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RESEARCH

Genome-wide patterns of diversity in the European midwife toad complex: phylogeographic and conservation prospects

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Abstract

Assessing how genetic diversity is spatially structured underlies many research questions in evolutionary ecology and contributes to understanding the factors implicated in population declines and extirpations, facilitating identification of conservation priorities and decision-making. In this study, we surveyed genomic diversity using genotyping by sequencing in the six subspecies of the midwife toad *Alytes obstetricans/almogavarii* complex, a group of amphibians from southwestern Europe threatened by habitat loss, climate change and chytridiomycosis. We first illustrate how the structure evident in mitochondrial DNA (mtDNA) and nuclear DNA microsatellites is discordant with the respective distributions of subspecies and patterns of admixture between them. We further document a deeply-divergent mtDNA haplogroup unique to Central Spain that is not reflected by the nuclear diversity, likely corresponding to a ghost mtDNA lineage. Patterns of genetic diversity and structure differ among and within subspecies. The Pyrenean endemics *A. a. almogavarii* and *A. a. inigoi* form homogenous genetic groups with high levels of heterozygosity, while the more widespread *A. o. pertinax*, *A. o. boscai* and *A. o. lusitanicus* are geographically structured across the Iberian Peninsula, comprising both genetically diverse and impoverished populations. Finally, *A. o. obstetricans* probably persisted in a composite glacial refugium north of the Pyrenees, from which it recently expanded across Western Europe, losing much of its genetic variation. Our results should be considered in future red list assessments, management unit delimitation, and ex-situ conservation efforts, and are also relevant to study chytrid epidemiology, for which *A. obstetricans* has been a model organism for nearly three decades.

Keywords *Alytes* · Chytridiomycosis · ddRAD-seq · Genetic structure · Heterozygosity · Iberian Peninsula

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Introduction

A primary goal in molecular ecology is to assess the genetic composition, diversity and structure of natural populations to understand demographic history and intraspecific phylogeography, and to inform conservation research. Such analyses are particularly pertinent to mapping the distributions of phenotypically similar, cryptic taxa, evaluating the genetic health of populations, and inferring patterns of population connectivity. These insights are crucial in risk assessments (e.g. Red List evaluations) and in designing conservation strategies (e.g. replenishing genetic diversity in bottlenecked populations, delineating management units, planning translocations and re-introductions). Adaptation to changing environmental conditions, either natural or caused by human activities, requires additive genetic variation, and accordingly, genetically impoverished small and fragmented populations are predicted to be more vulnerable to local extinction (Spielman et al. 2004; Frankham 2005; McMahon et al. 2014; Li et al. 2016).

Quantifying patterns of genetic diversity is particularly relevant in amphibians, a globally declining vertebrate group (Beebee 2005; Allentoft and O'Brien 2010). Low levels of heterozygosity can negatively affect survival (Hitchings and Beebee 1998), larval performance (Luquet et al. 2011), competitive potential (Rowe and Beebee 2005), tolerance to pesticides (e.g. Bridges and Semlitsch 2001) and resistance to infections by pathogens (e.g. Luquet et al. 2012; Banks et al. 2020). Genetic impoverishment and cessation of gene flow in amphibian populations have been shown to relate to major factors of decline, such as climate change, anthropogenic habitat degradation, and infection by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (e.g. Allentoft and O'Brien 2010; Horner et al. 2017; Banks et al. 2020; Fisher and Garner 2020; Lucati et al. 2022).

The link between genetic diversity and the susceptibility of populations to extrinsic stresses is largely taxon- and context-dependent (e.g. Savage and Zamudio 2016; Banks et al. 2020; Smith et al. 2022; Schmidt et al. 2023). One important factor is the baseline genetic variation of populations (both neutral and adaptive, Mable 2019), which can vary across the distribution range of species because of historical events, such as the Pleistocene climatic fluctuations (Hewitt 2000). In the Northern Hemisphere, more southernly populations that survived the Quaternary ice ages tend to have maintained higher genetic diversity than more northern populations, as the latter formed only after the last glacial maximum via sequential founder events (Hewitt 2000, 2011). These historical variations may thus predispose some populations to being more vulnerable to current threats (Schmitt and Hewitt 2004). In Europe, regardless of the intensity of human pressure, amphibian populations that occur within regions corresponding to glacial refugia are less threatened than those lying outside these regions (Dufresnes and Perrin 2015). Populations at the northern edges of species distributions are small and fragmented, with low neutral and immunogenetic variation (e.g. Zeisset and Beebee 2014; Höglund et al. 2015, 2022; Cortazar-Chinarro et al. 2017; Rödin-Mörch et al. 2019). Assessments of genetic diversity and structure should thus ideally include considerations of evolutionary history.

Amphibian genetic diversity has been extensively examined using various types of molecular markers (e.g. Milá et al. 2010; Shaffer et al. 2015; Rödin-Mörch et al. 2019; Höglund et al. 2022; Dufresnes et al. 2024), but most studies have analyzed only a small fraction of the genome, e.g., mitochondrial DNA (mtDNA) genes or small numbers of nuclear markers like DNA microsatellites (e.g. Palo et al. 2004; Dufresnes et al. 2013; Pröhl et al. 2021; Haugen et al. 2024). Such markers lack sufficient information to assess species that diversified in multiple lineages and comprise several taxa (i.e. "species complexes"). For instance, secondary contact and mitochondrial introgression resulting from hybridization can affect the identification of populations based on mtDNA. Furthermore, some populations may show mitochondrial homogeneity despite structure in nuclear markers (e.g. Milá et al. 2010) due to female-biased dispersal or selective sweeps (Toews and Brelsford 2012). Reciprocally, deeply-diverged mtDNA lineages may not correspond to commensurate divergence in the nuclear genome, i.e. so-called "ghost" lineages (e.g. Dufresnes et al. 2020a; Schultze et al. 2020; Wielstra et al. 2021). For DNA microsatellites, high mutation rates make them prone to allele homoplasy, i.e. convergence of independently-evolved alleles. Populations from distinct lineages may thus share identical microsatellite genotypes for some loci without gene flow, which compromises inferences of genetic structure and admixture (Miralles et al. 2024). Finally, large sample sizes and numbers of markers are necessary to obtain reliable population-average estimates of ancestry and diversity with microsatellite loci (Hale et al. 2012), and even then fine-scale geographic structure may not be detectable (Dufresnes et al. 2023a).

The above issues become evident from comparisons of mitochondrial and microsatellite data with results from new analyses conducted on hundreds/thousands of SNPs obtained by genotyping-by-sequencing (GBS) (e.g. Jeffries et al. 2016; Camacho-Sánchez et al. 2020; Sunde et al. 2020; Zimmerman et al. 2020; Dufresnes et al. 2023a). Genomic datasets obtained from double digest Restriction Associated DNA sequencing (RAD-seq; Peterson et al. 2012) or sequence-capture methods (Hutter et al. 2022; de Visser et al. 2024) offer far greater resolving power to distinguish

lineages and the structure therein, to accurately delineate their geographic boundaries, to quantify the extent of admixture, and to estimate population genetic diversity with fewer samples (Lexer et al. 2013; Vences et al. 2024).

Although significantly improving the accuracy of inferences, the shift from genetic to genomic methods poses challenges (Shaffer et al. 2015; Vences et al. 2024). Amphibian species are relatively weak dispersers among vertebrates (maximum a few kilometers per generation) and only occupy specific, sometimes disconnected habitats (Cayuela et al. 2020). Amphibian populations are thus expected to be locally structured, and analyses of thousands of loci may eventually recover every surveyed population as a distinct genetic cluster. Genetic assessments must then distinguish phylogeographic patterns that are the result of distinct evolutionary histories *vs.* local population processes (Rancilhac et al. 2023), so that designation of management units maximizes the amount of diversity captured in the fewest clusters possible (Mable 2019). Another issue to consider is introgressive hybridization between lineages that recently came into secondary contact (Hewitt 2011). As GBS data can be used to detect even subtle levels of admixture, genetic diversity indices (e.g. heterozygosity) in a given taxon/lineage may be inflated by the occurrence of foreign alleles from hybridizing parapatric populations (e.g. Dufresnes et al. 2020c). Finally, the phylogenetic scale of analysis also has implications for locus dropout. With ddRAD-seq, the more diverged the samples, the fewer the number of shared loci (e.g. Hodel et al. 2017; Ambu et al. 2023; van Riemsdijk et al. 2023). For more accurate inferences, restricting analyses to lineage-specific datasets increases the number of loci retained in analyses, but diversity estimates may then not be comparable among lineages if they were obtained from different loci.

Genomic data would be useful for assessing patterns of genetic diversity for phylogeography and conservation in the species complex of European midwife toads, subgenus *Alytes* (also called the *Alytes obstetricans* complex; Dufresnes and Hernandez 2021). European midwife toads are small terrestrial anuran amphibians that occupy a broad array of habitats in the Western Mediterranean region and exhibit parental care (Speybroeck et al. 2016; Dufresnes 2019). During mating, the female transfers the clutch to the male's hindlimbs, which carries the eggs until they are ready

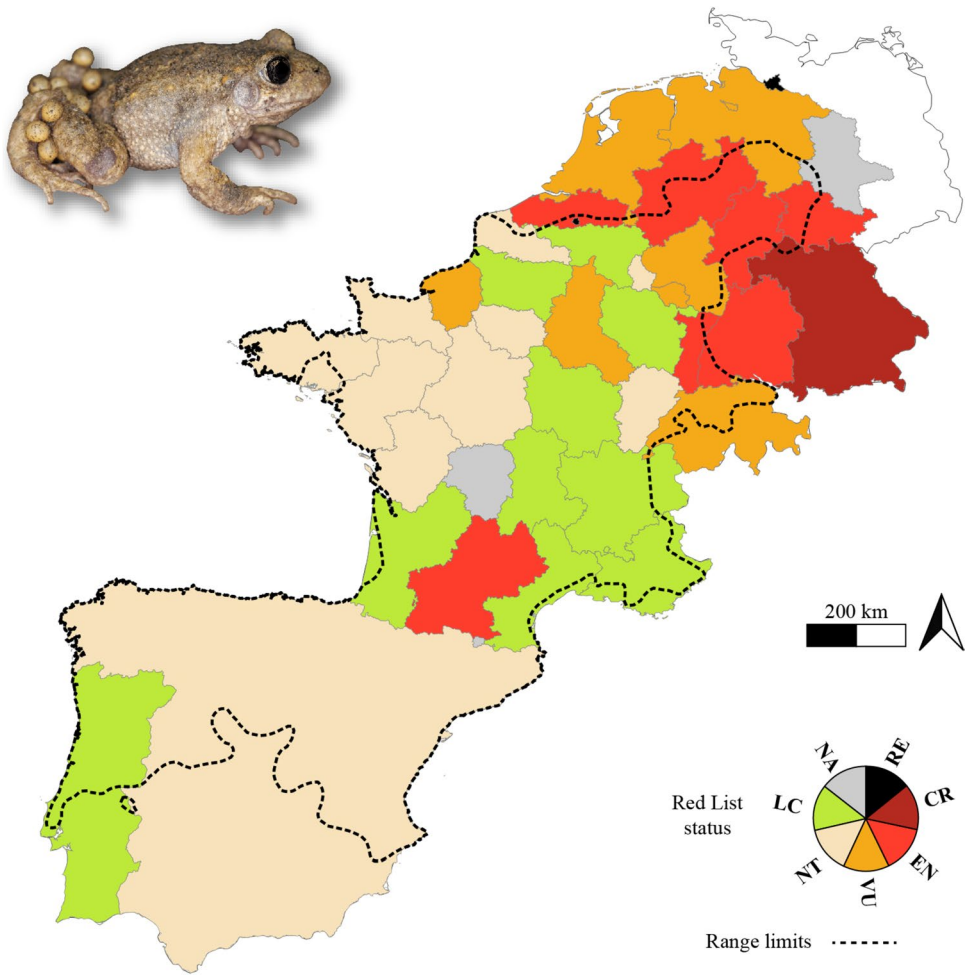

Fig. 1 Red list status of midwife toads from the *A. obstetricans* complex, according to national (Netherlands, Spain, Portugal, Luxemburg, Switzerland) and regional (France, Germany, Belgium) assessments. Sources: Dufresnes and Perrin (2015); Ambu and Dufresnes (2022) and references therein. Photo: CD

to hatch, ensuring protection and proper development in the critical first larval stages (illustrated in Fig. 1). Hatchlings are deposited in permanent water bodies or water bodies with a long-hydroperiod because tadpoles grow slowly, and metamorphosis can take more than a calendar year (Speybroeck et al. 2016; Dufresnes 2019). This peculiar lifestyle makes *Alytes* sensitive to the effect of global warming, including wildfires and early pond desiccation, to invasive predatory fishes in breeding sites and to the alteration and destruction of its habitats, particularly in lowland agricultural and urbanized areas (Pleguezuelos et al. 2002; Barrios et al. 2012). Moreover, midwife toads are both symptomatic and asymptomatic carriers of *Bd*, and chytrid outbreaks have led to well-studied mortality episodes in the 1990s and 2000s (Bosch et al. 2001; Walker et al. 2010). Due to these combined threats, populations have been declining and are of conservation concern in several regions, despite the wide distributions and often high local abundance of species (Fig. 1).

The *A. obstetricans* complex extends throughout the northern half of the Iberian Peninsula and across most of France, Switzerland, Luxemburg and Belgium, reaching western Germany and the southeastern Netherlands (Fig. 1). Molecular studies using allozymes (Artnzen and García-París 1995), mtDNA (Martínez-Solano et al. 2004; Maia-Carvalho et al. 2014; Gonçalves et al. 2015; Lucati et al. 2022), targeted nuclear sequences (Maia-Carvalho et al. 2014; Gonçalves et al. 2015), DNA microsatellites (Maia-Carvalho et al. 2018; Lucati et al. 2022) and more recently ddRAD-seq (Dufresnes and Martínez-Solano 2020; Ambu et al. 2023, 2024a, 2024b), have progressively unveiled complex patterns of diversity, showing a lineage-rich history of diversification initiated in the Pliocene, some 4 Mya. Presently, six taxa delimited within two species, *A. obstetricans* and *A. almogavarii*, are recognized (Speybroeck et al. 2020; Dufresnes and Hernandez 2021; Ambu et al. 2024a). In *A. obstetricans*, this includes *A. o. obstetricans* (extra-Iberian range), *A. o. pertinax* (northern and eastern Spain), *A. o. boscai* (northern Portugal and northwestern Spain), and *A. o. lusitanicus* (central Portugal and central western Spain). In *A. almogavarii*, this includes *A. a. almogavarii* (eastern French and Spanish Pyrenees) and *A. a. inigoi* (central Spanish Pyrenees). These lineages recurrently hybridized over the course of their evolution–the subspecies *A. o. pertinax* has a reticulate origin involving *A. o. obstetricans* and *A. almogavarii* ancestors (Ambu et al. 2023)–and they continue to exchange genes at range margins. Parapatric populations are admixed, sometimes with pervasive nuclear and mitochondrial introgression over hundreds of kilometers (Ambu et al. 2023, 2024b), which has complicated the identification of populations based on a few molecular markers (Dufresnes and Hernandez 2021). Moreover, populations may show genetic structure at the regional level, even in areas with low diversity, prompting for fine-scale assessments (Tobler et al. 2013; Barrat et al. 2024).

In this study, we revisit taxon-specific patterns of genetic diversity and structure in the *A. obstetricans* complex. Exploiting GBS data recently produced for phylogenomic (Ambu et al. 2023) and hybrid zone analyses (Dufresnes and Martínez-Solano 2020; Ambu and Dufresnes 2024; Ambu et al. 2024b), we first re-assess discrepancies in population identification between genomic SNPs, DNA microsatellites and mitochondrial sequences. Restricting our analyses to "pure" populations (i.e. without signatures of recent inter-lineage admixture), we then infer genetic structure within each subspecies through a combination of phylogenetic and population genetic analyses. Finally, we report on geographic variation in heterozygosity to identify hotspots of genetic diversity and areas with impoverished genetic diversity based on data from different phylogenetic scales. Our results showcase how using GBS data can help us to comprehensively decipher causes and consequences of genetic diversity and to delineate taxa when conventional markers struggle to do so.

Methods

Population identification and discrepancies between genetic markers

For the distribution of the six subspecies and the location of their contact zones, we used the population ancestry coefficients of Ambu et al. (2024b) for 181 populations (425 individuals) obtained by Bayesian clustering of two ddRAD-seq datasets: (1) 5111 SNPs genotyped in 163 populations (314 individuals) of the *A. obstetricans* complex (Ambu et al. 2024b); (2) 433 SNPs genotyped in 18 populations (111 individuals) from NE-Spain (Dufresnes and Martínez-Solano 2020). To assess discrepancies from previous studies, we gathered the following mitochondrial and microsatellite datasets.

For mtDNA, mitochondrial lineages were inferred for 402 populations (1000 individuals) based on 308 sequences of 16S, 688 sequences of ND4 and 4 sequences of both (File S1). These combine 222 sequences of 16S and 666 sequences of ND4 published by Gonçalves et al. (2015), Dufresnes and Martínez-Solano (2020), Lucati et al. (2022), Vliegenthart et al. (2023), Laorden-Romero et al. (2024), Ambu and Dufresnes (2024), with new sequences as follows. Ninety new 16S sequences were generated for 62 populations following Ambu and Dufresnes (2024). Twenty-six new ND4 sequences were generated for 13 populations following Vliegenthart et al. (2023). For each gene, sequences were manually aligned and trimmed in Seaview 5 (Gouy et al. 2021) to 515 base pairs (bp) for 16S and 574 bp for

ND4. Haplotypes were identified by building phylogenetic networks (phylonetwork) with SplitsTree (Huson and Bryant 2006), and given unique identifiers. For ND4, we used the haplotypes listed by Vliegenthart et al. (2023) as a starting reference.

For microsatellites, we re-used to the population ancestry coefficients from seven clusters identified by Bayesian clustering analyses in Dufresnes and Hernandez (2021), which were based on 12 loci genotyped in 142 populations (965 individuals) by Maia-Carvalho et al. (2018).

Nuclear structure and diversity using GBS

To measure genetic structure and diversity within the six taxa of the *A. obstetricans* complex, we re-used the ddRAD-seq de novo assembly of Ambu et al. (2024b), which contains 332 *Alytes* samples, including 314 samples of the *A. obstetricans* complex. In brief, these ddRAD-seq data were obtained by paired-end sequencing (Illumina Next-Seq 550) of four genomic libraries prepared with a protocol adapted from Brelsford et al. (2016), using enzyme restriction by *MseI* and *SbfI* and a size selection window of 400–500 bp. The bench workflow is available at https://dx.https://doi.org/https://doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1. Raw sequence reads were demultiplexed, stacked and cataloged with the *process_radtags* function and the *denovo_map.pl* pipeline of STACKS 2.59 (Catchen et al. 2013). Details on library preparation and assembly parameters can be found in the original publication (Ambu et al. 2024b).

To exclude introgressed and low-quality samples, we included only 170 samples (104 populations) assigned to one of the six subspecies of the *A. obstetricans* complex with ancestry coefficients above 0.95 in the Bayesian clustering analysis of Ambu et al. (2024b) and with < 50% of missing data in the SNP dataset used for their inference. New datasets were generated for the selected samples (File S2) with the *populations* module of STACKS, as follows.

To assess the relative patterns of divergence among and within subspecies, we first generated a concatenated sequence alignment (*–phylip-var-all*) of the RAD loci sequenced in all but five samples (*–p* 165). The alignment (282,247 bp) was used to build a phylonetwork with SplitsTree. Second, to estimate population diversity, we produced a SNP matrix (*–structure*) of the RAD loci sequenced in all populations (*–p* 104), present in at least half of the samples of each (*–r* 0.5), and randomly retaining only one SNP per locus to avoid physically linked SNPs (*–write-random-snp*). The dataset (678 SNPs) was used to estimate the expected heterozygosity (H_s, also known as gene diversity) and the observed heterozygosity (H_o) for each population with the R package *hierfstat* (Goudet 2005).

To assess genetic diversity and structure within each subspecies separately, we produced six SNP matrices (*–structure*) of the RAD loci sequenced in all individuals (*–r* 1 and *–p* as the number of populations), and randomly keeping only one SNP per tag (*–write-random-snp*). These filtering parameters avoid missing data and physically linked loci, as a prerequisite to some of the population genetic inferences conducted below. Table 1 summarizes the number of individuals, populations and SNPs obtained for each subspecies.

For each subspecies dataset, we estimated populational H_s and H_o with *hierfstat* and assessed genetic structure in two ways. First, we performed a Principal Component Analysis (PCA) on individual allele frequencies with the *R* package *adegenet* (Jombart 2008). Second, we used the *snmf* clustering algorithm implemented in the *R* package *LEA* (Frichot and François 2015). For each subspecies dataset, chains were run for K = 1–12 clusters, with 20 replicates for each K, retaining 10% of the data for the cross-entropy estimation (function *snmf*). To assess the number of clusters that best summarize the data, we examined the distribution of cross-entropy values across Ks (Frichot and François 2015). Low cross-entropy reflects the most likely Ks. To thoroughly explore the data, we reported all Ks with a cross-entropy lower than the cross-entropy of K = 1 (no structure) – except if K = 1 had the lowest cross-entropy, in which case K = 2 was reported. Individual ancestry coefficients from the best run (the one with the lowest cross-entropy among replicates) were extracted and averaged per population to obtain populational ancestry coefficients. For visualization, individual ancestry information was further reported on the other analyses conducted above (phylonetwork, PCA). Finally, we tested for isolation-by-distance (IBD) by computing pairwise population F_{st} with *hierfstat* and geographic distances *d* with *Geographic Distance Matrix Generator* (Ersts 2006), and correlating matrices of $F_{st}/(1\text{-}F_{st})$ and log(*d*) using Mantel tests with 1000 permutations (Rousset 1997).

Table 1 Number of samples and populations analyzed for each subspecies, number of loci obtained for each subspecies SNP sets, and most likely number of clusters (K) according to the cross-entropy approach (brackets: number of clusters more likely than K = 1)

	samples	populations	SNPs	K
A. a. almogavarii	30	17	2594	1*
A. a. inigoi	19	8	3325	1*
A. o. boscai	26	14	4848	2 (2–3)
A. o. lusitanicus	18	11	5909	2 (2–3)
A. o. obstetricans	33	18	2307	3 (2–4)
A. o. pertinax	44	36	1084	3 (2–6)

*K = 2 is shown on the dedicated figure

Results

Population identification and discrepancies between markers

The distributions of subspecies and their hybrid zones inferred from genomic SNPs are provided in Fig. 2A. Based on mitochondrial genes (Fig. 2B) and microsatellites (Fig. 2C), some populations are either misattributed or unattributable to a given subspecies.

Analysis of mitochondrial DNA sequences retrieved most of the main lineages/haplogroups previously identified in the *A. obstetricans* complex (Fig. 3), with a total of 44 haplotypes for 16S and 107 haplotypes for ND4. Populations assigned to *A. o. obstetricans* and *A. o. pertinax* share some identical 16S haplotypes and cannot be reliably distinguished with this marker (Fig. 3A). Two deeply-diverged

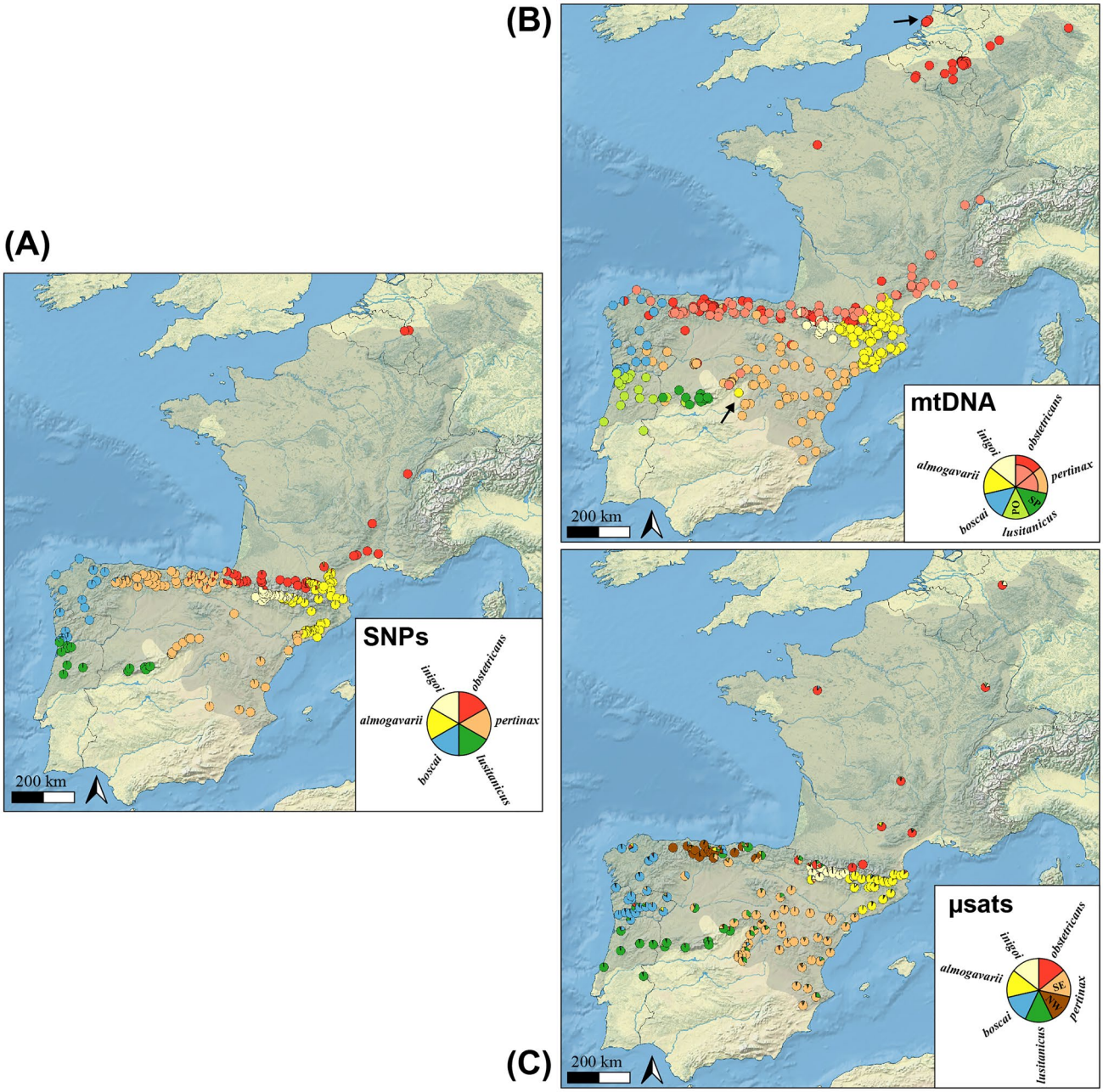

Fig. 2 Subspecies identification in the *A. obstetricans* complex based on **A** nuclear genomic SNPs; **B** mtDNA (16S and ND4); and **C** DNA microsatellites. Arrows point to introduced populations. For mtDNA, light red illustrates 16S barcoding for *obstetricans*/*pertinax* (which cannot be distinguished with this marker)

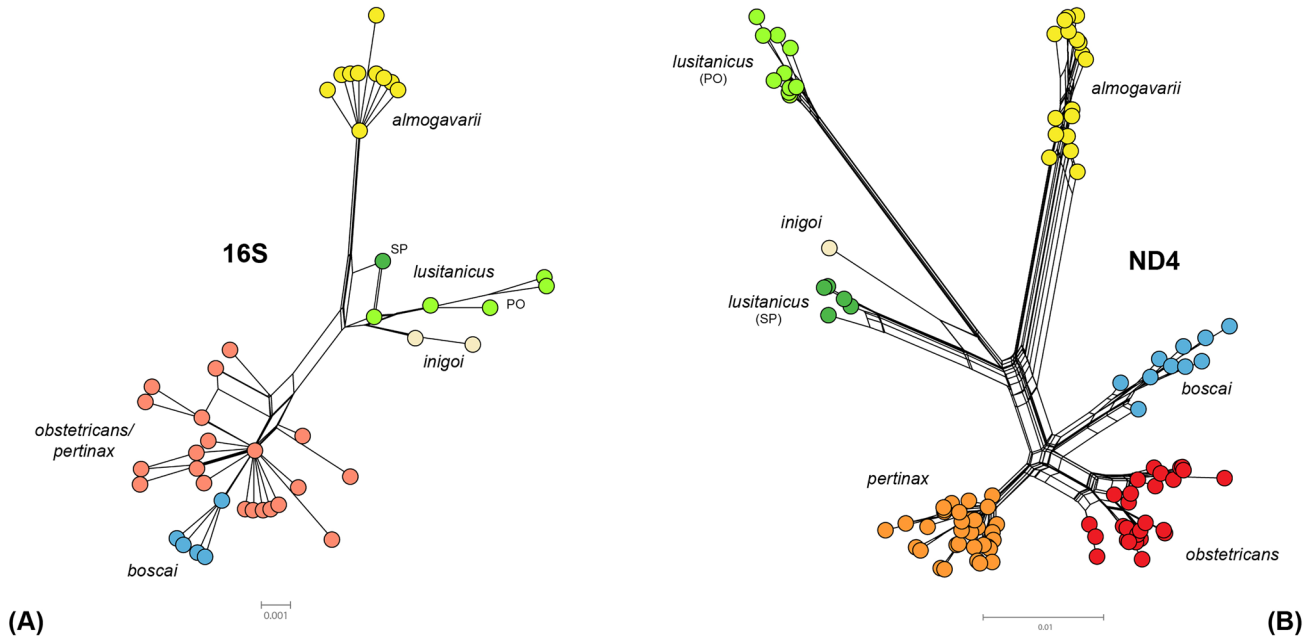

Fig. 3 Phylonetworks of: **A** the 44 16S haplotypes identified; **B** the 107 ND4 haplotypes identified. SP = Spanish lineage of *A. o. lusitanicus*; PO: Portuguese lineage of *A. o. lusitanicus*. Note that *A. o. obstetricans* and *A. o. pertinax* cannot be distinguished with 16S

ND4 haplogroups were retrieved in the populations assigned to *A. o. lusitanicus*: the one previously documented in Portugal and a few sites in Western Spain (Gonçalves et al. 2015; labelled PO), and a new one found exclusively in Western Spain (labelled SP). These lineages are as distant from each other as from the lineages of other taxa of the complex and correspond to distinct haplotypes at the less variable 16S (Fig. 3A).

The distributions of mtDNA lineages generally reflect the geographic ranges of the subspecies they have been associated with based on the genomic SNPs (Fig. 2A). The most striking discordance occurs in NW-Spain, where populations of *A. o. pertinax* and *A. o. boscai* bear *A. o. obstetricans* ND4 haplotypes. In *A. o. lusitanicus*, one population bears *A. o. pertinax* mtDNA despite the absence of contemporary contact with that subspecies. In a few contact zones, notably between the Pyrenean endemic *A. a. almogavarii* and *A. a. inigoi*, gene flow is more extensive than mtDNA suggests.

Geographic distributions of the seven microsatellite clusters (Maia-Carvahlo et al. 2018) mostly match the subspecies ranges inferred from the genomic SNPs (Fig. 2C); *A. o. pertinax* corresponds to two clusters that distinguish its eastern *vs.* northern ranges. However, many unadmixed populations based on the SNPs received mixed ancestry estimates based on microsatellites, and reciprocally, many admixed populations based on the SNPs received high ancestry estimates for a single microsatellite cluster (Fig. 2C). In particular, some *A. o. pertinax*, *A. o. obstetricans/pertinax* and *A. o. obstetricans* populations appear to exhibit introgression by *A. o. lusitanicus* based on microsatellites, but this observation is not supported by the results of the genomic SNPs.

Genetic structure

The phylonetwork constructed from ~ 282 kb of concatenated RAD sequences obtained from pure *A. obstetricans* samples retrieves the six subspecies as distinct phylogroups (Fig. 4), some featuring high diversity that matches the patterns of genetic structure retrieved by the clustering analyses (Fig. 4; see below).

The cross-entropy of *snmf* runs reveals more structure in the widespread than in the geographically restricted subspecies (Table 1, Fig. 5). When few clusters are considered (low Ks), these distinguish populations from distinct geographic areas, which are also evident in separation on the PCA. Clustering solutions with higher Ks tend to separate single local populations (Figs. 6–11).

The two *A. almogavarii* subspecies are not significantly structured based on our sampling. In both subspecies, the cross-entropy of $K > 1$ is higher than $K = 1$, and the first axis of the PCAs primarily distinguish a single sample from the others (Figs. 6–7). For *A. a. almogavarii*, analyses with $K = 2$ identify loose spatial differentiation between the Pyrenean foothills and the Aude Valley (Fig. 6). For *A. a. inigoi*, $K = 2$ separates three samples in the southernmost population (Fig. 7), which however branch close to other *inigoi* samples in the phylonetwork (Fig. 4).

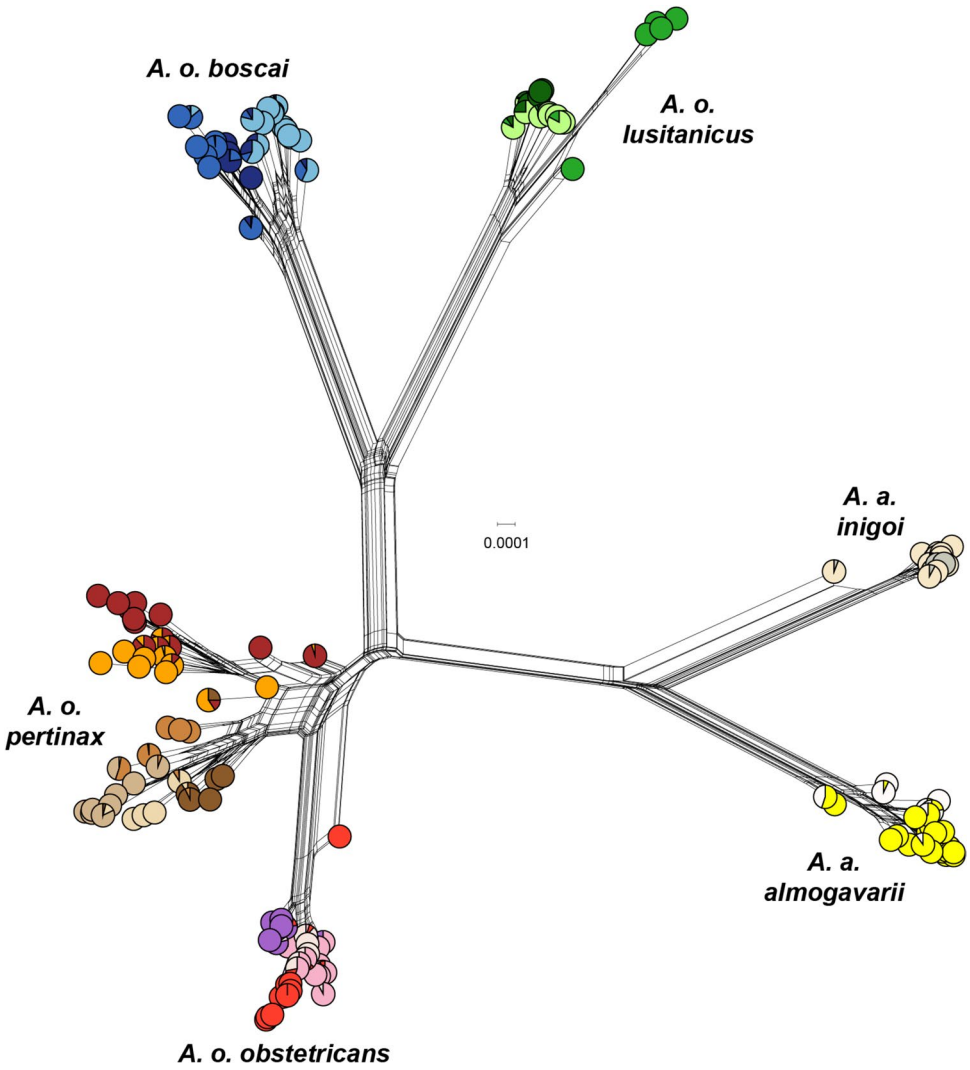

Fig. 4 Phylonetwork of ~ 282 kb of concatenated RAD loci genotyped in pure samples of each *A. obstetricans* taxon. Individual ancestries in the clustering analyses (highest K reported for each; Figs. 5–11) are displayed as pie charts

For both *A. o. boscai* and *A. o. lusitanicus*, runs with K = 2 feature the lowest cross-entropy (Fig. 5). The best of these runs reflects range-wide patterns of differentiation (Figs. 8–9) that correspond to distinct branches on the phylonetwork (Fig. 4). In *A. o. boscai*, the clustering analysis with K = 2 and the PCA separate the southern from the northern populations; K = 3 further identifies some substructure in the western Asturian mountains (Fig. 8). In *A. o. lusitanicus*, the analyses primarily separate the Spanish and the Portuguese populations and highlight the distinctiveness of the northernmost Portuguese population (Fig. 9).

For the widespread subspecies *A. o. obstetricans* and *A. o. pertinax*, cross-entropy suggests K = 3, although runs up to K = 4 and K = 6 are still better than no structure (Fig. 5). *Alytes o. obstetricans* is essentially structured across southern France, while the same cluster is found from the Massif Central to the Netherlands (Fig. 10). In *A. o. pertinax*, the analyses primarily separate populations from the southeastern vs. northern ranges (Cantabrian area). With higher Ks, populations from the Sistema Central mountains form a distinct cluster (K = 3), and the Cantabrian populations are divided into multiple regional groups (K = 4–6) (Fig. 11).

IBD was significant (Mantel tests of matrix correlations, $P < 0.05$) within *A. a. almogavarii*, *A. o. boscai*, and *A. o. pertinax*, but not within *A. a. inigoi*, *A. o. lusitanicus* and *A. o. obstetricans* (File S3).

Genetic diversity

Expected (H_s) and observed heterozygosity (H_o) are correlated (File S4) and here we only discuss the former (Fig. 12). H_s is highly variable between and within subspecies and depends on the datasets used; estimates are on average four times higher when computed from the markers obtained independently for each subspecies [subspecies-$H_s = 0.08 \pm 0.04$ (0.02–0.18)], than when computed from the 678 SNPs informative across all of them [global-$H_s = 0.02 \pm 0.01$ (0.0–0.05)] (Fig. 12, File S5). The

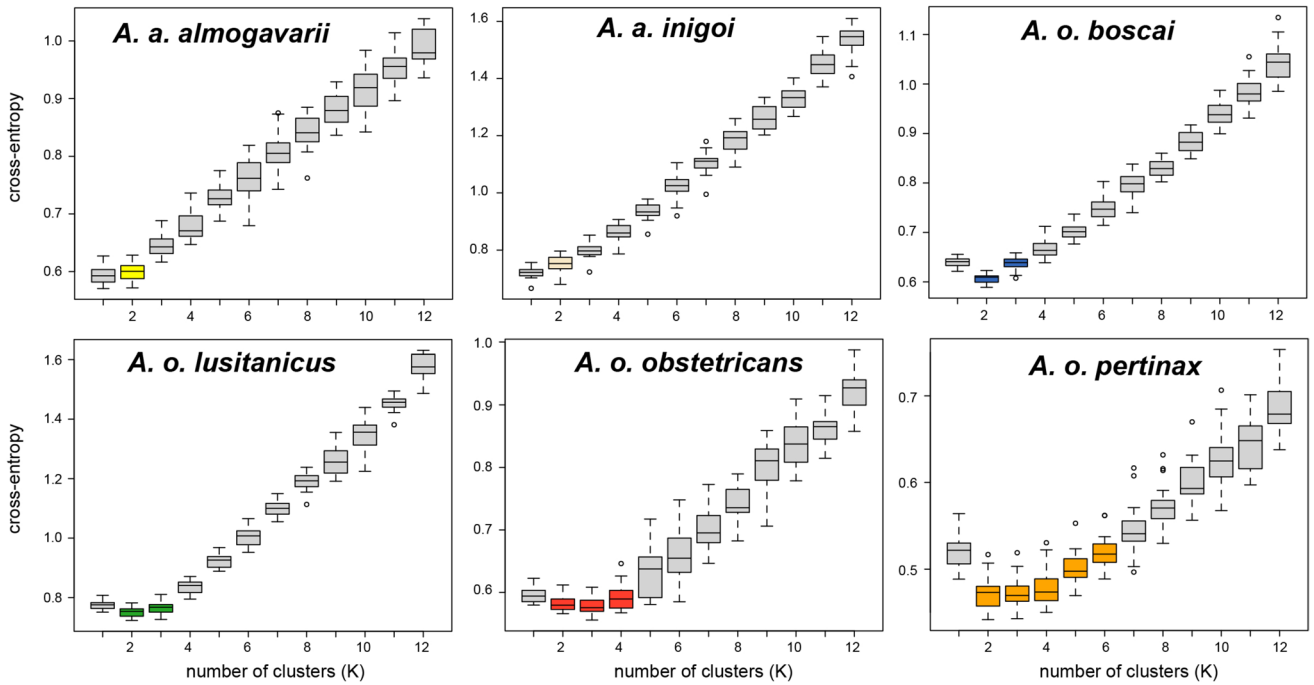

Fig. 5 Cross-entropy of the *snmf* runs for K = 1–12 for each subspecies. The Ks reported in the dedicated figures are emphasized by colors

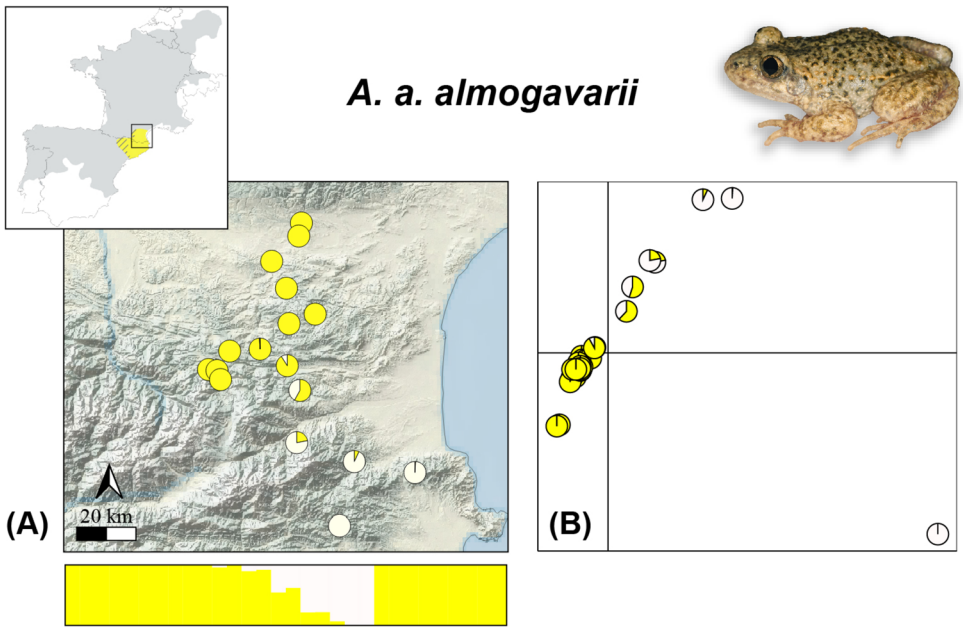

Fig. 6 Genetic clustering with spatial location of clusters **A** and PCA **B** of 30 individuals (17 populations) of *A. a. almogavarii* genotyped at 2594 SNPs for K = 2. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones)

subspecies-H_s and global-H_s are highly correlated, but the relationship (slope) differs among subspecies (File S6).

Comparisons of global-H_s indicate that *A. o. obstetricans* is the least genetically variable subspecies, and *A. o. boscai* and *A. o. lusitanicus* are the most variable, with noticeable variation across ranges (Fig. 12). Hotspots of gene diversity are evident in Portugal and in several mountain ranges, notably the Eastern Pyrenees, the western and eastern parts of the Cantabrian mountains, and the northeastern part of the Sistema Central mountains (Sierra de Guadarrama). Areas with lower diversity are found in coastal areas (notably Galicia and Valencia), in the southwestern parts of the Sistema Central mountains (Sierra de Gredos), and in the extra-Pyrenean ranges of *A. o. obstetricans*.

The subspecies-H_s estimates confirm the low diversity of the coastal populations of *A. o. boscai* (Galicia) and *A. o.*

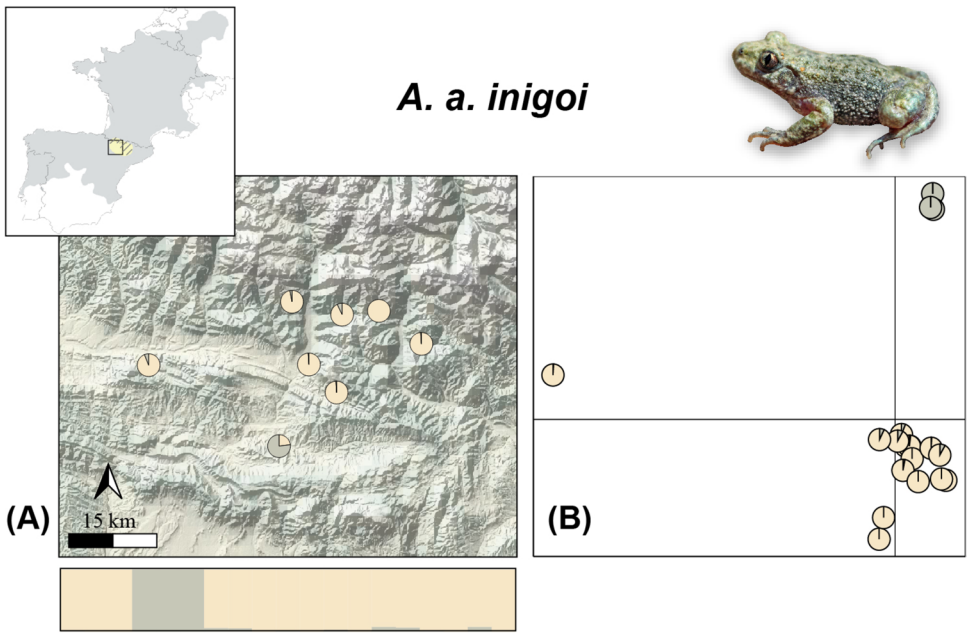

Fig. 7 Genetic clustering with spatial location of clusters **A** and PCA **B** of 19 individuals (8 populations) of *A. a. inigoi* genotyped at 3325 SNPs for K = 2. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones)

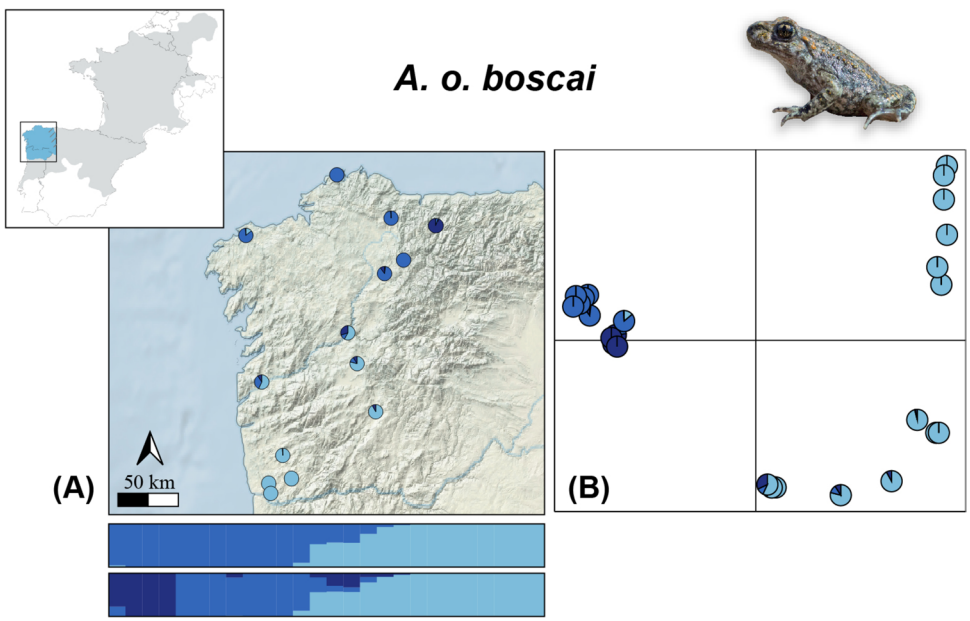

Fig. 8 Genetic clustering with spatial location of clusters **A** and PCA **B** of 26 individuals (14 populations) of *A. o. boscai* genotyped at 4848 SNPs for K = 2–3. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones). The highest K is shown on the map and PCA

pertinax (Valencia), the extra-Pyrenean populations of *A. o. obstetricans*, and the Spanish populations of *A. o. lusitanicus* (Fig. 12). In some subspecies, H_s seems to decrease along geographic gradients, to some extent in a northward direction in *A. o. obstetricans* (File S7A), and along an eastward axis in *A. o. lusitanicus* (File S7B). H_s is highly variable within the clusters of *A. o. pertinax* (Fig. 11).

Discussion

Clarifying taxon distribution and diversity with GBS data

Our study explores patterns of genetic diversity and

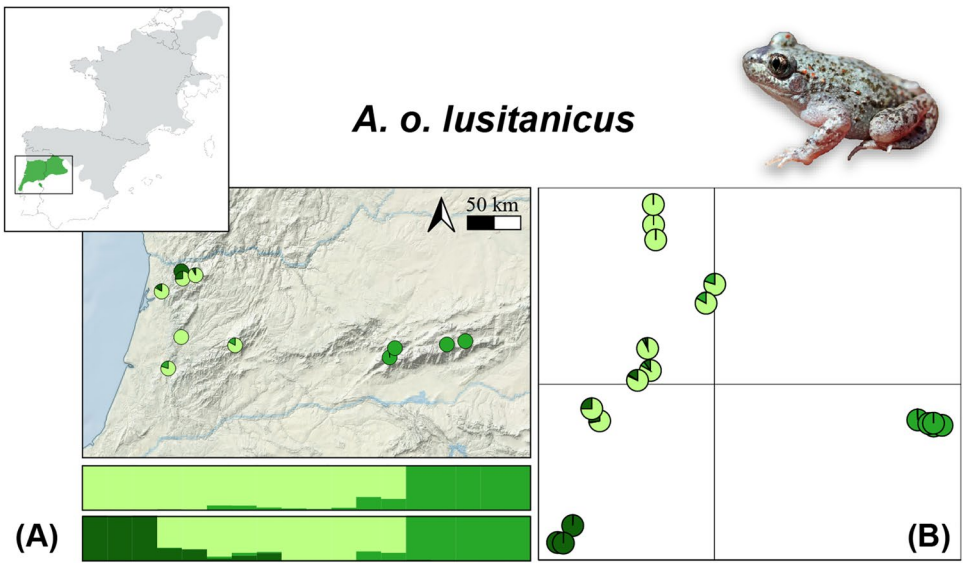

Fig. 9 Genetic clustering with spatial location of clusters **A** and PCA **B** of 18 individuals (11 populations) of *A. o. lusitanicus* genotyped at 5909 SNPs for K = 2–3. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones). The highest K is shown on the map and PCA

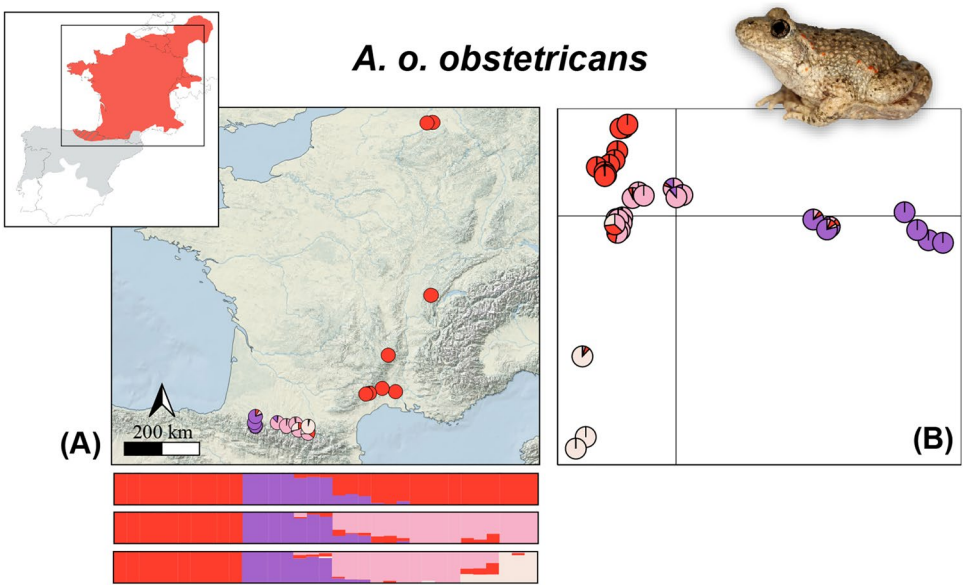

Fig. 10 Genetic clustering with spatial location of clusters **A** and PCA **B** of 33 individuals (18 populations) of *A. o. obstetricans* genotyped at 2307 SNPs for K = 2–4. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones). The highest K is shown on the map and PCA

structure among subspecies of the *A. obstetricans* complex, with special attention to three aspects that may affect GBS inferences: (1) accounting for admixture zones when delineating the distributions of taxa; (2) distinguishing genuine phylogeographic structure from local population differentiation or genetic artefacts (e.g. ghost lineages); and (3) comparing diversity estimates with the challenges posed by locus dropout.

Microsatellite markers are not always reliable in retrieving the ancestry and thus the taxonomic identity of *Alytes* populations (Dufresnes and Hernandez 2021; Ambu et al. 2023). In particular, Bayesian clustering of microsatellite genotypes inferred admixture where there is none (e.g. between the *lusitanicus* and *pertinax* clusters), and isolation where there is gene flow (e.g. at the *almogavarii*/*inigoi* transition). The same issues were evident in another study focused on NE-Iberia (Lucati et al. 2022), where microsatellite ancestry estimates were also not supported by the genome-wide inferences (see their Fig. 6 compared to our Fig. 2A). Concomitantly, mitochondrial-based identification may not be reliable in some regions due to mitochondrial introgression during past and present secondary contact (e.g. *obstetricans* mtDNA across northwestern Spain in *pertinax* and *boscai* populations) or retention of ancestral polymorphisms (e.g. *obstetricans*/*pertinax* at 16S, which share closely related mtDNA due to their history of reticulation, Ambu et al. 2023). These limitations led to misidentification of the Cantabrian populations, which formed a microsatellite

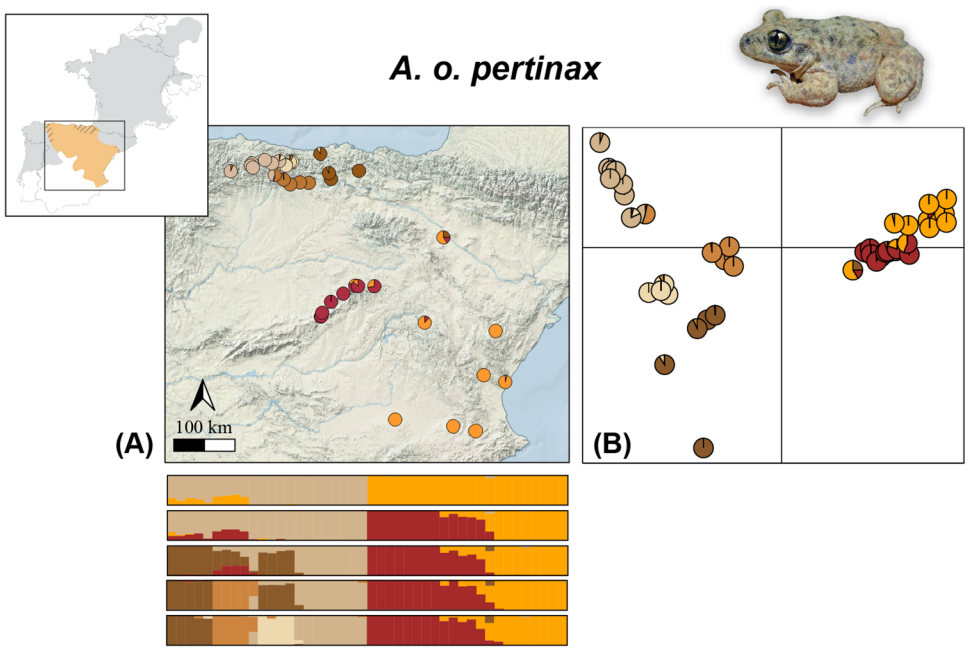

Fig. 11 Genetic clustering with spatial location of clusters **A** and PCA **B** of 44 individuals (36 populations) of *A. o. pertinax* genotyped at 1084 SNPs for K = 2–6. The inset map shows the displayed area and the distribution range of the taxon (dash line: admixture zones). The highest K is shown on the map and PCA

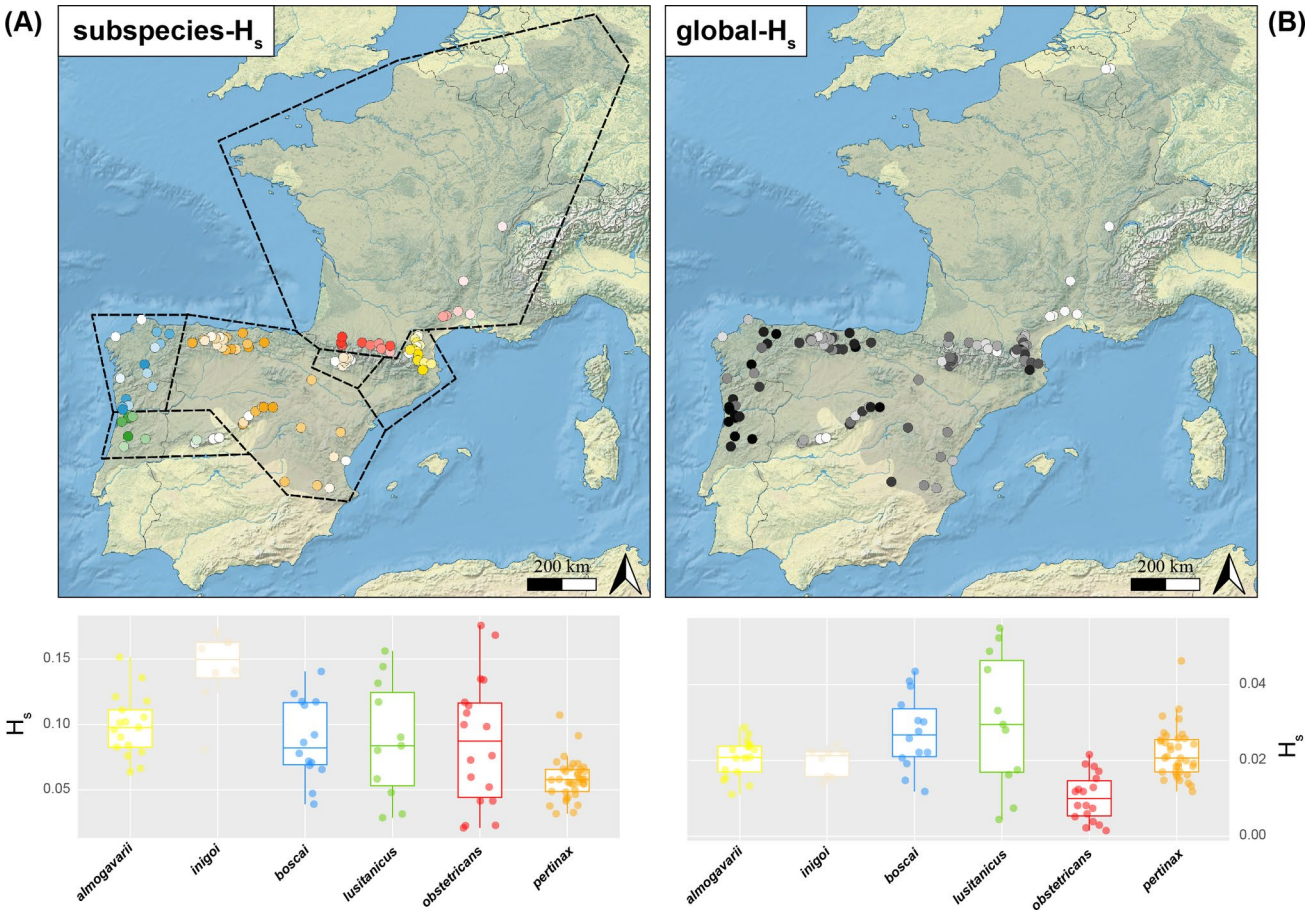

Fig. 12 Geographic and lineage variation and populational expected heterozygosity H_s, computed from subspecies-specific SNP sets **A** and a single SNP set informative in all subspecies **B**. On the maps, relative variations are shown by independent color gradients running from the lowest (white) to the highest (most colorful/darker) estimates. Note the different order of magnitudes of H_s values between the two datasets (y-axis)

cluster differentiated from any subspecies (Maia-Carvalho et al. 2018; Lucati et al. 2022) and was previously assigned to *A. o. obstetricans* based on mtDNA (Dufresnes and Hernandez 2021); in reality, these populations correspond to phylogeographic structure in *A. o. pertinax* (Fig. 11).

The deeply-diverged mitochondrial haplogroups found in Spain and Portugal within *A. o. lusitanicus* imply a phylogeographic break that is not apparent in the analyses of nuclear loci (Fig. 2). Instead, these populations are only slightly differentiated in their nuclear genome (Fig. 9), with much shallower divergence than in their mitochondrion (Fig. 4), potentially reflecting recent demographic events (see below). Accordingly, *A. o. lusitanicus* samples from Spain and Portugal belong to the same nuclear lineage in the phylogenomic tree (Ambu et al. 2023). The unexpected mitochondrial divergence in *A. o. lusitanicus* probably represents a "ghost" lineage, i.e. a mitochondrial lineage that does not correspond to an extant nuclear genome. Such mitochondrial ghost lineages are increasingly being documented (e.g. Hinojosa et al. 2019). This is particularly notable in Iberian amphibians (Dufresnes et al. 2020a) where they possibly represent the signatures of lineages that no longer persist, for instance, because they became genetically assimilated by other lineages through fusion (Garrick et al. 2019). Here, the position of the Portuguese mtDNA in the mitochondrial phylogeny matches the position of *A. o. lusitanicus* in phylogenomic analyses (Ambu et al. 2023). It is thus reasonable to assume that the Spanish mtDNA is the one with a foreign origin and might correspond to an extinct phylogeographic lineage that formerly existed within Central Iberia. Midwife toads appear to have diverged and admixed multiple times throughout their diversification (Ambu et al. 2023) and the Sistema Central mountains are both a hotspot of endemism (some species have been evolving in situ for millions of years, e.g. *Salamandra salamandra almanzoris*, Antunes et al. 2021; Gippner et al. 2024; *Iberolacerta cyreni*, Crochet et al. 2004) and a "melting pot" for herpetofauna (e.g. the hybrid swarm between *Discoglossus galganoi galganoi* and *D. g. jeanneae*, Martínez-Solano 2004; Vences et al. 2014; Dufresnes et al. 2020b). The presence of this ghost lineage is thus not surprising and reminds us that mitochondrial divergence should be interpreted with caution in phylogeographic and taxonomic studies (Dufresnes and Jablonski 2022).

The high resolving power of genomic analyses allows to detect subtle levels of structuring, some of which may be more artefactual (e.g. sampling effect) than biological, which implies the necessity to decide an upper limit when defining distinct populations, especially for operational conservation purposes (e.g. management units). As the real K number of genetic groups present in a genomic dataset should represent the number of distinct, panmictic populations, any deviation from panmixia can generate additional clusters. Such clustering methods perform poorly when genetic diversity varies along a geographic gradient (i.e. IBD). Under IBD, the results of clustering methods are strongly influenced by the sampling scheme (Schwartz and McKelvey 2009) and deciphering the contribution of IBD vs. ecological or geographic barriers to gene flow on genetic population structure becomes challenging (Renner et al. 2016; Bradburd et al. 2018). Regardless of clustering method (e.g. STRUCTURE, Pritchard et al. 2000; DAPC, Jombart et al. 2010) and method to find the most likely K (e.g. Evanno's ΔK, Evanno et al. 2005; BIC, Jombart et al. 2010), the choice ultimately depends on the mechanisms generating differentiation, the scale of analyses and the goals of the study. Here we found that the cross-entropy estimate of *snmf* is a useful statistic to evaluate range-wide structure (here revealed by the first K values, with the lowest cross-entropy) from local population structure (higher K values, with linearly increasing cross-entropy). Clustering with higher K values also retrieved meaningful patterns (e.g. different populations form separate clusters) but is not relevant here given the range-wide scale of our analyses.

The spatial variation in gene diversity within subspecies remained broadly similar whether computed from a few, more conserved loci (global-H_s), or from more, less conserved loci (subspecies-H_s), so both are useful for among-population comparisons. The subspecies-H_s values are more informative for inferring fine-scale patterns given their greater variation but are not appropriate for among-taxa comparisons because the datasets differ in their polymorphism (File S6). These differences can be explained by the conservativeness and thus mutation rates of loci retained in the analyses because of locus dropout. The more diverged the populations included in a dataset, the fewer the number of loci retrieved for downstream analyses, because only loci that are conserved across all samples pass SNP calling filters. As presumably these more conserved markers evolve more slowly, they would be expected to be less polymorphic within populations. Thus, to compare patterns among taxa, the global-H_s is more appropriate, although it is inherently less informative. Locus dropout is a well-known limitation of ddRAD-seq that inevitably constrains analyses to a "local" phylogenetic scale (e.g. a species group or a genus). For these reasons, comparisons of quantitative estimates of genetic diversity or divergence among distantly related taxa should be done with GBS methods that rely on predefined sets of phylogenetically conserved markers (e.g. sequence-capture, Hutter et al. 2022; de Visser et al. 2024).

Historical processes impacting genetic diversity

If one considers monophyly and deep phylogeographic divergence as the hallmark of valid taxa (Dufresnes et al. 2023b; Vences et al. 2024), the six subspecies do represent the evolutionary diversity of the *A. obstetricans* complex.

The additional shallow structure revealed by our analyses rather reflects IBD and recent population isolation during the Late Quaternary, eventually combined with more recent human-mediated declines (see below).

Reflecting earlier microsatellite analyses (Lucati et al. 2022), the most defined structure within subspecies maps to mountain ranges, notably the northern side of the Pyrenees (*A. o. obstetricans*) and the Cantabrian Mountains (*A. o. pertinax*). For *A. o. obstetricans* (Figs. 10, 12, File S7A), this pattern fits well with the expected signature of subdivided glacial refugia in southern ranges vs. genetically homogenous and impoverished populations in northern ranges (Hewitt 2000; Gómez and Lunt 2007; Schmitt 2007). This is also consistent with the negligible mitochondrial variation in Belgium and the Netherlands (Vliegenthart et al. 2023) resulting from recolonization and founder events. Pleistocene refugia in European herpetofauna are often attributed to Mediterranean Peninsulas (Iberia, the Apennine, the Balkans, Gómez and Lunt 2007; Paúl et al. 2023), but evidence for northern refugia have also been presented (Stewart and Lister 2001; Schmitt 2007; Wielstra et al. 2017), especially for cold-tolerant species with Euro-Siberian affinities (e.g. *Rana temporaria*, Vences et al. 2013; *Bufo bufo*, Arntzen et al. 2017). Based on mitochondrial and microsatellite analyses combined with projection of past climatic conditions, a glacial refugium in northern Spain was previously hypothesized for *A. o. obstetricans* (Maia-Carvalho et al. 2018), but these analyses inadequately retrieved the range of this subspecies (see above). Given its present distribution outside Iberia and high genetic variation in the Pyrenees, *A. o. obstetricans* could have persisted throughout the last glacial cycle in a fragmented refugium spanning southern France rather than northern Spain. The existence of refugia outside the Iberian Peninsula is also supported by genetic diversity in *Epidalea calamita*, which similarly features some phylogeographic structure in southern France, from where it presumably recolonized Western Europe after the last glacial maximum (Rowe et al. 2006).

The milder Pleistocene paleoclimates on the southern versus the northern side of the Pyrenees probably offered larger, more connected glacial refugia for *A. almogavarii*. This may underlie the lack of significant structure and the relatively high diversity of its subspecies. These taxa probably also had the opportunity for southern contractions at the peak of the glaciation, unlike other species that were trapped in several isolated pockets, e.g. *Calotriton asper* (Milá et al. 2010). Considered together, these observations emphasize two important roles of the Pyrenean region. First, that it acts as a hotspot of endemism where phylogeographic diversity is generated. Second, that it served as a sanctuary-type refugium (sensu Recuero and García-París 2011), where diversity persisted throughout the Pleistocene. For eastern Pyrenean taxa like *A. a. almogavarii*, the Mediterranean coast further favored connections between both sides of the Pyrenees, as evident in *Pelodytes punctatus* (Díaz-Rodríguez et al. 2015; Dufresnes et al. 2020b), *Pelobates cultripes* (Crottini et al. 2010; Gutiérrez-Rodríguez et al. 2017), and *Emys orbicularis* (Pöschel et al. 2018).

As in southern France, the shallow but complex population structure in *A. o. pertinax* probably reflects subdivision in separate micro-refugia during the final stages of the Pleistocene, resulting from the heterogenous topography of its geographic range (see also Lucati et al. 2022). The different massifs of the Cantabrian Mountains correspond to phylogeographic breaks in other amphibians, as e.g. in *Rana temporaria/parvipalmata* (Vences et al. 2017; Dufresnes et al. 2020a), *Lissotriton helveticus* (Recuero & García-París 2011), and *Salamandra salamandra* (Gippner et al. 2024). We further note that *A. o. pertinax* does not have notably higher levels of heterozygosity relative to the other subspecies, despite its composite ancestry (past introgression from *A. almogavarii*; Ambu et al. 2023). This implies that demographic processes (e.g. drift) involved in the Quaternary histories of populations had a stronger impact than reticulation on present-day genetic variability.

Variations in heterozygosity levels were highest for *A. o. lusitanicus* and *A. o. boscai* (Fig. 12B). Given the longitudinal gradient of diversity between Portugal and the Sistema Central mountains (File S7B), it is tempting to hypothesize an eastward expansion of *A. o. lusitanicus* from an Atlantic refugium, as in *D. g. galganoi* (Martínez-Solano 2004; Vences et al. 2014) and *Rana iberica* (Martínez-Solano et al. 2005). Alternatively, or in addition, the co-existence of the two mitochondrial lineages may indicate that midwife toads persisted in both geographic ranges, possibly maintaining some relative connectivity. The observed depleted genetic variation and genetic differentiation may then be the consequence of recent declines and isolation.

Implications for conservation

Genetic variation underlies all aspects of biodiversity and is key to the long-term persistence of populations, through its effect on individual fitness, adaptive potential to rapidly changing environments, and resilience in the face of inbreeding (Frankham 2005). The different levels of genetic diversity among populations of each *A. obstetricans* subspecies should be considered when evaluating their conservation (Schmidt et al. 2023). The northernmost populations of *A. o. obstetricans*, which are the most threatened (Fig. 1), are also the most genetically impoverished (Fig. 12), which may exacerbate their ongoing decline (Dufresnes and Perrin 2015).

In Spain, the conservation status of populations has been evaluated for subspecies rather than administrative regions (Pleguezuelos et al. 2002). It should be re-assessed in the

light of the classification and distributional updates. Notably, the recently described and geographically restricted *A. o. lusitanicus* has not been evaluated and the range of *A. o. boscai* is smaller than previously assumed, being restricted to northwestern Spain following this taxonomic revision (Ambu et al. 2024a). In this area, midwife toads have regressed over the past decades (M. Vences pers. comm.), and coastal populations have low genetic diversity (Fig. 12). In Central and eastern Spain (Castilla la Mancha, Madrid, Comunidad Valenciana), where some populations are genetically depleted, the subspecies *A. o. pertinax* faces a widespread shortage of breeding sites due to the abandonment of traditional farming and the concomitant deterioration and loss of water tanks associated with livestock and irrigation (Galvez et al. 2018), which has resulted in local population extinctions (Caballero-Díaz et al. 2020).

The results of our study should inform monitoring and conservation actions for improving the situation of these declining taxa. Specifically, we recommend to prioritize the surveillance of populations with depleted genetic diversity, which may collapse more rapidly following habitat perturbation or disease. Such populations could be reinforced by genetic rescue via ex-situ conservation programs, i.e. reintroductions sourced from nearby populations that belong to the same genetic cluster (Fig. 6–11) and feature higher levels of genetic diversity (Fig. 12) (see also Barratt et al. 2024). In dry regions inhabited by fragmented and bottlenecked populations (especially in Central and eastern Iberia), creating new water bodies and/or extending the hydroperiod of existing ones could increase larval survival and juvenile recruitment, and could promote population connectivity (Caballero-Díaz et al. 2022; Gutiérrez-Rodríguez et al. 2023). At the regional level, patterns of genetic structure should aid the delineation of management units and thus the identification of new protected areas that maximize the amount of diversity covered overall, to maintain the adaptive potential of each subspecies (Hanson et al. 2020, 2021). Moreover, the updated distributions of all subspecies offer a framework to evaluate the impact of climate change based on more precise, refined projections of environmental suitability.

Finally, midwife toads have experienced severe declines in Central Spain due to outbreaks of chytridiomycosis (Bosch et al. 2001). One population close to an infected area in Sierra de Guadarrama (Peñalara Natural Park) features the lowest heterozygosity among all *A. o. pertinax* populations, which could indicate past bottlenecks (see also Lucati et al. 2022), while adjacent ranges retained higher diversity (Fig. 12). In the nearby Sierra de Gredos, the striking reduction in the genetic diversity of *A. o. lusitanicus* is puzzling, and while *Bd* has not been widely reported in the area, its role in potential declines cannot be ruled out. Other fatal outbreaks have been monitored in the Central Pyrenees, notably in Valle del Aragon (Ibón de Acherito and surrounding sites, Walker et al. 2010; Bates et al. 2018). While we did not sample this area, we predict the presence of a contact zone between *A. obstetricans* (*obstetricans*) and *A. almogavarii* (*inigoi*), parallel to the one we examined in the Formigal area a few kilometers to the southeast (Ambu et al. 2024b). These two species show restricted admixture suggesting partial post-zygotic reproductive isolation (Dufresnes and Martínez-Solano 2020; Ambu et al. 2024b; Ambu and Dufresnes 2024). The prevalence of *Bd* in a putative contact zone thus offers exciting prospects to test for the consequences of hybridization on the susceptibility of populations to *Bd*. As a symptomatic and asymptomatic carrier of *Bd*, *Alytes obstetricans* has become a model species to identify the abiotic and biotic factors influencing chytrid epidemiology (e.g. Walker et al. 2010; Bates et al. 2018), including its resistance (Greener et al. 2020). Genetic variation has been hypothesized to affect the tolerance to *Bd* infection among populations (Tobler and Schmidt 2010), but phylogeography is seldom considered in classical chytrid assays (e.g. Walker et al. 2010; Bates et al. 2018; Greener et al. 2020). The genetic units that we have identified herein could be the focus of experimental tests to compare the effect of host genetic background on the virulence of infections.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10592-025-01673-7.

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Author contributions JA, IMS and CD planned the study. All authors conducted fieldwork. JA and GSM conducted labwork. JA analyzed the data and drafted the manuscript. CD edited the manuscript. All authors reviewed the manuscript.

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Data availability New mitochondrial sequences were uploaded on GenBank under accessions PQ741975-PQ742064 (16S) and PQ757935-PQ757960 (ND4). Datasets re-used or generated in this study are available on Zenodo (https://doi.org/https://doi.org/10.5281/zenodo.14411535).

Declarations

Competing Interests The authors declare no competing interests.

References

Allentoft ME, O'Brien J (2010) Global amphibian declines, loss of genetic diversity and fitness: a review. Diversity 2:47–71

Ambu J, Dufresnes C (2022) Aperçu spatial des niveaux de diversité et de menaces pour l'herpétofaune française et pays limitrophes. Bull Soc Herpetol Fr 140:4

Ambu J, Dufresnes C (2024) Genomic and bioacoustic variation in a midwife toad hybrid zone: a role for reinforcement? PLoS ONE 19:e0314477

Ambu J, Martínez-Solano Í, Suchan T, Hernandez A, Wielstra B, Crochet P-A, Dufresnes C (2023) Genomic phylogeography illuminates deep cyto-nuclear discordances in midwife toads (*Alytes*). Mol Phylogenet Evol 183:107783

Ambu J, Martínez-Solano Í, Dufresnes C (2024a) A new subspecies of midwife toad (Anura, Alytidae, *Alytes* Wagler, 1830) supported by genomic taxonomy. Alytes 41:18–39

Ambu J, Litvinchuk SN, Caballero-Díaz C, Nicieza A, Velo-Antón G, Gonçalves H, Martínez-Freiría F, Martínez-Gil H, Beltrán JF, Donaire-Barroso D, Hernandez A, Suchan T, Crochet P-A, Martínez-Solano Í, Dufresnes C (2024b) Genomic, phenotypic and environmental correlates of speciation in the midwife toads (*Alytes*). biorxiv 2024.10.24.619835

Antunes B, Velo-Antón G, Buckley D, Pereira RJ, Martínez-Solano Í (2021) Physical and ecological isolation contribute to maintain genetic differentiation between fire salamander subspecies. Heredity 126:776–789

Arntzen JW, García-París M (1995) Morphological and allozyme studies of midwife toads (genus *Alytes*), including the description of two new taxa from Spain. Contrib Zool 65:5–34

Arntzen JW, de Vries W, Canestrelli D, Martínez-Solano Í (2017) Hybrid zone formation and contrasting outcomes of secondary contact over transects in common toads. Mol Ecol 26:5663–5675

Banks SC, Scheele BC, Macris A, Hunter D, Jack C, Fraser CI (2020) Chytrid fungus infection in alpine tree frogs is associated with individual heterozygosity and population isolation but not population-genetic diversity. Front Biogeogr 12:e43875

Barratt CD, Preißler K, Jennert PR, Eckhardt F, Nadjafzadeh M, Steinfartz S (2024) A decision-making framework to maximise the evolutionary potential of populations-genetic and genomic insights from the common midwife toad (*Alytes obstetricans*) at its range limits. Heredity 133:249–261

Barrios V, Olmeda C, Ruiz E (2012) Action Plan for the Conservation of the Common Midwife Toad (*Alytes obstetricans*) in the European Union. European Commission (The N2K Group).

Bates KA, Clare FC, O'Hanlon S, Bosch J, Brookes L, Hopkins K, McLaughlin EJ, Daniel O, Garner TWJ, Fisher MC, Harrison XA (2018) Amphibian chytridiomycosis outbreak dynamics are linked with host skin bacterial community structure. Nat Commun 9:693

Beebee T (2005) Conservation genetics of amphibians. Heredity 95:423–427

Bosch J, Martínez-Solano Í, García-París M, (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biol Conserv 97:331–337

Bradburd GS, Coop GM, Ralph PL (2018) Inferring continuous and discrete population genetic structure across space. Genetics 210:33–52

Brelsford A, Dufresnes C, Perrin N (2016) High-density sex-specific linkage maps of a European tree frog (*Hyla arborea*) identify the sex chromosome without information on offspring sex. Heredity 116:177–181

Bridges CM, Semlitsch RD (2001) Genetic variation in insecticide tolerance in a population of southern leopard frogs (*Rana sphenocephala*): implications for amphibian conservation. Copeia 1:7–13

Caballero-Díaz C, Sánchez-Montes G, Butler HM, Vredenburg VT, Martínez-Solano Í (2020) The role of artificial breeding sites in amphibian conservation: a case study in rural areas in central Spain. Herpetol Conserv Bio 15:87–104

Caballero-Díaz C, Sánchez-Montes G, Gómez I, Díaz-Zúñiga A, Martínez-Solano Í (2022) Artificial water bodies as amphibian breeding sites: the case of the common midwife toad (*Alytes obstetricans*) in central Spain. Amphib-Reptil 43:395–406

Camacho-Sanchez M, Velo-Antón G, Hanson JO, Veríssimo A, Martínez-Solano Í, Marques A, Moritz C, Carvalho SB (2020) Comparative assessment of range-wide patterns of genetic diversity and structure with SNPs and microsatellites: a case study with Iberian amphibians. Ecol Evol 10:10353–10363

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA (2013) Stacks: an analysis tool set for population genomics. Mol Ecol 22:3124–3140

Cayuela H, Valenzuela-Sánchez A, Teulier L, Martínez-Solano Í, Léna JP, Merilä J, Muths E, Shine R, Quay L, Denoël M, Clobert J, Schmidt BR (2020) Determinants and consequences of dispersal in vertebrates with complex life cycles: a review of pond-breeding amphibians. Q Rev Biol 95:1–36

Cortazar-Chinarro M, Lattenkamp EZ, Meyer-Lucht Y, Luquet E, Laurila A, Höglund J (2017) Drift, selection or migration? processes affecting genetic differentiation along a latitudinal gradient in an amphibian. BMC Evol Biol 17:189

Crochet P-A, Chaline O, Surget-Groba Y, Debain C, Cheylan M (2004) Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Mol Phylogenet Evol 30:860–866

Crottini A, Galán P, Vences M (2010) Mitochondrial diversity of Western spadefoot toads, *Pelobates cultripes*, in northwestern Spain. Amphib-Reptil 31:443–448

Díaz Rodríguez J, Gonçalves H, Sequeira F, Sousa-Neves T, Tejedo M, Ferrand N, Martínez-Solano Í (2015) Molecular evidence

for cryptic candidate species in Iberian *Pelodytes* (Anura, Pelodytidae). Mol Phylogenet Evol 83:224–241
Dufresnes C (2019) Amphibians of Europe, North Africa and the Middle East: a photographic guide. Bloomsbury Publishing, London
Dufresnes C, Hernandez A (2021) Phylogeographic advances in midwife toads (*Alytes*) support the existence of a novel taxon endemic to the Central Pyrenees. J Zool Syst Evol Res 59:2170–2179
Dufresnes C, Jablonski D (2022) A genomic revolution in amphibian taxonomy. Science 377:1272
Dufresnes C, Martínez-Solano Í (2020) Hybrid zone genomics supports candidate species in Iberian *Alytes obstetricans*. Amphib-Reptil 41:105–112
Dufresnes C, Perrin N (2015) Effect of biogeographic history on population vulnerability in European amphibians. Conserv Biol 29:1235–1241
Dufresnes C, Wassef J, Ghali K, Brelsford A, Stöck M, Lymberakis P, Crnobrnja-Isailovic J, Perrin N (2013) Conservation phylogeography: does historical diversity contribute to regional vulnerability in European tree frogs (*Hyla arborea*)? Mol Ecol 22:5669–5684
Dufresnes C, Nicieza AG, Litvinchuk SN, Rodrigues N, Jeffries DL, Vences M, Perrin N, Martínez-Solano Í (2020a) Are glacial refugia hotspots of speciation and cyto-nuclear discordances? answers from the genomic phylogeography of Spanish common frogs. Mol Ecol 29:986–1000
Dufresnes C, Pribille M, Alard B, Gonçalves H, Amat F, Crochet P-A, Dubey S, Perrin N, Fumagalli L, Vences M, Martínez-Solano Í (2020b) Integrating hybrid zone analyses in species delimitation: lessons from two anuran radiations of the Western Mediterranean. Heredity 124:423–438
Dufresnes C, Litvinchuk SN, Rozenblut-Kościsty B, Rodrigues N, Perrin N, Crochet P-A, Jeffries DL (2020c) Hybridization and introgression between toads with different sex chromosome systems. Evol Lett 4:444–456
Dufresnes C, Dutoit L, Breslford A, Goldstein-Witsenburg F, Clément L, López-Baucells A, Palmeirim J, Pavlinić I, Scaravelli D, Ševčík M, Christe P, Goudet J (2023a) Inferring genetic structure when there is little: population genetics vs genomics of the threatened bat *Miniopterus schreibersii* across Europe. Sci Rep 13:1523
Dufresnes C, Poyarkov N, Jablonski D (2023b) Acknowledging more biodiversity without more species. PNAS 120:e2302424120
Dufresnes C, Ambu J, Galán P, Sequeira F, Viesca L, Choda M, Álvarez D, Alard B, Suchan T, Künzel S, Martínez-Solano Í, Vences M, Nicieza A (2024) Delimiting phylogeographic diversity in the genomic era: application to an iberian endemic frog. Zool J Linn Soc 202:zlad170
Ersts PJ (2006) Geographic distance matrix generator (version 1.2.3). American Museum of Natural History, Center for Biodiversity and Conservation. http://biodiversityinformatics.amnh.org/open_source/gdmg.
Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol Ecol 14:2611–2620
Fisher MC, Garner TWJ (2020) Chytrid fungi and global amphibian declines. Nat Rev Microbiol 18:332–343
Frankham R (2005) Genetics and extinction. Biol Conserv 125:131–140
Frichot E, François O (2015) *lea*: An r package for landscape and ecological association studies. Methods Ecol Evol 6:925–929
Galvez A, McKnight DT, Monrós González JS (2018) Habitat preferences of breeding amphibians in eastern Spain. Herpetol Conserv Bio 13:453–463
Garrick RC, Banusiewicz JD, Burgess S, Hyseni C, Symula RE (2019) Extending phylogeography to account for lineage fusion. J Biogeogr 46:268–278
Gippner S, Strowbridge N, Šunje E, Capstick M, Amat F, Bogaerts S, Merabet K, Preißler K, Galán P, Martínez-Solano Í, Bonato L, Steinfartz S, Velo-Antón G, Dufresnes C, Elmer KR, Vences M (2024) The effect of hybrids on phylogenomic delimitation of subspecies in *Salamandra*, a highly polymorphic amphibian genus. Salamandra 60:105–128
Gómez A, Lunt DH (2007) Refugia within refugia: Patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N (eds) Phylogeography of Southern European refugia. Springer, Amsterdam, pp 155–188
Gonçalves H, Maia-Carvalho B, Sousa-Neves T, García-París M, Sequeira F, Ferrand N, Martínez-Solano Í (2015) Multilocus phylogeography of the common midwife toad, *Alytes obstetricans* (Anura, Alytidae): contrasting patterns of lineage diversification and genetic structure in the Iberian refugium. Mol Phylogenet Evol 93:363–379
Goudet J (2005) *hierfstat*, a package for r to compute and test hierarchical F-statistics. Mol Ecol Notes 5:184–186
Gouy M, Tannier E, Comte N, Parsons DP (2021) Seaview version 5: a multiplatform software for multiple sequence alignment, molecular phylogenetic analyses, and tree reconciliation. Methods Mol Biol 2231:241–260
Greener MS, Verbrugghe E, Kelly M, Blooi M, Beukema W, Canessa S, Carranza S, Croubels S, De Troyer N, Fernandez-Giberteau D, Goethals P, Lens L, Li Z, Stegen G, Strubbe D, van Leeuwenberg R, van Praet S, Vila-Escale M, Vervaeke M, Pasmans F, Martel A (2020) Presence of low virulence chytrid fungi could protect European amphibians from more deadly strains. Nat Commun 11:5393
Gutiérrez-Rodríguez J, Barbosa AM, Martínez-Solano Í (2017) Present and past climatic effects on the current distribution and genetic diversity of the Iberian spadefoot toad (*Pelobates cultripes*): an integrative approach. J Biogeogr 44:245–258
Gutiérrez-Rodríguez J, Gonçalves J, Civantos E, Maia-Carvalho B, Caballero-Díaz C, Gonçalves H, Martínez-Solano Í (2023) The role of habitat features in patterns of population connectivity of two Mediterranean amphibians in arid landscapes of central Iberia. Landsc Ecol 38:99–116
Hale ML, Burg TM, Steeves TE (2012) Sampling for microsatellite-based population genetic studies: 25 to 30 individuals per population is enough to accurately estimate allele frequencies. PLoS ONE 7:e45170
Hanson JO, Marques A, Veríssimo A, Camacho-Sanchez M, Velo-Antón G, Martínez-Solano Í, Carvalho SB (2020) Conservation planning for adaptive and neutral evolutionary processes. J App Ecol 57:2159–2169
Hanson JO, Veríssimo A, Velo-Antón G, Marques A, Camacho-Sanchez M, Martínez-Solano Í, Gonçalves H, Sequeira F, Possingham HP, Carvalho SB (2021) Evaluating surrogates of genetic diversity for conservation planning. Cons Biol 35:634–642
Haugen H, Dervo BK, Østbye K, Heggenes J, Devineau O, Linløkken A (2024) Genetic diversity, gene flow, and landscape resistance in a pond-breeding amphibian in agricultural and natural forested landscapes in Norway. Evol Appl 17:e13633
Hewitt GM (2000) The genetic legacy of Quaternary ice ages. Nature 405:907–913
Hewitt GM (2011) Quaternary phylogeography: the roots of hybrid zones. Genetica 139:617–638
Hinojosa JC, Koubínová D, Szenteczki MA, Pitteloud C, Dincă V, Alvarez N, Vila R (2019) A mirage of cryptic species: Genomics uncover striking mitonuclear discordance in the butterfly *Thymelicus sylvestris*. Mol Ecol 28:3857–3868

Hitchings SP, Beebee TJC (1998) Loss of genetic diversity and fitness in common toad (*Bufo bufo*) populations isolated by inimical habitat. J Evol Biol 11:269–283

Hodel RG, Chen S, Payton AC, McDaniel SF, Soltis P, Soltis DE (2017) Adding loci improves phylogeographic resolution in red mangroves despite increased missing data: comparing microsatellites and RAD-Seq and investigating loci filtering. Sci Rep 7:1–14

Höglund J, Wengström Å, Rogell B, Meyer-Lucht Y (2015) Low MHC variation in isolated island populations of the Natterjack toad (*Bufo calamita*). Conserv Genet 16:1007–1010

Höglund J, Bolender L, Cortazar-Chinarro M, Meurling S, Laurila A, Hermaniuk A, Dufresnes C (2022) Low neutral and immunogenetic diversity in northern fringe populations of the green toad *Bufotes viridis*: implications for conservation. Conserv Genet 23:139–149

Horner AA, Hoffman EA, Tye MR, Hether TD, Savage AE (2017) Cryptic chytridiomycosis linked to climate and genetic variation in amphibian populations of the southeastern United States. PLoS ONE 12:e0175843

Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267

Hutter CR, Cobb KA, Portik DM, Travers SL, Wood PL Jr, Brown RM (2022) FrogCap: a modular sequence capture probe-set for phylogenomics and population genetics for all frogs, assessed across multiple phylogenetic scales. Mol Ecol Resour 22:1100–1119

Jeffries DL, Copp GH, Lawson Handley L, Olsén KH, Sayer CD, Hänfling B (2016) Comparing RAD seq and microsatellites to infer complex phylogeographic patterns, an empirical perspective in the Crucian carp, *Carassius carassius*, L. Mol Ecol 25:2997–3018

Jombart T (2008) *adegenet*: a R package for the multivariate analysis of genetic markers. Bioinformatics 24:1403–1405

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11:94

Laorden-Romero D, Caballero-Díaz C, Sánchez-Montes G, Ambu J, Dufresnes C, Martínez-Solano Í (2024) Alien amphibian introductions via the plant trade: a breeding population of the Catalonian midwife toad (*Alytes almogavarii*) in Central Spain. Amphib-Reptil 45:357–363

Lexer C, Mangili S, Bossolini E, Forest F, Stölting KN, Pearman PB, Zimmermann NE, Salamin N (2013) 'Next generation' biogeography: towards understanding the drivers of species diversification and persistence. J Biogeogr 40:1013–1022

Li H, Xiang-Ju J, Dai G, Gu Z, Ming C, Yang Z, Ryder OA, Li W-H, Fu Y-X, Zhang Y-P (2016) Large numbers of vertebrates began rapid population decline in the late 19th century. PNAS 113:14079–14085

Lucati F, Miró A, Bosch J, Caner J, Jowers MJ, Rivera X, Donaire-Barroso D, Rebelo R, Ventura M (2022) New insights on patterns of genetic admixture and phylogeographic history in Iberian high mountain populations of midwife toads. PLoS ONE 17:e0277298

Luquet E, David P, Léna JP, Joly P, Konecny L, Dufresnes C, Perrin N, Plenet S (2011) Heterozygosity-fitness correlations among wild populations of European tree frog (*Hyla arborea*) detect fixation load. Mol Ecol 20:1877–1887

Luquet E, Garner TW, Léna JP, Bruel C, Joly P, Lengagne T, Grolet O, Plénet S (2012) Genetic erosion in wild population makes resistance to a pathogen more costly. Evolution 66:1942–1952

Mable BK (2019) Conservation of adaptive potential and functional diversity: integrating old and new approaches. Conserv Genet 20:89–100

Maia-Carvalho B, Gonçalves H, Ferrand N, Martínez-Solano Í (2014) Multilocus assessment of phylogenetic relationships in *Alytes* (Anura, Alytidae). Mol Phylogenet Ecol 79:270–278

Maia-Carvalho B, Vale CG, Sequeira F, Ferrand N, Martínez-Solano Í, Goncalves H (2018) The roles of allopatric fragmentation and niche divergence in intraspecific lineage diversification in the common midwife toad (*Alytes obstetricans*). J Biogeogr 45:2146–2158

Martínez-Solano Í (2004) Phylogeography of Iberian *Discoglossus* (Anura: Discoglossidae). J Zool Syst Evol Res 42:298–305

Martínez-Solano Í, Gonçalves HA, Arntzen JW, García-París M (2004) Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: *Alytes*). J Biogeogr 31:603–618

Martínez-Solano Í, Rey I, García-París M (2005) The impact of historical and recent factors on genetic variability in a mountain frog: the case of *Rana iberica* (Anura: Ranidae). Anim Conserv 8:431–441

McMahon BJ, Teeling EC, Höglund J (2014) How and why should we implement genomics in conservation? Evol Appl 7:999–1007

Milá B, Carranza S, Guillaume O, Clobert J (2010) Marked genetic structuring and extreme dispersal limitation in the Pyrenean brook newt *Calotriton asper* (Amphibia: Salamandridae) revealed by genome-wide AFLP but not mtDNA. Mol Ecol 19:108–120

Miralles A, Secondi J, Pabijan M, Babik W, Lemaire C, Crochet P-A (2024) Inconsistent estimates of hybridization frequency in newts revealed by SNPs and microsatellites. Conserv Genet 25:215–225

Palo JU, Schmeller DS, Laurila A, Primmer CR, Kuzmin SL, Merilä J (2004) High degree of population subdivision in a widespread amphibian. Mol Ecol 13:2631–2644

Paúl MJ, Rosauer D, Tarroso P, Velo-Antón G, Carvalho SB (2023) Environmental and topographic drivers of amphibian phylogenetic diversity and endemism in the Iberian Peninsula. Ecol Evol 13:e9666

Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double Digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. PLoS ONE 7:e37135

Pleguezuelos JM, Márquez R, Lizana M (2002) Atlas y libro rojo de los anfibios y reptiles de españa (2ª impresión). Dirección General de Conservación de la Naturaleza-Asociación Herpetologica Española, Madrid

Pöschel J, Heltai B, Graciá E, Quintana MF, Velo-Antón G, Arribas O, Valdeón A, Wink M, Fritz U, Vamberger M (2018) Complex hybridization patterns in European pond turtles (*Emys orbicularis*) in the Pyrenean region. Sci Rep 8:15925

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Pröhl H, Auffarth J, Bergmann T, Buschmann H, Balkenhol N (2021) Conservation genetics of the yellow-bellied toad (*Bombina variegata*): population structure, genetic diversity and landscape effects in an endangered amphibian. Conserv Genet 22:513–529

Rancilhac L, Miralles A, Geniez P, Mendez-Aranda D, Beddek M, Brito JC, Leblois R, Crochet P-A (2023) Phylogeographic breaks and how to find them: an empirical attempt at separating vicariance from isolation by distance in a lizard with restricted dispersal. Peer Community J 3:e74

Recuero E, García-París M (2011) Evolutionary history of *Lissotriton helveticus*: multilocus assessment of ancestral vs. recent colonization of the Iberian Peninsula. Mol Phylogenet Evol 60:170–182

Renner SC, Suarez-Rubio M, Wiesner KR, Drögemüller C, Gockel S, Kalko EKV, Ayasse M, Frantz AC (2016) Using multiple landscape genetic approaches to test the validity of genetic clusters in a species characterized by an isolation-by-distance pattern. Biol J Linn Soc 118:292–303

Rödin-Mörch P, Luquet E, Meyer-Lucht Y, Richter-Boix A, Höglund J, Laurila A (2019) Latitudinal divergence in a wide-spread

amphibian: contrasting patterns of neutral and adaptive genomic variation. Mol Ecol 28:2996–3011
Rousset F (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. Genetics 145:1219–1228
Rowe G, Beebee TJC (2005) Intraspecific competition disadvantages inbred natterjack toad (*Bufo calamita*) genotypes over outbred ones in a shared pond environment. J Anim Ecol 74:71–76
Rowe G, Harris DJ, Beebee TJC (2006) Lusitania revisited: a phylogeographic analysis of the natterjack toad *Bufo calamita* across its entire biogeographical range. Mol Phylogenet Evol 39:335–346
Savage AE, Zamudio KR (2016) Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. Proc R Soc B 30:283
Schmidt C, Hoban S, Hunter M, Paz-Vinas I, Garroway CJ (2023) Genetic diversity and IUCN Red List status. Conserv Biol 7:e14064
Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. Front Zool 4:1–13
Schmitt T, Hewitt GM (2004) The genetic pattern of population threat and loss: a case study of butterflies. Mol Ecol 13:21–31
Schultze N, Spitzweg C, Corti C, Delaugerre M, Di Nicola MR, Geniez P, Lapini L, Liuzzi C, Lunghi E, Novarini N, Picariello O, Razzetti E, Sperone E, Stellati L, Vignoli L, Asztalos M, Kindler C, Vamberger M, Fritz U (2020) Mitochondrial ghost lineages blur phylogeography and taxonomy of *Natrix helvetica* and *N. natrix* in Italy and Corsica. Zool Scr 49:395–411
Schwartz M, McKelvey KS (2009) Why sampling scheme matters: the effect of sampling scheme on landscape genetic results. Conserv Genet 10:441–452
Shaffer HB, Gidiş M, McCartney-Melstad E, Neal KM, Oyamaguchi HM, Tellez M, Toffelmier EM (2015) Conservation genetics and genomics of amphibians and reptiles. Annu Rev Anim Biosci 3:113–138
Smith D, O'Brien D, Hall J, Sergeant C, Brookes LM, Harrison XA, Garner TWJ, Jehle R (2022) Challenging a host-pathogen paradigm: susceptibility to chytridiomycosis is decoupled from genetic erosion. J Evol Biol 35:589–598
Speybroeck J, Beukema W, Bok B, Van Der Voort J (2016) Field guide to the amphibians and reptiles of Britain and Europe. Bloomsbury Publishing, London
Speybroeck J, Beukema W, Dufresnes C, Fritz U, Jablonski D, Lymberakis P, Martínez-Solano Í, Razzetti E, Vamberger M, Vences M, Vörös J, Crochet P-A (2020) Species list of the European herpetofauna–2020 update by the taxonomic committee of the Societas Europaea Herpetologica. Amphib-Reptil 41:139–189
Spielman D, Brook BW, Frankham R (2004) Most species are not driven to extinction before genetic factors impact them. PNAS 101:15261–15264
Stewart JR, Lister AM (2001) Cryptic northern refugia and the origins of the modern biota. Trends Ecol Evol 16:608–613
Sunde J, Yıldırım Y, Tibblin P, Forsman A (2020) Comparing the performance of microsatellites and RADseq in population genetic studies: analysis of data for pike (*Esox lucius*) and a synthesis of previous studies. Front Genet 11:218
Tobler U, Schmidt BR (2010) Within- and among-population variation in chytridiomycosis-induced mortality in the toad *Alytes obstetricans*. PLoS ONE 5:e10927
Tobler U, Garner TWJ, Schmidt BR (2013) Genetic attributes of midwife toad (*Alytes obstetricans*) populations do not correlate with degree of species decline. Ecol Evol 3:2806–2819
Toews DPL, Brelsford A (2012) The biogeography of mitochondrial and nuclear discordance in animals. Mol Ecol 21:3030–3907
van Riemsdijk I, Arntzen JW, Bucciarelli GM, McCartney-Melstad E, Rafajlović M, Scott PA, Toffelmier E, Shaffer HB, Wieltra B (2023) Two transects reveal remarkable variation in gene flow on opposite ends of a European toad hybrid zone. Heredity 131:15–24
Vences M, Hauswaldt JS, Steinfartz S, Rupp O, Goesmann A, Künzel S, Orozco-terWengel P, Vieites DR, Nieto-Roman S, Haas S, Laugsch C, Gehara M, Bruchmann S, Pabijan M, Ludewig AK, Angelini C, Borkin LJ, Crochet P-A, Crottini A, Dubois A, Ficetola GF, Galán P, Geniez P, Hachtel M, Jovanovic O, Litvinchuk SN, Lymberakis P, Ohler A, Smirnov NA (2013) Radically different phylogeographies and patterns of genetic variation in two European brown frogs, genus *Rana*. Mol Phylogenet Evol 68:657–670
Vences M, de Pous P, Nicolas V, Díaz-Rodríguez J, Donaire D, Hugemann K, Hauswaldt JS, Amat F, Barnestein JAM, Bogaerts S, Bouazza A, Carranza S, Galán P, de la Vega JPG, Joger U, Lansari A, El Mouden EH, Ohler A, Sanuv D, Slimani T, Tejedo M (2014) New insights on phylogeography and distribution of painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula. Amphib-Reptil 35:305–320
Vences M, Sarasola-Puente V, Sanchez E, Amat F, Hauswaldt JS (2017) Diversity and distribution of deep mitochondrial lineages of the common frog, *Rana temporaria*, in northern Spain. Salamandra 53:25–33
Vences M, Miralles A, Dufresnes C (2024) Next-generation species delimitation and taxonomy: implications for biogeography. J Biogeogr 51:1709–1722
de Visser MC, France J, McCartney-Melstad E, Bucciarelli GM, Theodoropoulos A, Shaffer HB, Wielstra B (2024) NewtCap: an efficient target capture approach to boost genomic studies in Salamandridae (True Salamanders and Newts). bioRxiv 2024.10.25.620290
Vliegenthart C, van de Vrede M, den Boer I, Gilbert MJ, Lemmers P, France J, de Visser MC, Struijk RPJH, Wielstra B (2023) The limits of mtDNA analysis for determining the provenance of invasive species: a midwife toad example. Amphib-Reptil 44:27–33
Walker SF, Bosch J, Gomez V, Garner TWJ, Cunningham AA, Schmeller DS, Ninyerola M, Henk DA, Ginestet C, Arthur CP, Fisher MC (2010) Factors driving pathogenicity vs. prevalence of amphibian panzootic chytridiomycosis in Iberia. Ecol Lett 13:372–382
Wielstra B, Zieliński P, Babik B (2017) The Carpathians hosted extra-Mediterranean refugia-within-refugia during the Pleistocene Ice Age: genomic evidence from two newt genera. Biol J Linn Soc 122:605–613
Wielstra B, Salvi D, Canestrelli D (2021) Genetic divergence across glacial refugia despite interglacial gene flow in a crested newt. Evol Biol 48:17–26
Zeisset I, Beebee TJC (2014) Drift rather than selection dominates MHC class II allelic diversity patterns at the biogeographical range scale in natterjack toads *Bufo calamita*. PLoS ONE 9:e100176
Zimmerman SJ, Aldridge CL, Oyler-McCance SJ (2020) An empirical comparison of population genetic analyses using microsatellite and SNP data for a species of conservation concern. BMC Genomics 21:1–16