 2009; Lindgren et al., 2011).

#### 2.5. Spatial prioritization of genetic diversity

To identify priority areas for the conservation of intraspecific genetic diversity, we first generated species-specific maps of nucleotide diversity and phylogenetic originality, based on median coefficient estimates for both fixed and spatial random effects; projections were limited to species ranges. Information on species ranges was retrieved from the published Areas of Habitat maps (Nania et al., 2022); for the species not considered by Nania and colleagues (*D. montalentii*, *P. bergeri*, *E. montanus*, *S. corsica*) we instead used the IUCN Red List maps. For some taxa (e.g. *R. dalmatina* or *I. alpestris apuana* and *inexpectata*), species ranges were clipped to represent single haplogroups, using available literature information (Supplementary Fig. 1 and Supplementary Table 1); range maps were rasterized to 1 km cell size (EPSG:32632) and then aggregated to 5 km using the maximum value. Species-specific maps of genetic diversity were then used as inputs to the spatial prioritization algorithm Zonation v.4.0 (Moilanen et al., 2014), as implemented in the *rzonation* R package (Morris, 2022). Zonation is a commonly used tool that allows ranking areas based on their

conservation values; the algorithm maximizes feature representation and the potential for conservation while limiting protection costs (Moilanen et al., 2005). The procedure starts by removing least valuable cells from the landscapes one by one, using minimization of marginal loss (i.e. under a “Basic core-area”, removing the cell with the minimum value for the most valuable occurrence over all the species) to select the cells of lower conservation value.

We used the “Basic core-area” cell removal rule, with uniform weights assigned to species and equal costs for all cells. To decrease computation times, we used the edge removal rule (i.e. select the cell with minimum marginal loss from the edges of the remaining landscape); still, we added 5000 randomly chosen edge points to allow for the detection of low-value patches surrounded by areas of high conservation priority. To evaluate the efficiency of protected areas in preserving genetic diversity, we i) downloaded the most up-to-date version of the current network of protected areas for the study area (UNEP-WCMC, IUCN, 2022); ii) rasterized polygons (5 km cell size), calculating the percent protected surface of each raster cell, and iii) used the obtained map as a mask layer (after setting to 0 all cells with protection  $\leq 0.5$  %). Zonation allows implementing mask layers with ordered values to identify areas of differential importance; during the removal process the algorithm proceeds from the lower values, so that the higher levels (in our case, the cells with high percentages of protection) are forced to be in the top fraction of the priority ranking. To evaluate the surrogacy value of species diversity, we performed an additional run of Zonation with the same setting described above, but using as features the ranges of the whole set of species present in the study area (45 species). We then used the obtained species-based prioritization as a mask layer; in this way, the most valuable areas for preserving species-diversity are removed later during the genetic-based prioritization. To evaluate the efficiency of the obtained prioritization, we replicated 1000 times the run on genetic diversity changing no setting but the removal rule (Random instead of “Basic core-area”), and comparing the relative efficiency of the solutions found. All the analyses were performed with R v.4.2.2 (R Core Team, 2022).

### 3. Results

We obtained sufficient data for 21 out of 45 amphibian species (10 urodeles and 11 anurans; 2596 individuals in 102 cells), representing 47 % of the whole amphibian diversity of the study area. The complete list of the species considered, with the criteria for the inclusion is provided

**Table 1**

Details on the markers, number of haplotypes, number of individuals and occupied Voronoi cells, as well as mean and standard deviation for the genetic indices. The values only refer to the species  $\times$  sites used in the analyses (77). Five species (*S. terdigitata*, *L. vulgaris*, *P. fuscus*, *B. balearicus* and *B. siculus*) were excluded as we never found  $>1$  cells with  $\geq 10$  individuals; in the same way, several sampling stations from each species were excluded when they did not contain  $>10$  individuals. mtDNA marker abbreviations refer to cytochrome-b (cytb), cytochrome-oxidase subunit 1 (cox1), displacement-loop (D-loop), NADH dehydrogenase subunit 2 and 4 (ND2 and ND4, respectively), 12S and 16S ribosomal RNA subunits (12S and 16S, respectively).

Species	mtDNA marker(s)	length (bp)	N haplotypes	N individuals	N cells	Nucleotide diversity		Originality	
						Mean	SD	Mean	SD
<i>S. salamandra</i>	cytb - cox1	1220	10	48	3	0.001	0.000	0.062	0.016
<i>S. corsica</i>	cytb - cox1	1292	36	165	4	0.003	0.002	0.022	0.018
<i>S. perspicillata</i>	12S - 16S - cytb	1344	10	80	4	0.001	0.000	0.053	0.018
<i>E. montanus</i>	cytb - cox1	1285	97	193	4	0.019	0.014	0.008	0.007
<i>E. platycephalus</i>	D-loop	491	14	95	3	0.003	0.002	0.069	0.030
<i>L. italicus</i>	ND2 - ND4	1897	34	162	10	0.003	0.007	0.022	0.012
<i>I. alpestris</i>	cytb - ND2	1277	12	136	10	0.000	0.001	0.068	0.061
<i>T. carnifex</i>	ND2 - ND4	1273	19	106	7	0.003	0.003	0.011	0.011
<i>D. montalentii</i>	12S - cytb	1383	14	64	2	0.004	0.005	0.058	0.024
<i>B. pachypus</i>	cytb	598	10	88	7	0.001	0.001	0.059	0.031
<i>B. bufo</i>	16S - cytb	1234	10	20	2	0.004	0.004	0.011	0.007
<i>H. sarda</i>	cytb - ND1	1229	45	122	5	0.004	0.003	0.012	0.012
<i>R. dalmatina</i>	cytb - cox1	1394	28	151	6	0.002	0.002	0.028	0.008
<i>R. italica</i>	cytb	615	17	135	6	0.002	0.003	0.044	0.052
<i>R. temporaria</i>	cox1	569	8	39	3	0.002	0.001	0.056	0.016
<i>P. bergeri</i>	cytb	620	4	30	3	0.001	0.001	0.064	0.069

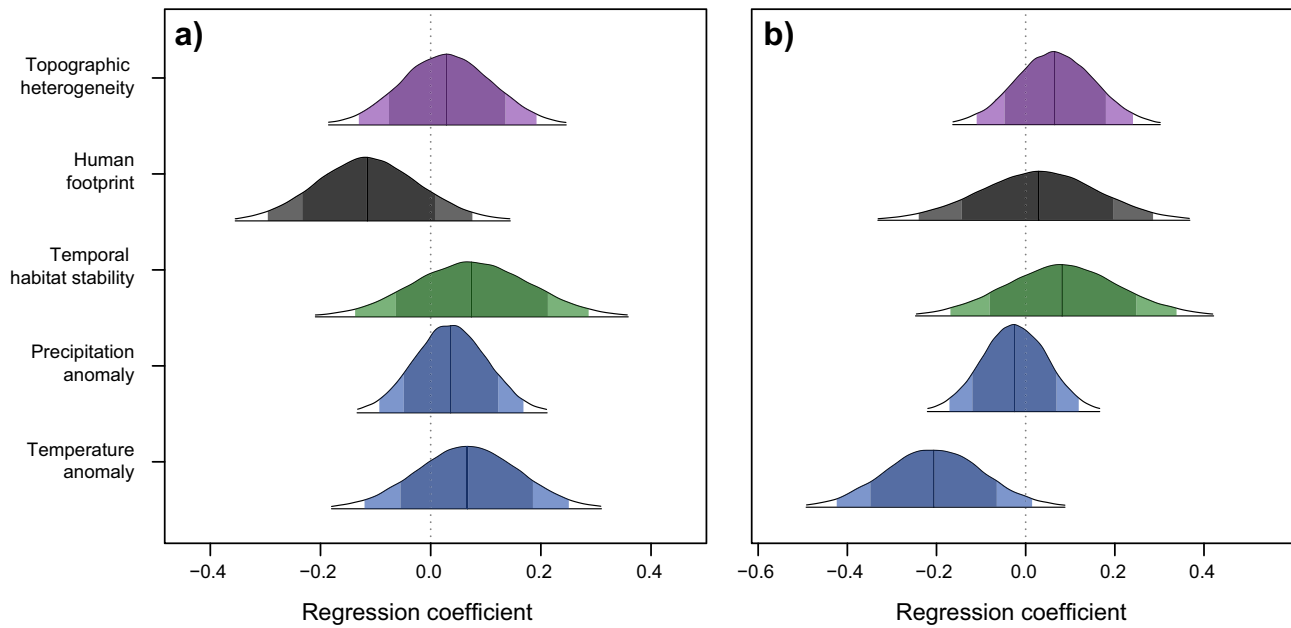
in Supplementary Table 1. For five species (*S. terdigitata*, *L. vulgaris*, *P. fuscus*, *B. balearicus* and *B. siculus*), the number of cells containing  $\geq 10$  individuals was lower than 2, thus these species were excluded from analyses. The final dataset included data from 16 species and 1634 individuals (per-species mean = 102.13; sd = 52.84) in 77 cells (per-species mean = 4.94; sd = 2.54; Table 1). Nucleotide diversity ranged between 0 and 0.036 (mean = 0.003; sd = 0.004), with the maximum value recorded in *E. montanus*. Per-cell average originality ranged between 0.002 and 0.226 (mean = 0.040; sd = 0.023), with the minimum value recorded in *T. carnifex* and the maximum in *I. alpestris*. Maps of sampling coverage for each species are reported in Supplementary Fig. 1.

#### 3.1. Modelling genetic diversity

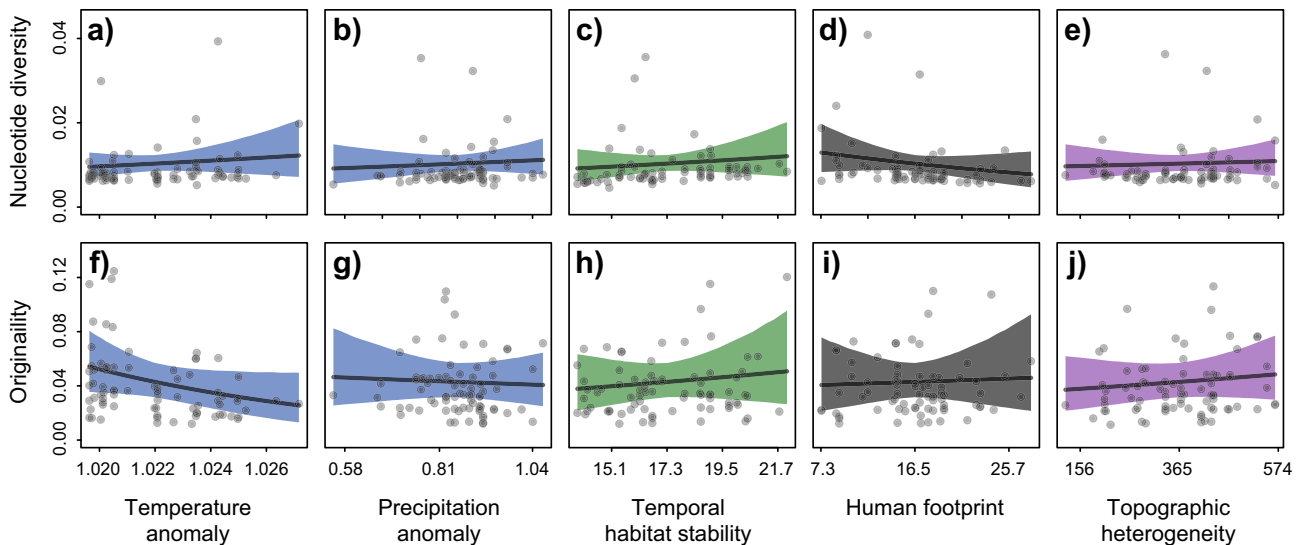
Environmental variables showed relationships with both nucleotide diversity and phylogenetic originality. For nucleotide diversity we only detected a weak relationship with human footprint, with confidence intervals marginally overlapping zero (Figs. 2a and 3a–e), and a model explaining a low amount of the observed variation ( $pseudo-R^2$ : 0.15). Conversely, the considered variables explained very well the spatial variation of phylogenetic originality ( $pseudo-R^2$ : 0.61). In particular, originality was negatively related to temperature anomaly, while relationships with the other predictors were weak (Figs. 2b and 3f–j).

Posterior estimates of the spatial effect had comparable peak values for the two independent variables (nucleotide diversity: 131 km; originality: 142 km) and range (i.e. the distance beyond which correlation is  $\leq 0.1$ ; Lindgren et al., 2011; nucleotide diversity: 575 km; originality: 626 km), but large 95 % confidence intervals (nucleotide diversity: 22–1624 km; originality: 25–2013 km). When we compared the median estimates of model coefficients with the distribution of the medians obtained after resampling, we found small differences (90 % absolute difference on coefficient estimates were  $\leq 0.02$  - Supplementary Fig. 3), suggesting limited model instability due to differences in sample size.

When we assessed the sensitivity of model outputs to the spatial scale of analysis (Supplementary Fig. 4), we found small differences. For nucleotide diversity, the  $pseudo-R^2$  values and coefficients were extremely similar; the relationship with human footprint was slightly stronger if a limited number of clusters was considered. Models were extremely similar also for originality. A smaller number of clusters yielded models with higher fit ( $pseudo-R^2$  values: 0.59 vs. 0.61 vs. 0.76 with  $k = 450, 150$  and 50 clusters respectively) and detected a stronger



**Fig. 2.** Posterior distribution of the regression coefficients for the models on nucleotide diversity (a) and phylogenetic originality (b). Median value for regression coefficients (vertical lines), and 80 % (dark fill), 90 % (light fill) and 99 % (outlines) credible intervals.



**Fig. 3.** Relationships between environmental predictors and nucleotide diversity (a-e) and phylogenetic originality (f-j). Effect of temperature anomaly (a and f), precipitation anomaly (b and g), temporal habitat stability (c and h), human footprint (d and i) and topographic heterogeneity (e and j). Conditional regression plots of Bayesian mixed models are reported; points represent partial residuals, shaded areas represent the 95 % confidence interval of the average estimates.

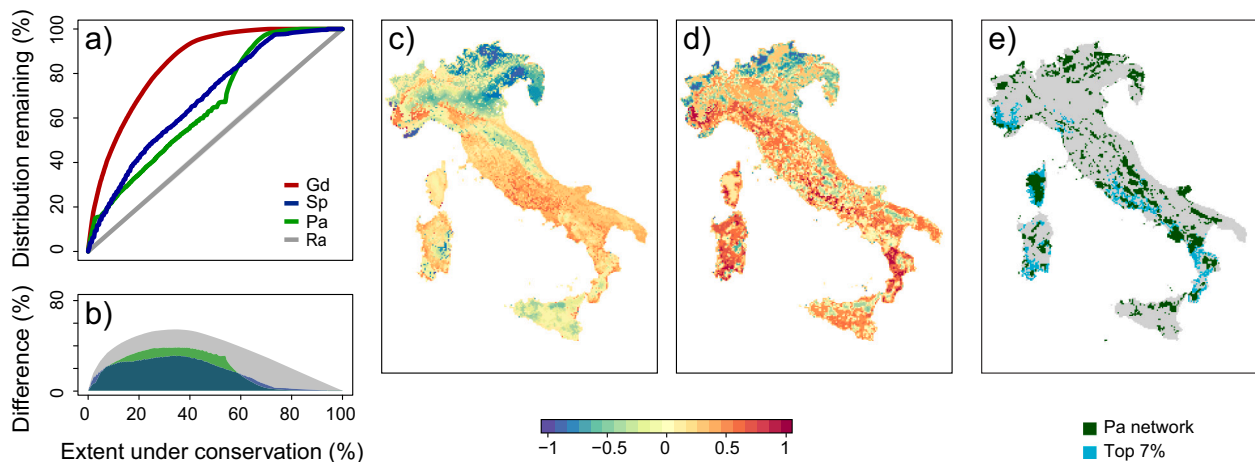
relationship between originality and temperature anomaly. The analysis with  $k = 50$  also detected a slightly significant and positive relationship between temporal habitat stability and originality. When we removed insular species, we obtained very consistent results (Supplementary Table 2).

The mapping of model predictions returned divergent spatial patterns. Nucleotide diversity was particularly high in the northern portions of the study area (i.e. the Alps) and Corsica (Supplementary Fig. 5a), while originality was highest along the coast of south-western Italy, the western coasts of central Italy, the main islands and the foothills of the western Alps (Supplementary Fig. 5b). When analysing the cumulative diversity maps (accounting for both genetic and species diversity; Supplementary Fig. 5d-e), some common patterns emerged between nucleotide diversity and originality, such as the high diversity in

the peninsular south, and the lower diversity in the main Italian islands and the northern areas.

### 3.2. Spatial prioritization of genetic diversity

Given the limited fit of the model for nucleotide diversity, spatial prioritization was focused on phylogenetic originality. Spatial solutions showed that a high conservation performance may be achieved already by a relatively low effort (Fig. 4). As an example, 50 % and 90 % protection targets can be achieved by protecting 10.8 and 35.8 % of the total study area, respectively (Fig. 4a - Gd scenario). Targeting originality while accounting for the current network of protected areas, or the species-based prioritization, always returned suboptimal solutions (Fig. 4a - Pa and Sp scenarios, Fig. 4b, Supplementary Fig. 6), with much



**Fig. 4.** Spatial prioritization of intraspecific genetic differentiation (phylogenetic originality). Lines in (a) show the relative efficiency obtained when aiming to protect genetic diversity (Gd) for each proportion of landscape under conservation, and the solutions found when informing the models on (i.e. masking with) the current distribution of protected areas (Pa) or the priority areas for species diversity (Sp). The solutions obtained with 1000 replicates under a random removal strategy are also reported (Ra). Polygons in (b) represent the percent difference between the target solution (Gd) and the Pa (green), Sp (blue) and Ra (grey) approaches. Maps in (c) and (d) report the differences in ranking between (c) genetic diversity and species diversity, and (d) genetic diversity and protected areas (UNEP-WCMC, IUCN, 2022) solutions. Positive values indicate areas with high priority for intraspecific genetic diversity not included in the Pa or Sp solutions. (e) Extent of the current network of protected areas and potential integrative areas allowing to reach the 30 % target (EC, 2020), as obtained including the top 7 % ranking in the Gd solution (after excluding cells with >50 % of protection). Map projection: UTM zone 32 N (EPSG: 32632); resolution = 5 km. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

higher percentages of area under protection needed to reach the same objectives. When mapping the differences in priority ranking between the different scenarios, some mismatches become evident (Fig. 4c and d). Some large protected areas are of low value for the conservation of originality (negative values - blue areas in Fig. 4d); similarly, areas with high priority for the conservation of species diversity host genetically poor amphibian communities (blue areas in Fig. 4c). All solutions performed significantly better than the random selection of conservation areas (Fig. 4a - Ra scenario, and Fig. 4b).

#### 4. Discussion

By focusing on proxies of palaeoenvironmental stability as predictors of phylogeographic structures, we found evidence that the current intraspecific spatial patterns of genetic diversity (in particular, the distribution of phylogenetic originality within species) represent, at least in part, the outcome of consistent responses to environmental changes across multiple amphibian species. This allowed producing spatial predictions of originality for the whole set of taxa included in the analysis, and identifying priority areas for the conservation of intraspecific genetic differentiation.

When evaluating the effects of environmental changes on the distribution of genetic diversity, long-term environmental stability (low temperature anomaly, high habitat stability) emerged as the main determinant of phylogenetic originality. This is consistent with the general hypothesis that highly divergent lineages tend to persist in climatically stable areas (Graham et al., 2006; Sandel et al., 2011). Conversely, relationships between nucleotide diversity and tested predictors (i.e. habitat stability, topographic heterogeneity and anomalies) were generally weak. The lack of relationships might occur because our analysis missed key drivers and/or because a consistent pattern may not emerge when attempting to cumulate information from different species, suggesting idiosyncratic responses to palaeoenvironmental features. Indeed, elevated nucleotide diversity can result from both persistence in stable areas and colonization of a previously uninhabited area from multiple refugia (secondary contact zones, often in areas that underwent important environmental changes, where mixing of highly divergent genetic lineages occurs; e.g. Petit et al., 2003; Havrdová et al., 2015). This might explain why the effects of environmental predictors

on nucleotide diversity did not fit our expectations.

Nucleotide diversity showed negative, albeit weak, relationship with human footprint, suggesting a negative impact of present-day human impacts on intraspecific diversity, partially agreeing with a global analysis of amphibian genetic diversity (Miraldo et al., 2016). Nevertheless, the occurrence of broad-scale relationships between genetic diversity and human impacts remains controversial (Millette et al., 2020). Despite a long history of human modifications has affected Mediterranean regions (Blondel and Aronson, 1999), the demographic decline and fragmentation imposed by human activities on the amphibian populations of the Italian peninsula were likely limited and local in extent until recent times (Ficetola et al., 2007). This is particularly true for the kind of modification that may be revealed by a quantification of current anthropic pressure, which will be more intense where industrial development and urbanization are highest, and not necessarily where anthropogenic modification of habitats was already intense in a more distant past (deforestation in Italy was widespread and intense since the Roman age; see Giguet-Covex et al., 2023). Therefore, although current human footprint may impact the overall nucleotide diversity of local populations (e.g. Schlaepfer et al., 2018), it is unlikely to have had relevant effects at the meso-scale (mean Voronoi cell size - 2124 km<sup>2</sup>) within a short timeframe (Wang, 2010). The limited fit of the model of nucleotide diversity was also confirmed by the mapping of model predictions, where the inner alpine areas unexpectedly showed the highest diversity (Supplementary Fig. 5a), probably because of the low human footprint on these environments, despite their limited suitability for amphibians (Supplementary Fig. 5d and c, respectively).

Conversely, the spatial distribution of phylogenetic originality remarkably matched refugial areas previously recognized based on comparative phylogeographic studies or the occurrence of micro-endemic species (Supplementary Fig. 5b). For instance, the area with very high originality in the Western Alps strikingly corresponds to the distribution range of *Salamandra lanzai* (Lanza's alpine salamander), a micro-endemic species that was not included in our analysis because of lack of data. Additionally, the foothills of the Western and Central Italian Alps stand out for their high originality, when compared with the inner valley and the Po Plain, and acted as refugia for many alpine plant species (Schönswetter et al., 2005). Similarly, the high originality areas in Southern Italy well match the range of the Southern spectacled

salamander, *Salamandrina terdigitata* (not included in our analysis because of the limited number of genotyped individuals), and the location of most plant refugia (Médail and Diadema, 2009). This stresses the importance of climatically stable areas for both the persistence of intraspecific diversity, and the occurrence of micro-endemic species of very high conservation priority (IUCN, 2022).

Obtaining broad-scale, generalizable information on the distribution of intraspecific diversity requires merging a large amount of data, that allow covering multiple species across a large number of sites (Miraldo et al., 2016; Millette et al., 2020). However, the integration of data collected by several authors for diverse aims can be challenging because of multiple processes, including complex spatial patterns, methodological differences among studies, and subjective choices performed during data integration (Gratton et al., 2017b). Failing to account complex spatial pattern is a major determinant of spurious macroecological relationships (Dormann, 2007; Gratton et al., 2017b). Nevertheless, our analyses were based on spatially-explicit methods that are robust to the issues related to spatial autocorrelation (Beale et al., 2010). Furthermore, alternative methodological choices (e.g. grid size, minimum sample size per cell, exclusion of insular species) yielded highly consistent results, suggesting that our conclusions are robust.

The increasing availability of genome-wide data, coupled with robust approaches to comparative phylogeography, will provide opportunities for more and more effective description and conservation of genetic diversity in the near future (Andrello et al., 2022; Formenti et al., 2022). Even though neutral markers may fail to capture adaptive genetic diversity (McKay and Latta, 2002), mtDNA-based estimates of genetic diversity and phylogeographic structure have been shown to relate to the extinction probability of species, and proved to be useful tools in assessing conservation status and priorities (Dufresnes and Perrin, 2015; Carvalho et al., 2017). In fact, some authors suggested that mtDNA variation does not reflect expected variation in effective population size across species (e.g., Bazin et al., 2006). However, this effect seems to be essentially due to variation in generation time (Allio et al., 2017), and the general correlation between demographic and evolutionary processes and mtDNA variation at the intraspecific level has been largely confirmed (see, e.g., Piganeau and Eyre-Walker, 2009; de Kort et al., 2021). Using neutral genetic data, we targeted intraspecific originality to identify conservation strategies maximizing representation of amphibian intraspecific genetic diversity. As expected, the prioritization scheme adopted here showed that all scenarios (i.e. Gd, Sp and Pa) performed significantly better than the random selection of conservation areas (Ra) in preserving intraspecific diversity (Fig. 4a and b). However, solutions found when targeting intraspecific originality differed significantly when accounting for the current distribution of protected areas, or for species-based prioritizations (Fig. 4). For instance, the 50 % conservation target might easily be achieved by protecting just 10.8 % of study area (Fig. 4a - Gd scenario), but the conservation effort (measured as the percentage of the study area under protection) should be 2.4 times larger than for Gd when driving cell selection using species-based prioritizations (Fig. 4a - Sp scenario and Fig. 4b). These results are in line with previous studies showing that species-based prioritizations have limited surrogacy values when targeting intraspecific genetic diversity (Carvalho et al., 2017). When we evaluated the efficiency of the current network of protected areas in preserving intraspecific genetic diversity, we found similar results, with required conservation effort even larger than in the Sp scenario (Fig. 4a - Pa scenario and Fig. 4b, 50 % target with 33.9 % of area under protection). When only accounting for areas under strict or intermediate protection (IUCN categories Ia, Ib and II, only covering 5 % of the study area; Supplementary Fig. 7), the conservation effort needed to reach the 50 % target was lower and close to the Gd scenario (15.1 %), mainly indicating the looser constraints imposed to Zonation by the mask layer during the selection of priority areas.

Protected areas currently cover >73,000 km<sup>2</sup> of emerged lands within the study area (23.0 %); still, from a genetic perspective, many

areas hosting populations with unique genetic features lack protection. The mismatches between the distribution of genetic diversity and currently protected areas are evident when looking at the difference between the two solutions (Fig. 4d): in fact, most of large protected areas are of low conservation values for amphibians intraspecific diversity. This result was not unexpected, as protected areas in Italy (as elsewhere) have been established in the past without accounting for genetic diversity. Moreover, protected areas in Italy and other regions of the world often focus on high-elevation ecosystems, where human impact is generally lowest and charismatic species occur (e.g. large carnivores), but key biodiversity components, including many micro-endemic species and lineages, are poorly represented (Martínez et al., 2006).

## 5. Conservation implications

The EU Biodiversity Strategy for 2030 sets the target of protecting 30 % of land by 2030 (EC, 2020). Around 23 % of study area is currently under protection; further adding the top 7 % areas in the Gd solution might allow reaching this objective by targeting biodiversity components that are rarely considered in conservation planning (Fig. 4e). In some cases, these areas represent large territories that currently receive very limited protection. For instance, in the western Alps protected areas mostly occur at high elevations while the foothills currently receive little protection. This area is a hotspot of genetic diversity, but is currently threatened by multiple factors including the spread of invasive species (e.g. the American crayfish *Procambarus clarkii*), agricultural intensification and spread of pathogens, which are determining the decline of many amphibians living there (Giovannini et al., 2014; Andreone, 2015; Lo Parrino et al., 2020; Sindaco et al., *in press*). A different situation occurs in lowland areas of Central and Southern Italy (Latium, Campania and Calabria), where several protected areas occur, but are often scattered, small and interspersed within human-dominated areas (Battisti and Gippoliti, 2004). In this case, management strategies should favour the integration between natural and semi-natural areas within complex landscapes that enable population persistence and enhance connectivity and gene flow (Kremen and Merenlender, 2018). These landscapes should be actively managed to mitigate the impacts of invasive species and climate change (e.g. droughts), and this can be facilitated by traditional agricultural practices promoting the persistence of small landscape elements (e.g. semi-artificial ponds, hedgerows, stone walls) that are the microhabitat of many amphibians (Canessa et al., 2013; Romano et al., 2014; Guerra and Aráoz, 2015; Valdez et al., 2021).

Priority areas identified in our analyses might differ when implementing datasets based on different markers, and/or taxonomic groups with different characteristics (e.g., dispersal ability or generation length), still amphibians stand out as one of the most endangered animal taxa (Cox et al., 2022), thus their specificities should be prioritized during conservation planning. We also highlight that the conservation of areas identified by spatial prioritizations does not necessarily mean establishing new strictly protected areas; in fact, several other actions can be taken, from the restoration of natural habitats to allow the persistence of target species and/or enhance inter-population connectivity, to the control of invasive alien species (predators, competitors, vector of pathogens). Whatever the approach, our results stress the importance for decision-makers to take into account prioritization analyses based on both species- and intraspecific genetic diversity for the long-term conservation of biodiversity.

## CRedit authorship contribution statement

**Silvio Marta:** Conceptualization, Methodology, Software, Formal analysis, Visualization, Writing; **Daniele Druella:** Conceptualization, Investigation, Writing - Review & Editing; **Lorenzo Talarico:** Investigation, Writing - Review & Editing; **Gentile Francesco Ficetola:** Conceptualization, Supervision, Funding acquisition, Methodology, Writing - Review & Editing; **Paolo Gratton:** Conceptualization,



Supervision, Methodology, Writing - Review & Editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocon.2023.110179>.

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