



Present and past climatic effects on the current distribution and genetic diversity of the Iberian spadefoot toad (*Pelobates cultripipes*): an integrative approach

Jorge Gutiérrez-Rodríguez¹, A. Márcia Barbosa² and Íñigo Martínez-Solano^{3,4,*}

¹Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid, Spain, ²Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO/InBIO) – Universidade de Évora, 7004-516 Évora, Portugal, ³CIBIO/InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, 4485-661 Vairão, Portugal, ⁴Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM), Ronda de Toledo, s/n, 13071 Ciudad Real, Spain

ABSTRACT

Aim Predicting species responses to global change is one of the most pressing issues in conservation biogeography. A key part of the problem is understanding how organisms have reacted to climatic changes in the past. Here, we use species distribution modelling to infer the effects of climate changes since the Last Interglacial (LIG, c. 130,000 yr BP) on patterns of genetic structure and diversity in the western spadefoot toad (*Pelobates cultripipes*) in combination with spatially explicit phylogeographical analyses.

Location Iberian Peninsula and mainland France.

Methods Five hundred and twenty-four individuals from 54 populations across the species range were sampled to document patterns of genetic diversity and infer their evolutionary history based on data from mtDNA and 14 polymorphic microsatellites. Generalized linear models based on distribution data were used to infer climatic favourability for the species in the present and in palaeoclimatic simulations for the LIG, the mid-Holocene and the Last Glacial Maximum (LGM).

Results Estimates of genetic diversity show a decreasing trend from south to north, suggesting persistence of high historical population sizes in the southern Iberian Peninsula. Species distribution models show differences in climatic favourability through time, with significant correlations between historically stable favourable areas and current patterns of genetic diversity. These results are corroborated by Bayesian skyline plots and continuous diffusion phylogeographical analyses.

Main conclusions The results indicate the presence of southern refugia, with moderate recent expansions at the northern end of the species' range. Toads at the northern range margin exhibit the lowest genetic diversity and occupy areas of high past climate variability, classified as marginal in terms of favourability, rendering these populations most vulnerable to climate-mediated changes in the long term.

Keywords

amphibians, Holocene, microsatellites, mtDNA, phylogeography, Pleistocene, species distribution models

*Correspondence and current address: Íñigo Martínez-Solano, Department of Wetland Ecology, Doñana Biological Station, CSIC, c/Américo Vespucio, s/n, 41092 Seville, Spain. E-mail: inigomsolano@gmail.com

INTRODUCTION

One of the most pressing issues in conservation biogeography is predicting species responses to global change. Given projected scenarios of climate warming, the survival of many taxa will be determined by plastic responses, genetic

adaptation or range shifts tracking favourable conditions (Williams *et al.*, 2008; Duputié *et al.*, 2012). Initial approaches to this complex problem have involved characterization of the fundamental niche of species and projection under a variety of future scenarios (Araújo *et al.*, 2006). These attempts have been fundamentally based on correlative

models of individual species (Thuiller *et al.*, 2008), which have several limitations, such as coarse spatial resolution (Moritz & Agudo, 2013) or static niche stability (Gavin *et al.*, 2014). Thus, more realistic models need to take into account other factors, such as the evolutionary history of the organisms under study (Gavin *et al.*, 2014). Critical information can be obtained using the past to predict the future, that is, by understanding how organisms have reacted to climatic changes in the past in order to predict how they are likely to be affected by changes in the future. For example, the combination of species distribution modelling and spatially explicit phylogeographical analyses is emerging as a powerful means to predict how species will react to future climatic changes (Dawson *et al.*, 2011; Schmidt *et al.*, 2014).

The Quaternary Period, encompassing the past 2.6 Myr, is characterized by a succession of marked, well-studied climatic changes, with 'cold' and 'warm' periods repeated in cyclic intervals. These oscillations have affected patterns of genetic diversity across species through the processes of demographic contraction, expansion and extinction, ultimately shaping current species ranges (Hewitt, 1996, 2004; Provan & Bennett, 2008). From the perspective of several independent disciplines, scientists have documented range shifts in the flora and fauna of this period based on the fossil record, palaeoclimatic reconstructions, and patterns of species distributions (Devender *et al.*, 1976; Hoffmann, 1981; Ashworth, 1996; Sket, 1997; Morales Pérez & Serra, 2009). More recently, disciplines like phylogeography and tools like species distribution models (SDMs) have been applied to reconstruct historical processes. Phylogeography identifies refugial areas based on the presence of exclusive lineages or patterns of higher genetic diversity (Médail & Diadema, 2009; Keppel *et al.*, 2012). Refugial areas are important in predicting species responses to climate change, because they represent the fractions of the distribution range where species have persisted for longer in the face of geological and climatic events. This approach is, however, prone to some bias due to incomplete sampling and/or the random extinction of lineages (Weisrock & Janzen, 2000; Cusimano *et al.*, 2012). SDMs allow the prediction of the extent of past distribution ranges based on palaeoclimatic reconstructions [with simulations available back to the Last Interglacial (LIG), up to *c.* 120–140 ka]. The combination of phylogeographical data and SDMs has been shown to produce robust inferences about past demographic changes in a variety of taxa (Fitzte *et al.*, 2011; Todisco *et al.*, 2012; Wilson & Pitts, 2012; Hegna *et al.*, 2015; Martínez-Freiría *et al.*, 2015; Tsuda *et al.*, 2015). Understanding how these demographic changes have affected patterns of population structure and genetic diversity in relation with past climatic changes is key to predicting how future changes will affect population viability.

Southern European peninsulas have been long studied as textbook examples of glacial refugia that have allowed the survival of species and well-differentiated intraspecific lineages across a variety of taxa. However, many phylogeographical studies in this region have focused on the role of

these refugia in preserving deeper phylogenetic history, with many taxa originating in the Pliocene-Miocene and surviving as more or less isolated population groups until the present (Engbrecht *et al.*, 2000; Martínez-Solano *et al.*, 2006; Velo-Antón *et al.*, 2012; Recuero *et al.*, 2014). Comparative approaches have also emphasized the existence of 'refugia within refugia', with several areas functioning as allopatric refugia within each major peninsula (Gómez & Lunt, 2007). These studies have focused mainly on the possibility of incipient or cryptic speciation. However, within each major peninsula, there are examples of taxa that have evolved *in situ* for millions of years without showing any genetic signature of deep phylogeographical structure. These species have also been affected by geological and climatic changes throughout the Pliocene and Pleistocene, and thus their study can provide clues about how species cope with such wide-range environmental perturbations.

Here, we use an integrative approach, including population genetics, phylogeographical analyses and modelling of current and past [Holocene, Last Glacial Maximum (LGM) and LIG] climatic favourability, to quantify changes in favourability through time and analyse the relationship between climatic stability and current patterns of genetic diversity and structure. As a model species, we focused on the Iberian spadefoot toad, *Pelobates cultripes* (Cuvier, 1829). This species belongs to the family Pelobatidae (order Anura) and is a typical inhabitant of Mediterranean habitats across the Iberian Peninsula (IP), extending along the Mediterranean coast of France, with some disjunct, isolated populations in the French Atlantic coast (Duguet & Melki, 2003; García-París *et al.*, 2004; Loureiro *et al.*, 2008). Its range is mostly continuous in areas with sandy siliceous substrate, dominant in the western half of the IP, and fragmented in calcareous areas in the eastern IP (García-París *et al.*, 2004). The species breeds mostly in long-hydroperiod seasonal ponds, which allow the completion of its relatively long larval development (up to 6 months) (Talavera & Sanchiz, 1987; Tejedo, 1993; Buchholz & Hayes, 2002; Díaz-Paniagua *et al.*, 2005). This dependence makes it vulnerable to changes in future water availability under global climate change (Corn, 2005). Similarly, past hydroclimatic variation in the Holocene (Magny *et al.*, 2013) may have affected this species' historical demography and population structure through changes in seasonal rainfall patterns limiting water availability. Currently, the species is listed as Near Threatened by the IUCN (Beja *et al.*, 2009), as its populations are declining range-wide due to habitat loss and the negative impact of invasive species (Tejedo & Reques, 2002).

Previous phylogeographical studies based on limited data and sampling have shown low mitochondrial diversity across this species' range (Crottini *et al.*, 2010; Fitó *et al.*, 2011). These authors have hypothesized a range reduction during the Pleistocene glaciations to explain this pattern, with glacial refugia inferred to have been located in the southern IP. In that case, we would expect to detect a genetic signature of demographic decline, followed by recent population growth,

a pattern of increasing genetic diversity along a south–north axis, and little genetic structure across the species range. Corroboration of this hypothesis would indicate that the species can recover from climatically induced demographic declines, tracking newly available habitats when environmental conditions ameliorate. Here we test this hypothesis, document range-wide patterns of genetic diversity and structure based on a geographically comprehensive multilocus data set, reconstruct historical demography and describe changes in environmental favourability through time, in order to infer how past climatic changes have affected patterns of genetic structure and diversity in this species.

MATERIALS AND METHODS

Sampling and DNA extraction

Tissue samples from 524 individuals of *P. cultripipes* were collected from 54 localities covering all of the species' range (Fig. 1b, see Table S1 in Supporting Information). Samples were obtained from tail tips of larvae and toes of adults, preserved in absolute ethanol, and subsequently kept in a freezer at -20°C . Genomic DNA was extracted using NucleoSpin Tissue-Kits (Macherey-Nagel, Düren, Germany) and later stored at 4°C .

Mitochondrial DNA

A fragment of mitochondrial DNA (872 bp) including the NADH dehydrogenase subunit 4 gene and flanking tRNAs was amplified and sequenced using primers ND4 and Leu (Arévalo *et al.*, 1994). A total of 197 individuals from 54 populations were sequenced. This subsampling is justified by the low level of genetic divergence in mtDNA, including cases in which all individuals sampled in a locality share the same mtDNA haplotype (i.e. nucleotide diversity = 0), which makes sample aggregation a good solution for representation purposes. PCR conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30s, annealing at 53°C for 30 s and extension at 72°C for 1.30 min, with a final extension step at 72°C for 10 min. Each reaction was performed in a final volume of 25 μL containing 0.2 μM of each primer, 0.4 mM dNTP, 1 mM MgCl_2 and 2.5 μL of 5 \times GoTaq Flexi buffer (PROMEGA, Madison, WI, USA), 0.5 U GoTaq Flexi DNA polymerase (PROMEGA) and 25 ng of template DNA. PCR amplifications were run on 1% agarose gels to check for successful amplification and possible contaminations using negative controls. PCR products were purified using sodium acetate and ethanol, and then resuspended in 20 μL of ultrapure water prior to sequencing in an automatic sequencer (ABI PRISM 3730, Applied Biosystems, Foster City, CA, USA).

Sequences of mtDNA were edited with SEQUENCHER 4.10.1 (GeneCodes Corp., Ann Arbor, MI, USA) and aligned by eye with MESQUITE 3.03 (Maddison & Maddison, 2015). DNASP 5.10 (Librado & Rozas, 2009) was used to estimate

haplotype and nucleotide diversity (π) for each population. Localities within 25 km of each other were grouped together to estimate nucleotide diversity using the software SPADS 1.0 (Dellicour & Mardulyn, 2014). These values were subsequently interpolated in a map, using the universal kriging function and a spherical semivariogram model in ArcGIS 10 (ESRI Inc., Redlands, CA, USA).

A haplotype network was generated with HAPLOVIEWER (Available from: <http://www.cibiv.at/~greg/haploviewer>). We also tested for molecular signatures of demographic expansion by calculating mismatch distributions, and several statistics: Fu's F_s (Fu, 1997), Tajima's D (Tajima, 1989) and Ramos-Onsins and Rozas' R_2 (Ramos-Onsins & Rozas, 2002). Significance of these statistics was assessed through coalescent simulations with 10,000 replicates using DNASP 5.10 (Librado & Rozas, 2009). Mean genetic distances (Kimura-2 parameter, K2p-corrected) between and within lineages were calculated with MEGA 6.06 (Tamura *et al.*, 2013).

We used phylogeographical continuous diffusion models as implemented in BEAST 1.8 (Drummond *et al.*, 2012) to reconstruct range dynamics through time in *P. cultripipes* based on mtDNA sequences. This analysis simultaneously reconstructs gene trees, ancestral population sizes, and the geographical ranges of inferred nodes, which can be visualized through time provided appropriate calibrations or substitution rates are supplied. As no reliable estimates of substitution rates or calibrations based on fossils or geological events are available for *Pelobates*, we estimated a substitution rate for the mtDNA fragment used (ND4+ adjacent tRNAs) in a family-level phylogenetic analysis including representatives of the families Scaphiopodidae (*Scaphiopus couchii* Baird, 1854, accession: JX564894, and *Spea bombifrons* (Cope, 1863): JX564896, Zhang *et al.*, 2013), Pelodytidae (*Pelodytes caucasicus* Boulenger, 1896: KP166769, *P. ibericus* Sánchez Herráiz, Barbadillo-Escrivá, Machordom & Sanchiz, 2000: KP166779, and *P. punctatus* (Daudin, 1802): KP166116, Díaz-Rodríguez *et al.*, 2015), Megophryidae (*Leptobrachium montanum* Fischer, 1885: EU180967, *Scutigera cf. mammatum* (Günther, 1896): EU180974, and *Oreolalax jingdongensis* Ma, Yang & Li, 1983: EU180972, Rao & Wilkinson, 2008), and Pelobatidae (including one sequence of *P. cultripipes* – voucher PC935-, and another one of its sister taxon *P. varaldii* Pasteur & Bons, 1959 – voucher IMS1148-). Based on the estimates of Roelants *et al.* (2007) (153.7 Myr for the split between *Pelobates* and *Spea*) and Wiens (2007) (127 Myr for the same split), we specified a prior for the age of the root of the tree with a lognormal distribution (offset: 125 Myr; log(mean): 1.5; log(SD): 1.0), encompassing values between 125 and 157 Myr. We selected the optimal nucleotide substitution model based on jMODELTEST 2.5 (Darriba *et al.*, 2012) results, choosing the best-ranked model available in BEAST, in this case HKY+G (3rd ranked model based on the Bayesian information criterion). Analyses were run under a strict molecular clock and using the Yule speciation model as the coalescent prior. All families were constrained to be monophyletic in the analyses. Convergence was assessed through inspection of the logfile in TRACER 1.6

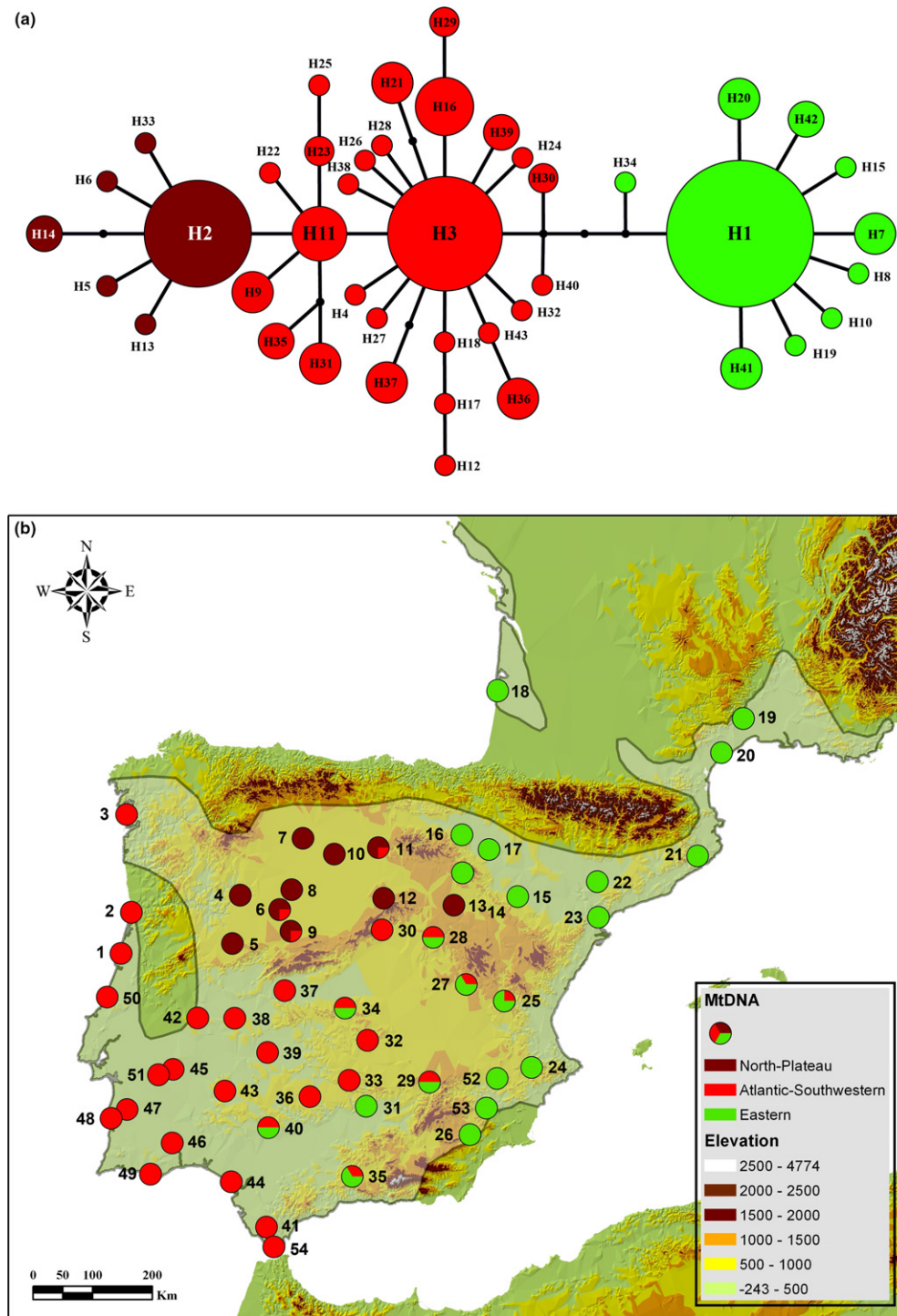


Figure 1 (a) Haplotype network of mtDNA sequences in *P. cultripes*. Three main haplogroups were identified, each of them including one haplotype found in high frequency and several closely related haplotypes. (b) Geographical distribution of the three major haplogroups identified in the analyses, including their relative frequencies when co-occurring in the same locality.

(Rambaut *et al.*, 2014) and a maximum clade credibility tree (MCCT) was reconstructed with TREEANNOTATOR (distributed as part of the BEAST package).

For the continuous diffusion analysis, we used all 197 *P. cultripes* sequences and selected the optimal partitioning strategy and corresponding nucleotide substitution models

with PARTITIONFINDER 1.1.1 (Lanfear *et al.*, 2012). Geographical coordinates for each sequence were included, with a jitter module generating random coordinates (window size: 0.1) for individuals from the exact same location. Analyses were run for 300 hundred million generations under a strict molecular clock (with the clock rate specified based on the

results of the previous analysis, with a normal distribution with mean = 0.005 substitutions/site/million years, and a SD of 0.001, and using the Bayesian skyline plot (linear, number of groups = 5) as the coalescent prior. The Cauchy model was used to describe the geographical diffusion process through time across branches of the inferred gene tree (Lemey *et al.*, 2010). Convergence was assessed through inspection of the log file in TRACER and a MCCT was reconstructed with TREEANNOTATOR. This tree was used to generate time-calibrated reconstructions of the diffusion process using the modules 'Continuous Tree' and 'Time Slicer' in SPREAD 1.0.7 (Bielejec *et al.*, 2011). BEAST analyses were run in the Cipres Science Gateway (Miller *et al.*, 2010).

Microsatellites (SSR)

A set of 16 recently characterized microsatellite markers (Gutiérrez-Rodríguez & Martínez-Solano, 2013) were used to genotype 517 samples from 49 localities (Table S1). Microsatellites were amplified with PCR conditions described in Gutiérrez-Rodríguez & Martínez-Solano (2013). Amplified fragments were run in an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). Alleles were visualized and scored manually using GENEMAPPER 4.0 (Applied Biosystems).

We used MICROCHECKER 2.2.3 (Van Oosterhout *et al.*, 2004) to test for null alleles, stuttering and large allele dropout in the data set, using a 99% confidence interval and 1000 randomizations. Additionally, we performed a genetic parentage analysis with the software COLONY 2.0.5.1 (Jones & Wang, 2010) to remove full-siblings from the sample collected at each locality, to minimize possible biases (Goldberg & Waits, 2010). We assumed a mating system with monogamous females and polygamous males, acknowledging the possibility of multiple mating by males, and implemented the full-likelihood method of Wang (2004), with 10 independent runs and updated allele frequencies. Evidence of linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were tested using GENEPOP on the web (<http://genepop.curtin.edu.au/>; Raymond & Rousset, 1995). Significance values for multiple tests were adjusted applying a sequential Bonferroni correction (Rice, 1989).

The number of alleles (N_a), observed (H_o) and expected heterozygosity (H_e), and F_{is} (inbreeding coefficient) for each population were estimated in GENALEX 6.5b5 (Peakall & Smouse, 2012). Private alleles (P) and allelic richness (A_r) were calculated with ADZE (Szpiech *et al.*, 2008) for each population and mapped using ARCINFO (ESRI, Redlands, CA, USA) with the interpolation universal kriging function and a spherical semivariogram model. ADZE uses a rarefaction approach to allow comparison of populations with different sample sizes. A principal components analysis was carried out using PAST 3 (Hammer *et al.*, 2001), to summarize the variation of nucleotide diversity, observed heterozygosity, private alleles and allelic richness. The first principal component (PC1) was then mapped following the steps above.

We used two Bayesian clustering algorithms to describe population structure based on microsatellite data: STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) and TESS 2.3.1 (Chen *et al.*, 2007). STRUCTURE assigns each multilocus genotype to different clusters, minimizing departures from Hardy–Weinberg and LD, while TESS includes a spatially explicit prior distribution based on a hierarchical mixture model (Chen *et al.*, 2007; Durand *et al.*, 2009). In STRUCTURE, analyses were run assuming correlated allele frequencies between populations (Falush *et al.*, 2003) and the admixture model. Ten runs were conducted for each value of K , testing values between 1 and 10, with each run consisting of a burn-in of 500,000 generations followed by 1,000,000 MCMC iterations. The best value of K was selected based on the log probability of the data ($\ln(\text{Pr}(X^K))$) and the ΔK method (Evanno *et al.*, 2005) using STRUCTURE HARVESTER 0.6.94 (Earl & vonHoldt, 2012), although other K values showing consistent, biologically significant results were also considered for further discussion. CLUMPP 1.1 (Jakobsson & Rosenberg, 2007) was used to summarize results from different runs for the best values of K and graphs of assignment probabilities were produced with DISTRUCT 1.1 (Rosenberg, 2004). Spatially explicit cluster analyses were performed with TESS. The analyses assumed the conditional autoregressive Gaussian model of admixture with linear trend surface (Durand *et al.*, 2009), and set the admixture parameter to 1 and the interaction parameter (α) to 0.6 as starting values. Ten independent runs were carried out with K values between 2 and 10, each run with a burn-in of 50,000 iterations followed by 250,000 additional iterations.

Distribution modelling

An environmental favourability model (Real *et al.*, 2006) was built with the FUZZYSIM package (Barbosa, 2015) under R 3.1 (R Core Team, 2014), based on presence/absence data on the UTM 10 × 10 km cells of mainland Portugal (Loureiro *et al.*, 2008), Spain (MAGRAMA, 2014) and France (MNHN, 2014), and on the 19 bioclimatic variables available in the WorldClim database (Hijmans *et al.*, 2005), which had an appropriate temporal and spatial scale for our study. Variables were pre-selected based on their direct (bivariate) relationship with the species' occurrence, using the false discovery rate (Benjamini & Hochberg, 1995). They were then included in a multivariate model, with a forward-stepwise procedure based on Akaike's information criterion. Any non-significant variables left in the model were further removed (Crawley, 2007).

The model was evaluated using several performance measures, of both discrimination and calibration (Jiménez-Valverde *et al.*, 2013), with the MODEVA R package (Barbosa *et al.*, 2013). It was then applied to the climatic conditions projected for the LIG, the LGM and the mid-Holocene, the latter two including the three hypothetical simulations (CCSM4, MIROC-ESM and MPI-ESM-P) that were available based on the same current climate values used for model building.

We used fuzzy logic (Barbosa & Real, 2012) to calculate the intersection between LIG and LGM favourability, which

represents the maintenance of relatively favourable conditions throughout the glaciations. We then correlated these intersected favourability values with the current pattern of genetic diversity within the species' distribution range. We also quantified and represented the general changes in favourability between past simulations and current climate, using the fuzzy range change measures (including overall proportional gain, loss and stability) available in the FUZZY-SIM package.

RESULTS

Mitochondrial analyses

The final mitochondrial data set included sequences of 872 bp for 197 specimens from 54 localities (GenBank accession numbers: KU670432-KU670630). The sequences contained 45 variable and 28 parsimony-informative sites and were collapsed into 43 unique haplotypes (Table S1). Three haplotypes (H1, H2 and H3) showed relatively high frequency, while the remaining ones were present at relatively low frequency (Fig. 1a). The haplotype network shows two main haplogroups (Eastern and Western), corresponding to populations in the eastern and western Iberian Peninsula. Based on the geographical spread of haplotypes, the western haplogroup can be further subdivided into a North-Plateau and an Atlantic Southwestern haplogroup (Fig. 1b). Mean (K2-p) genetic distance between the two main haplogroups was 0.4% (SD = 0.2), and intra-group genetic distances were 0.1% (SD = 0.0) and 0.3% (SD = 0.1) for the Eastern and Western haplogroups respectively. Neutrality tests (Fu's F_s , Tajima's D and R_2) and Mismatch distributions support scenarios of historical demographic expansion for the species and the two main haplogroups, with all test statistics yielding significant values (see Table 1 and Fig. S1 in Supporting Information). Nucleotide diversity ranged from 0 to 0.00382 (Table S1), with a tendency to increase from south to north (see Fig. S2a).

BEAST results produced largely unresolved trees, although some nodes were strongly supported (Bayesian posterior probabilities, BPPs > 0.90). These included haplogroup East (BPP: 0.99), as well as four clades including haplotypes from haplogroup West (which was not supported as a major clade). Three of these clades had relatively restricted ranges (pops. 41, 43 and 44; pops. 27, 29 and 30; and pops. 32 and 39), but the fourth clade included populations in the North Plateau (BPP: 1.0), with localities 4–13 (see Fig. S3). Continuous diffusion analyses produced good results, with ESSs values > 200 for all parameters. The root of the tree was inferred to date back to the Pleistocene, *c.* 600 ka. An ancestral area was inferred to be located between the basins of the Tajo and Guadiana rivers, with subsequent expansions to the south, east and north. The species would have been present in most of its current range in the LIG, with the exception of coastal Atlantic Galician and French populations, which would have originated in the last 20 ka (Fig. 2). The Bayesian Skyline Plot showed a sustained population increase in

Table 1 Results of tests for demographic expansion in *P. cultripes*, including the Western (W), Eastern (E) and North Plateau (NP) haplogroups. Values of Fu's F_s , Tajima's D and R_2 are provided, including their associated P values. All results are significant, indicating demographic expansions.

	Fu's F_s	P	Tajima's D	P	R_2	P
Pc	-35.3579	0.000	-1.9018	0.006	0.0291	0.016
W	-27.0992	0.000	-1.9819	0.004	0.0300	0.007
E	-8.0847	0.000	-1.9453	0.000	0.0383	0.033
NP	-3.3125	0.009	-1.6993	0.011	0.0604	0.047

the last 250 ka, with a recent decline *c.* 25 ka and subsequent recovery *c.* 5 ka (see Fig. S1).

Microsatellite analyses

Genotypes were amplified with a success rate of >99%. Based on results of MICROCHECKER, two microsatellite loci (Pc4.3 and Pc4.7) were discarded because of the inferred presence of null alleles in several populations. Besides, some individuals were removed from the data set after the sibship analyses performed in COLONY, leaving a single representative per sibship group (Table S1). We found no evidence for significant deviations from HWE or LD.

Descriptive statistics of genetic diversity in *P. cultripes* are shown in Table S1. The average number of alleles and observed heterozygosity per locus and population were 2.71 and 0.37 respectively. The lowest values of observed heterozygosity were found north of the Sistema Central Mountains, as well as in the north-eastern IP and in France (see Fig. S2d). Values of allelic richness across loci showed a decreasing trend from south to north, ranging from 6.09 to 1.45, whereas the highest values for private alleles were found in south-western IP (see Figs S2b and S2c).

The first principal component (PC1) of genetic diversity (summarizing the variables π , H_o , A_r and P) accounted for 97.56% of the variance. Mapping PC1 across the species range revealed a clear increasing trend towards the south (see Figs 3 and S2).

The results of the two Bayesian clustering algorithms were congruent. The optimal K value in STRUCTURE and TESS runs based on the method of Evanno and the DIC, respectively, was 2. At $K = 2$, the two genetic clusters correspond to a west-east division. In addition, at $K = 3$ and $K = 6$ there were significant increases of ΔK too (Fig. 4b). These levels of hierarchical structure showed a strong, clear relationship with geography. For $K = 3$, the western cluster is subdivided into two clusters, corresponding to populations in the north-west (coastal northern Portugal and Galicia and North Plateau) and on the Atlantic south-western region (Fig. 4). Finally, results of STRUCTURE for $K = 6$ show strong geographical association and consistency across replicate runs (Fig. 4). The eastern cluster is subdivided into north and east subgroups, and the western cluster into Atlantic and North Plateau subgroups.

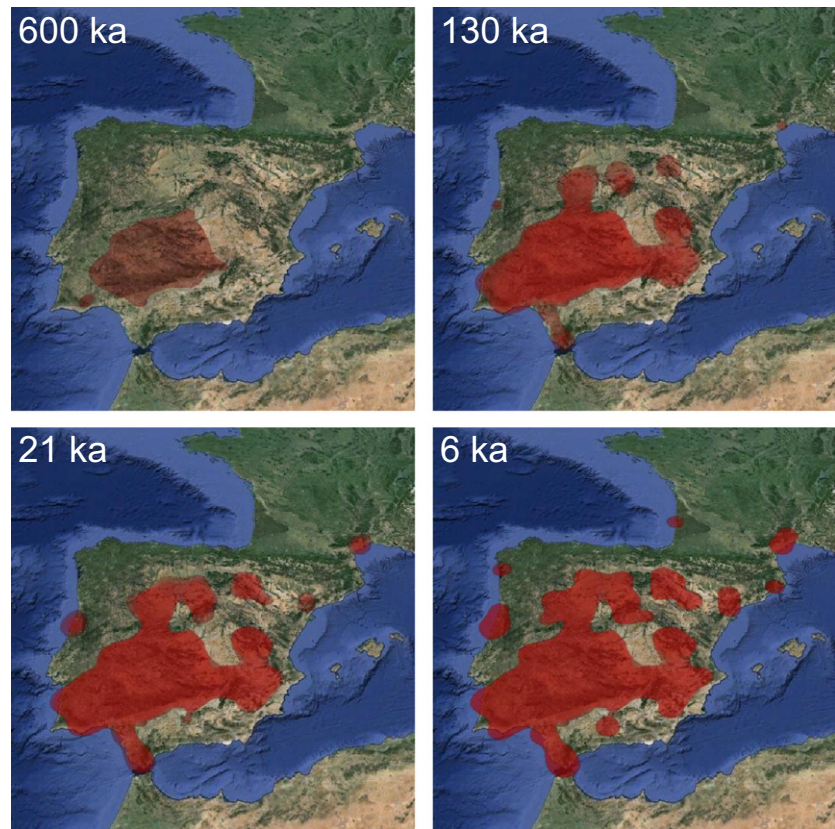


Figure 2 Continuous diffusion phylogeographical reconstruction in *Pelobates cultripes* at four time slices: 600, 130, 21 and 6 ka. Areas in red represent the inferred ancestral location at nodes in each temporal 'slice'.

Distribution modelling

Model predictions (see Fig. S4) had good overall evaluation measures (see Fig. S5). For example, they had an area under the receiver operating characteristic curve (AUC) of 0.85, which is generally considered 'good' (Swets, 1988), and a McFadden's pseudo- R^2 of 0.26, which is considered 'excellent fit' (McFadden, 1978).

Overall mean climatic favourability (given by the model based on the species' current distribution) was significantly higher during the LIG than it is currently, and significantly lower during the LGM according to two of the three climatic simulations (MIROC-ESM and MPI-ESM-P). Under the CCSM4 simulation, favourability was slightly higher during the LGM than currently. Regardless of this uncertainty, past favourability values were generally highly correlated with current ones, that is, the most and least favourable areas, within the available climatic conditions, were largely the same as today, especially during the LGM and the mid-Holocene (see Fig. S6).

The intersections between favourability in the LIG and under each LGM simulation, suggesting the maintenance of favourable conditions through the glaciations (Fig. 3), were significantly correlated with genetic diversity within the species' range (Pearson's $r = 0.63$ under LGM simulation CCSM4, $r = 0.71$ under MIROC-ESM and $r = 0.78$ under MPI-ESM-P; $P < 0.001$ with 5540 d.f.).

Favourability change between time periods included both increases and decreases, with south-eastern France and north-eastern Spain showing the highest and most consistent

increases in favourability since past climate simulations (see Fig. S7). Overall change was more notable with respect to the LIG, with a c. 20% fuzzy range contraction (as given by climatic favourability) towards current favourable areas, whereas a slighter increase occurred after the LGM (Table 2).

DISCUSSION

The integration of results from different disciplines such as phylogeography, population genetics and species distribution modelling allowed us to reconstruct demographic changes in *P. cultripes* since the Pleistocene, including an approximation to potential climate-induced range shifts occurring during the last 130,000 years. Overall, we found low genetic variation and relatively shallow genetic structure, but the results obtained by different approaches have provided relevant and congruent insights about the recent evolutionary history of the species that may help predict population responses to future environmental changes.

Our estimates of phylogeographical structure based on mitochondrial DNA data are consistent with previous studies showing low genetic variability and structure across the species' range (Crottini *et al.*, 2010; Fitó *et al.*, 2011). There is a deep temporal gap between our estimates of the time to the most recent common ancestor (TMRCA) of all extant haplotypes in *P. cultripes* around 500 ka (median value: 535 ka, 95%HPDi: 230–994 ka) and the inferred divergence time from its sister species *P. varaldii* in the Miocene, around 13 Ma (median: 13.26 Ma, 95%HPDi: 9.19–17.91 Ma). This split

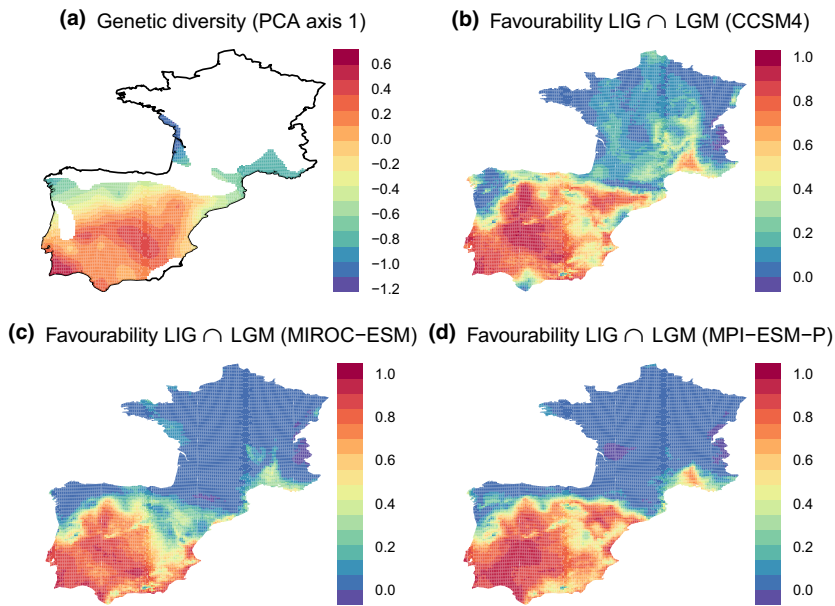


Figure 3 (a) Genetic diversity of *Pelobates cultripes*, based on the first axis of a principal components analysis (PCA) of nucleotide diversity (π), observed heterozygosity (H_o), allelic richness (A_r), and number of private alleles (P); and the intersection between climatic favourability for this species during the Last Inter-Glacial period (LIG) and each of the three simulations for the Last Glacial Maximum (LGM): (b) CCSM4, (c) MIROC-ESM and (d) MPI-ESM-P.

time is older than those estimated by Busack *et al.* (1985) and García-París *et al.* (2003), at 8–11 Ma and 5.5 Ma, respectively, but all estimates indicate speciation occurred in the Miocene. Our estimate at 13 Ma is consistent with the separation of the Betic-Rifean Massif during the upper Tortonian period (Martín *et al.*, 2009). This split coincides with other diversification events in Iberian amphibians, such as the genus *Alytes* (Maia-Carvalho *et al.*, 2014). Despite the inferred long evolutionary history of *P. cultripes* in Iberia, all extant haplogroups share a common ancestor within the last 1 Myr (95% HPDI: 230–994 ka). This contrasts with most other Iberian amphibians, for which geographically structured, old phylogroups provide evidence of survival in allopatric refugia across several Ice Ages (Díaz-Rodríguez *et al.*, 2015; Gonçalves *et al.*, 2015; Teixeira *et al.*, 2015). This may result from massive extinction of lineages during the glaciations of the Pliocene and Pleistocene, although other processes, such as selective sweeps, cannot be ruled out.

In spite of the low mitochondrial variability, two main haplogroups were recovered in our analysis. These groups were not recovered in previous studies using sparser sampling and more slowly evolving markers (Crottini *et al.*, 2010; Fitó *et al.*, 2011). The main split is across an east–west axis, as previously found in other species, such as *Discoglossus galganoi* Capula, Nascetti, Bullini, Lanza & Crespo, 1985 and *Pleurodeles waltli* Michahelles, 1830 (García-París & Jockusch, 1999; Carranza & Arnold, 2004; Martínez-Solano, 2004; Veith *et al.*, 2004). However, whereas in these taxa the vicariant event associated with the eastern–western haplogroup split was inferred to be related to the Guadalquivir basin formation or the Messinian salinity crisis, in the Miocene and Pliocene, vicariance in *P. cultripes* was much more recent, most probably in the Pleistocene.

This east–west division of *P. cultripes* is also apparent from the analyses of SSR data. STRUCTURE and TESS both recovered these groups at $K = 2$. The presence of wide admixture zones

between groups suggests high migration rates (Figs 1b and 4a). The interpolation of genetic diversity indices shows that genetic diversity is lower in the north, including several groups recovered by STRUCTURE: Atlantic, North Plateau and North-Eastern (Figs 3a and 4a). This low diversity contrasts with the higher values observed in southern populations and can be explained as resulting from a recent expansion of these populations from the south. The different analyses carried out to detect genetic signatures of demographic expansion, including F_s , D and R_2 statistics and the Bayesian skyline plot, consistently identify a signal of population growth across all major haplogroups, as well as for the species as a whole (Table 1, Fig. S1). However, the different northern groups seem to derive from different source populations. The North Plateau group was recovered in both mtDNA and SSR-based analyses, whereas the others were only identified by the faster evolving SSRs (Figs 1a and 4a). This may indicate an older origin for the North Plateau group with respect to the Atlantic and North-Eastern groups, as suggested by continuous diffusion analyses, which infer their presence at that area well before the LIG (Fig. 2 and S3).

The fossil record is also consistent with a long but discontinuous presence of the species in the North Plateau, since there are fossil remains in several levels of the Atapuerca formation that date back to the lower Pleistocene (Martín & Sanchiz, 2013; Lobo *et al.*, 2016), and are thus older than the estimated TMRCA for the species. This suggests cycles of colonization/extinction in areas north of the Sistema Central Mountains during the Pleistocene. This mountain range probably acted as a significant barrier to gene flow, limiting migration between populations in the south and north. This is in accordance with the species' life history traits: while the species reaches up to 1770 m of elevation in the Sistema Central (Cejudo, 1990), it is generally not abundant above 1000 m (Tejedo & Reques, 2002).

Species distribution models suggested an overall stability of climatically favourable conditions for *P. cultripes* over the last

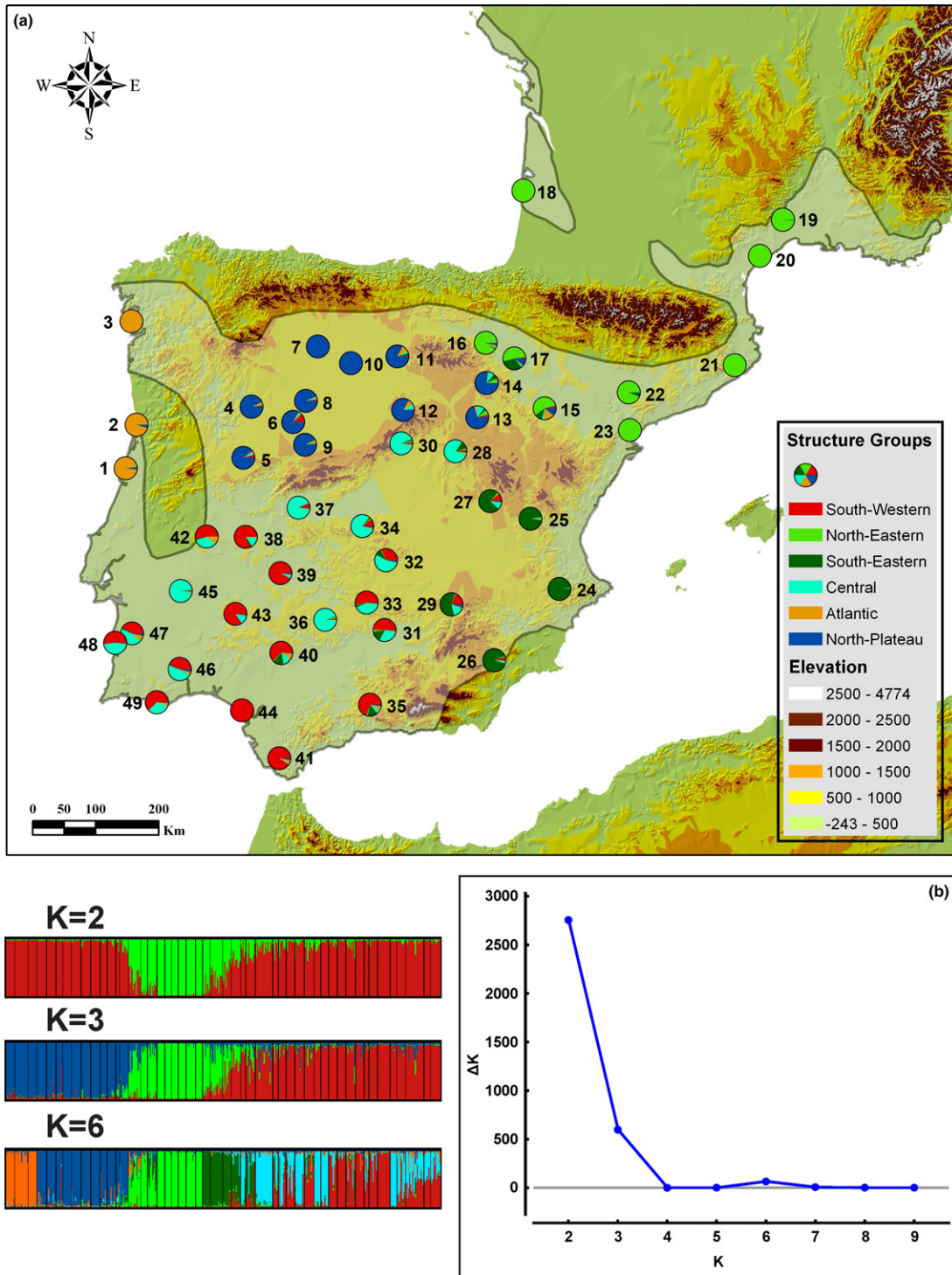


Figure 4 (a) Results of STRUCTURE, showing the spatial distribution of genetic clusters for $K = 6$ for *Pelobates cultripes*. Alternative values of K are shown for comparison ($K = 2$ and 3). (b) Values of ΔK , showing peaks at $K = 2, 3$ and 6 .

130 ka, but with some significant changes through time (see Figs S4, S6 and S7). The geographical extent of climatically favourable areas during the LIG (assuming stable species-environment relationships) was greater than in the present (Table 2), whereas significant decreases were detected in south-eastern France and north-eastern Iberia during the LGM (Figs. 3, S4 and S7). Climatically stable areas for the species,

that is, those resulting from the intersection between favourable areas in the LIG and LGM, are mostly located in the southern half of the Iberian Peninsula, the Northern Plateau and south-eastern France (see Fig. S3). Fossil records support our finding, providing evidence for the presence of the species in southern, south-western, central and northeastern Iberia during the upper Pleistocene (Lobo *et al.*, 2016).

Table 2 Fuzzy range change measures (fuzzy equivalents of the proportion of gained, lost, maintained and changed presences) for *Pelobates cultripes* based on climatic favourability in different time periods, from the Last Inter-Glacial (LIG), to the Last Glacial Maximum (LGM), the mid-Holocene and the present, under the three hypothetical climatic simulations currently available across periods on WorldClim.

Time periods	Gain	Loss	Stable	Balance
LIG to LGM (CCSM4)	0.07	0.32	0.06	-0.25
LIG to LGM (MIROC-ESM)	0.01	0.49	0.03	-0.49
LIG to LGM (MPI-ESM-P)	0.06	0.41	0.03	-0.35
LGM to Holocene (CCSM4)	0.13	0.25	0.08	-0.12
LGM to Holocene (MIROC-ESM)	0.45	0.12	0.09	0.32
LGM to Holocene (MPI-ESM-P)	0.22	0.09	0.04	0.13
Holocene (CCSM4) to present	0.11	0.1	0.1	0.01
Holocene (MIROC-ESM) to present	0.1	0.11	0.08	-0.01
Holocene (MPI-ESM-P) to present	0.1	0.19	0.06	-0.09

Our different approaches showed highly consistent results, suggesting the existence of at least three main refugial areas for *P. cultripes* in Iberia, in the south-west, south-east and the Northern Plateau. These areas coincide with climatically stable areas through the LIG and LGM, and were significantly correlated with current patterns of genetic diversity across the species range. A possible fourth refugial area may have existed in south-eastern France, from which both current Atlantic and Mediterranean French populations would descend. This hypothesis is supported by models of climatic favourability based on simulations CCSM4 and MPI-ESM-P (Figs. 3 and S4), and by the existence of fossil remains dated 220–130 ka in this region (Hanquet *et al.*, 2010). In addition, we found two exclusive haplotypes in Mediterranean French populations (H41, H42; see Table S1). SDMs and molecular evidence, including results of tests of neutrality and mismatch distributions, are thus consistent with the hypothesis that Eastern, Western and Northern Plateau populations have experienced demographic expansions after the LGM, resulting in colonization of northern areas across the current species' range (Table 1, Fig. S1). According to these findings, more recently during the Holocene, the Atlantic and North-Eastern groups may have colonized the North Atlantic coast of the Iberian Peninsula and the French Atlantic Coast respectively (Fig. 2). The French Atlantic coast may have been colonized from an additional refugium in south-eastern France. In any case, migration through a corridor along the Garonne river from the Mediterranean to the Atlantic coast of France is likely, as previously suggested for another anuran, *P. punctatus* (Díaz-Rodríguez *et al.*, 2015). Our mid-Holocene projections, showing some continuity of relatively favourable areas between the Atlantic and Mediterranean coasts, provide support for this hypothesis, although the evidence is not conclusive in terms of the temporal framework involved, as similar continuity was also inferred in previous time intervals (see Fig. S4). In addition, Holocene hydroclimatic variability in the Mediterranean region (Magny *et al.*, 2013) may have affected the species'

distribution and genetic diversity beyond what mid-Holocene simulations are able to predict.

The major goal of our study was to provide information to help predict the long-term species response to climate-mediated changes. In this respect, our integrative approach supports the idea that populations of *P. cultripes* can recover from climatically induced demographic declines, and allowed the identification of historically unstable areas in terms of climatic favourability. Among these stand out the Atlantic coasts of France and the north-western Iberian Peninsula, which are also characterized by relatively low values of current favourability. In addition, the low genetic diversity of these populations, along with their location in peripheral areas of the species' range, makes them the most vulnerable to climate-mediated changes in the medium to long term. On the other hand, our results suggest that refugial sites, assuming that they remain climatically stable in the future, ought to be a major conservation priority, since, as long as they are not destroyed by human activities, the species might be able to safely maintain viable populations there in the long run. While past refugia are not guaranteed to remain as favourable in the future, fine-scale environmental heterogeneity and microevolutionary adaptations often allow relict populations to persist under generally harsh climatic conditions, and climate is often a less important threat than direct anthropogenic impacts (Hampe & Jump, 2011). Conservation efforts in historical refugial areas may thus have high potential to ensure the persistence of species in a warmer world.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary tables and figures.

BIOSKETCHES

Jorge Gutiérrez Rodríguez is a PhD candidate in the Department of Biodiversity and Evolutionary Biology at the National Museum of Natural Sciences (MNCN-CSIC). This paper is part of his thesis, supervised by **Iñigo Martínez-Solano**, and focusing on the influence of demography, landscape and historical factors in the evolutionary history of Iberian amphibians.

A. Márcia Barbosa is a post-doctoral fellow at Évora University, Portugal. Her research interests include biogeography, macroecology, species distribution modelling, comparative phylogeography, biodiversity patterns and conservation.

Author contributions: JGR, AMB and IMS conceived the ideas; JGR collected the data; JGR, AMB and IMS analysed the data; and JGR led the writing with input from all authors.

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