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Genomic, Phenotypic and Environmental Correlates of Speciation in the Midwife Toads (*Alytes*)

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ABSTRACT

Speciation, i.e., the formation of new species, implies that diverging populations evolve genetic, phenotypic or ecological factors that promote reproductive isolation (RI), but the relative contributions of these factors remain elusive. Here we test which of genomic, bioacoustic, morphological, and environmental differences best predicts RI across a continuum of divergence in the midwife toads (genus *Alytes*), a group of Western Mediterranean amphibians, using a total evidence approach. We found that, without strong geographic barriers to dispersal, the extent of introgression across hybrid zones between phylogeographic lineages, which should reflect the strength of RI, predominantly covaries with genomic divergence. Overall phenotypic differentiation becomes substantial only between well established, fully isolated species. These results suggest that speciation in midwife toads initially involve cryptic lineages, which probably evolve RI through intrinsic (genetic) hybrid incompatibilities. As they continue to diverge, these nascent species eventually differentiate externally, which potentially enforces pre-mating barriers and facilitates sympatry. This speciation scenario has practical implications for species delimitation, notably when using hybrid zones and divergence thresholds as proxies for reproductive isolation.

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1 | Introduction

Speciation is a multifaceted process involving an array of behavioural, ecological, geographic and genetic factors, and understanding how these factors interact through time and space in shaping Earth's amazing biodiversity is one of the oldest and most fascinating topics in evolutionary biology (Coyne and Orr 2004; Nosil 2012; Mérot et al. 2017; Nosil et al. 2017; Nosil et al. 2021). What best characterises a species is also a very practical issue, as it conditions how populations are considered in taxonomy and thereby acknowledged in conservation policies and by society in general (Thomson et al. 2018; Vences et al. 2024). The diversity and complexity of speciation mechanisms are partly why scientists struggle to agree on the way to operationally delimit species (Zachos 2016; Conix et al. 2023; Dufresnes et al. 2023).

It is generally accepted that species correspond to bifurcating genealogical lineages (de Queiroz 2007), which may not fuse back even when given the chance to hybridise during secondary contact (Hillis 2020; Stankowski and Ravinet 2021; Dufresnes et al. 2023; Vences et al. 2024). Accordingly, taxonomic concepts and criteria have either brought the emphasis on the process leading to speciation (e.g., reproductive barriers; "biological" species, Mayr 1942; Coyne and Orr 2004) or on its perceived outcomes (e.g., phylogenetic divergence: "phylogenetic" species, de Queiroz 1998; phenotypic divergence: "morphological" species). Reproductive isolation (RI) and divergence are expected to increase with time, and speciation is accordingly viewed as a gradual phenomenon across the whole animal kingdom (Hedges et al. 2015; Roux et al. 2016; Coughlan and Matute 2020; but see Ronco and Salzburger 2021). Jointly documenting patterns of RI and divergence can thus provide clues on the pace of the speciation clock (e.g., Streicher et al. 2024), and on the mechanisms ticking at various stages of the continuum, especially across the "grey zone" of speciation, i.e., the window of divergence during which reproductive barriers are still permeable (Roux et al. 2016).

In particular, different hypotheses for the buildup of RI imply different expectations for the relationships between RI and divergence. On the one hand, if RI starts with pre-zygotic or extrinsic post-zygotic barriers, i.e., through selection on locally adapted phenotypes, the amount of interbreeding between nascent species should primarily relate to their divergence at the relevant phenotypic traits (Schluter 2009; Servedio et al. 2011; Nosil 2012; Arnegard et al. 2014). On the other hand, if RI starts as a side effect of genome-wide divergence, through the accumulation of intrinsic post-zygotic barriers, such as Dobzhansky –Muller incompatibilities (DMIs) (Muller 1940; Turelli and Orr 1995; Orr 1995; Orr and Turelli 2001; Matute et al. 2010; Moyle and Nakazato 2010), the amount of interbreeding between nascent species should primarily relate to their phylogenetic distances. Given that local adaptation continuously occurs during the geographic isolation of populations (Butlin and Faria 2024), and that post-zygotic barriers can trigger pre-mating barriers (e.g., reinforcement), genomic and phenotypic divergence may also jointly covary with RI (e.g., Streicher et al. 2024). Determining whether nascent species are better characterised by genetics or phenetics can in turn inform taxonomists on the most relevant criteria when delimiting candidate species under integrative taxonomy (Padial et al. 2010), and the RI-divergence relationships may be used in predictive inferences on species status when RI cannot be directly assessed (Dufresnes, Brelsford et al. 2021; Dufresnes et al. 2023; Vences et al. 2024).

Hybrid zones, i.e., geographic areas where diverging lineages meet and admix their genomes, offer open world experiments to quantify RI, notably by modelling the spatial extent of introgression with sigmoid clines, provided a dense transect sampling (Hewitt 1988; Barton and Hewitt 1989; Harrison 1993; Payseur 2010). Given the amount of resources needed to analyse just a single hybrid zone, however, introgression-divergence relationships based on hybrid-zone data usually rely on meta-analyses, both to increase sample sizes and to provide a broader taxonomic scope (McEntee et al. 2020; Dufresnes, Brelsford et al. 2021). In turn, these must cope with combining cline estimates measured at various types and numbers of loci, from organisms that differ in their dispersal capabilities (which affects introgression alongside RI), and from divergence metrics obtained with independent methods and sources (e.g., species ages inferred from molecular clocks; Kumar et al. 2022). As a consequence, introgression-divergence correlations often rely on ordinal rather than quantitative statistics (e.g., Dufresnes, Strachinis et al. 2019; Dufresnes, Mazepa et al. 2019; Dufresnes et al. 2020) or expectedly leave a large proportion of variance unexplained (e.g., Morgan-Richards and Wallis 2003; Arntzen et al. 2014; McEntee et al. 2020; Pulido-Santacruz et al. 2020; Dufresnes, Brelsford et al. 2021). The best way to alleviate this "background noise" is to relate introgression and divergence at a local taxonomic scale, and compute them with the same methodology, providing that enough pairs of lineages can be studied (e.g., Singhal and Moritz 2013; Pabijan et al. 2017; Streicher et al. 2024).

Midwife toads of the genus *Alytes* offer the opportunity to explore the genetic, ecological, and phenotypic determinants of reproductive isolation and speciation along a continuum of divergence, from Pleistocene phylogeographic lineages forming secondary contact zones to Miocene species sustaining in sympatry. Widely distributed from low to high elevations (above 2000 m) across the Western Mediterranean region, *Alytes* are known for their parental care behaviour—the male carries the eggs on its back for several weeks until the larvae are ready to hatch (Speybroeck et al. 2016). High variation in habitat use, climatic tolerance, mating calls, and morphology has been reported across the genus (Arntzen and García-París 1995; Reino et al. 2017; Rodríguez-Rodríguez et al. 2020; Martínez-Gil et al. 2022), but how this variation relates to the phylogeographic diversity and speciation processes remains largely unaddressed, in part because the distribution and diversification of taxa have been studied based on discordant molecular markers (Maia-Carvalho et al. 2014; Dufresnes and Hernandez 2021; Ambu et al. 2023).

Phylogeographic (Martínez-Solano et al. 2004; Gonçalves et al. 2007; Gonçalves et al. 2015; Maia-Carvalho et al. 2018; Dufresnes and Hernandez 2021; Lucati et al. 2022), and more recently phylogenomic analyses (Ambu et al. 2023), have confirmed the existence of ten evolutionary lineages, currently delimited into six species, which fall into three major clades considered as distinct subgenera (summarised in Figure 1). In

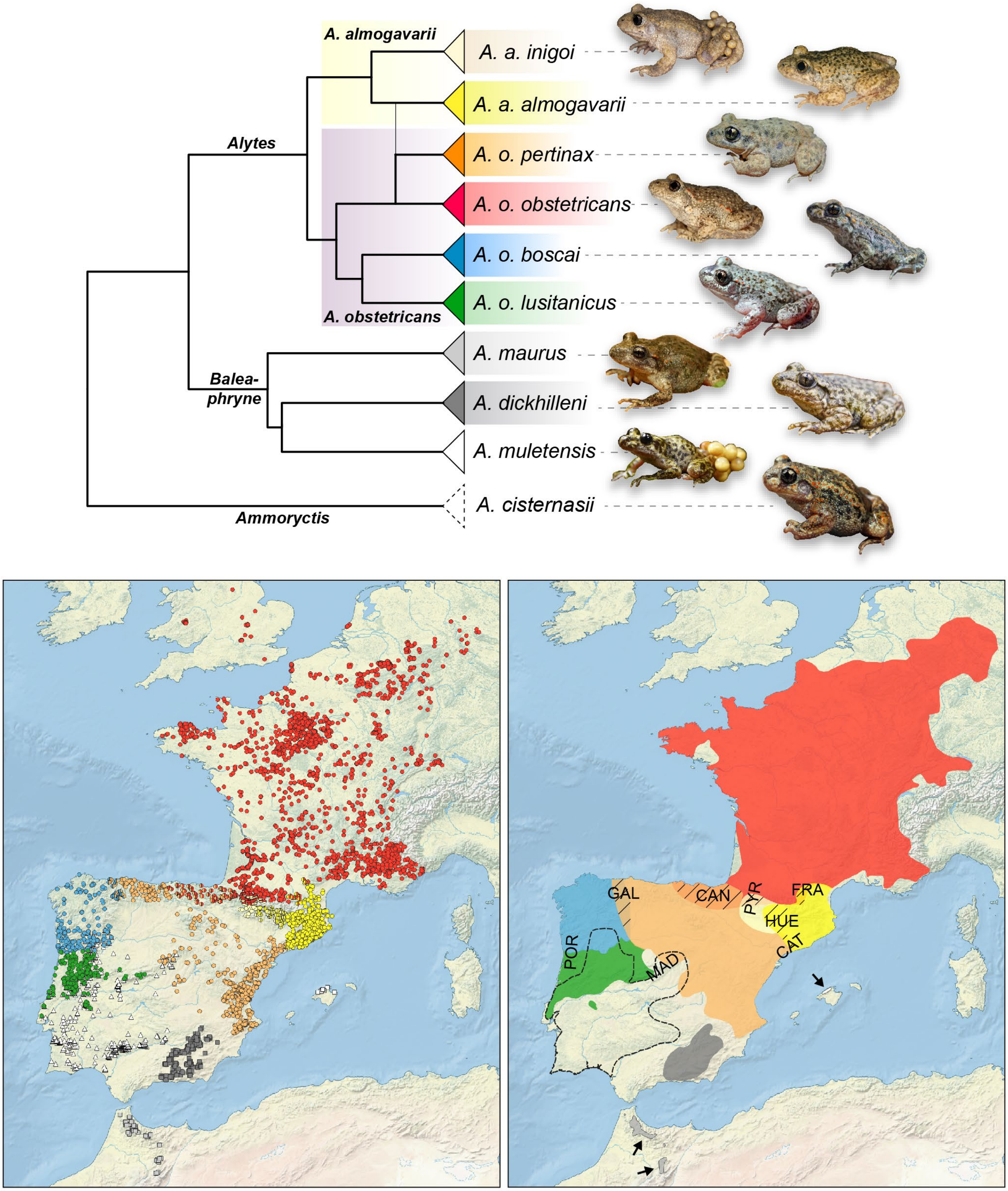

FIGURE 1 | Evolution, distribution and contact zones in midwife toads. The tree reflects phylogenomic analyses adapted from Ambu et al. (2023); the putative hybrid origin of *A. o. pertinax* is emphasised. Branch labels identify the three subgenera and the two species of the subgenus *Alytes*. The bottom left map shows occurrence records assigned to taxa based on our results (mixed circles: admixed localities). The bottom right map shows their distributions, with emphasis on the analysed contact zones. Arrows point to the geographically restricted *A. maurus* and *A. muletensis*.

particular, the subgenus *Alytes* features six phylogeographic lineages of Plio-Pleistocene origin (Figure 1) with mostly continuous distributions and multiple potential contact zones (Maia-Carvalho et al. 2018). These lineages have long been regrouped as a single species, *A. obstetricans*, based on phenotypic resemblance, young divergence, and instances of introgressive hybridisation (Gonçalves et al. 2007; Maia-Carvalho et al. 2018). However, genomic analyses have reported that admixture between the most diverged lineages is seemingly constrained to narrow hybrid zones, which is consistent with partial reproductive isolation and suggests that *A. obstetricans* represents a species complex (Dufresnes and Martínez-Solano 2020; Ambu and Dufresnes 2024). Accordingly, the subgenus *Alytes* is now divided into two species, *A. obstetricans* and *A. almogavarii*, which respectively consist of four and two phylogeographic lineages considered as subspecies (Speybroeck et al. 2020; Frost 2024; Ambu et al. 2024; Figure 1). Midwife toads thus present a gradient of divergence spanning intra- and interspecific lineages hybridising at various degrees (within the subgenus *Alytes*), which live nearby phylogenetically more distant species from other subgenera (e.g., *A. cisternasii*).

This study investigates how RI, genomic, phenotypic, and ecological differentiation correlate in the midwife toads through a total evidence approach. We first analyse population genomics data obtained with double-digest restriction-associated DNA sequencing (ddRAD-seq) to identify and examine the putative contact zones between eight pairs of admixing lineages from the subgenus *Alytes*, and further assess whether these lineages still hybridise with sympatric/parapatric species from the other subgenera. We then compiled morphometric measurements, audio recordings of mating calls (one of the primary reproductive cues of anurans, Köhler et al. 2017), and occurrence datasets for species distribution modelling to compare the general phenotypes and environmental envelopes of the ten species and subspecies of the genus in respect to their permeability to gene flow and phylogenetic divergence. If speciation in midwife toads is primarily initiated by intrinsic post-zygotic barriers, namely hybrid genetic incompatibilities, the geographic extent of admixture, as measured from sigmoid clines, should decrease with the genome-wide divergence of the interacting lineages, and nascent species may remain behaviorally, morphologically, and ecologically similar.

2 | Methods

2.1 | Hybrid Zone Location and Analyses

We processed ddRAD-seq data for 332 samples (File S1), including 205 new ones and 127 included in our previous studies (Ambu et al. 2023; Ambu and Dufresnes 2024). Among them, 314 correspond to the subgenus *Alytes*, with a dense coverage of seven contact zones. For the eighth contact zone, we reused the ddRAD-seq results obtained for another 111 samples from northeastern Spain (Dufresnes and Martínez-Solano 2020). In total, the study thus included 443 genetic samples.

Genomic DNA was isolated from buccal swabs (adults; stored dry at −20°C) and tail tips (tadpoles; stored in 70% ethanol) using an *ad hoc* salt protocol (Bruford et al. 1992), and checked for yield and integrity on agarose gels. Four genomic libraries were prepared following a custom protocol (dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1) as fully described in Dufresnes et al. (2025). Libraries were sequenced paired-end on an Illumina NextSeq 550, using either the 2 × 75 bp or 2 × 150 bp High-Output kit. Paired-end reads were demultiplexed with STACKS 2.59 (Catchen et al. 2013) and trimmed to 65 bp for all samples. We then used the denovo_map.pl pipeline of STACKS for RAD loci construction, assembly, and cataloguing (default −*m*, −*n*, and −*M* values), with PCR duplicate removal (−*rm-pcr-duplicates*). The STACKS catalogue contained 540,223 loci, with an average coverage of 17.6 reads (5.4–45.4, Standard deviation = 5.9). The population module of STACKS was used to export SNP and allele frequency datasets for the downstream analyses detailed below. File S1 highlights the samples used in each analysis. All datasets and raw results are available online (Ambu and Dufresnes 2025).

To examine lineage distribution and locate contact zones in the subgenus *Alytes*, we obtained a SNP matrix for the 314 samples (163 localities) by retaining RAD tags sequenced in at least 150 localities (−*p* 150), in at least half of the samples from each locality (−*r* 0.5), and by randomly choosing a single SNP per RAD tag (−*write-random-snp*). This dataset comprises 5111 SNPs, with 9.8% ± 2.9% of missing data and a coverage of 70.0 ± 44.7 reads (mean ± standard deviation). The dataset was analysed in STRUCTURE 2.3.4 (Pritchard et al. 2000) through runs of 200,000 iterations (20,000 burnin) for $K = 1$–10, with a particular emphasis for $K = 6$ to distinguish the six subspecies and infer the ancestry of samples to each of them. In addition, the STRUCTURE results of Dufresnes and Martínez-Solano (2020), which were based on 433 SNPs, provided the ancestry to *A. a. almogavarii* and *A. o. pertinax* for the 111 samples (18 localities) of northeastern Spain.

To investigate RI between hybridising lineages, we conducted a series of analyses on sample sets taken from eight areas where different lineages presumably come into contact, named Cantabria (CAN), Huesca (HUE), Portugal (POR), Madrid (MAD), Galicia (GAL), Pyrenees (PYR), Catalonia (CAT) and France (FRA) (Figure 1). The first six consist of new analyses, while for CAT and FRA, we reused the results obtained with the same approach by Dufresnes and Martínez-Solano (2020) and Ambu and Dufresnes (2024). For each pair of lineages, we first selected samples from allopatric and parapatric localities, featuring less than 50% of missing data in the global dataset above. A SNP matrix was obtained by retaining the RAD tags sequenced in all localities of the subset (−*p* number of localities), in at least half of the samples from each locality (−*r* 0.5), and by randomly choosing a single SNP per RAD tag (−*write-random-snp*). The matrix was analysed in a STRUCTURE run (same parameters as above) with $K = 2$ to compute the average population ancestry (Q_{pop}) for each lineage of the pair. Second, the geographic extent of gene flow was quantified by fitting sigmoid clines to Q_{pop} for populations sampled along transects. To this end, we used the *R* package *hzar* (Derryberry et al. 2014), choosing the cline model with two parameters (width *w* and center *c*) to compare estimates across the different hybrid zones without overparameterisation. Third, we characterised the heterogeneity of gene flow throughout the genome by fitting sigmoid clines to allele frequency data at lineage-diagnostic SNPs, i.e., with fixed alleles between the pure populations of each subspecies.

To frame the upper edge of the grey zone of *Alytes* speciation, we also examined whether the southern lineages of the subgenus *Alytes* (*A. o. lusitanicus*, *A. o. pertinax*) hybridise and admix with species from other subgenera that occur within or close to their ranges (subgenus *Ammoryctis*: *A. cisternasii*; subgenus *Baleaphryne*: *A. dickhilleni*). For this, we selected two additional subsets of samples from which we obtained two SNP matrices: one restricted to the RAD tags shared between *A. cisternasii*, *A. o. lusitanicus*, and *A. o. pertinax* from Central Spain (−*p* 3), present in at least half of the samples in each group (−*r* 0.5), and randomly choosing a single SNP per RAD tag (−*write-random-snp*); and one restricted to the RAD tags shared between *A. dickhilleni* and *A. o. pertinax* from SE-Spain (−*p* 2), present in at least half of the samples in each group (−*r* 0.5), and also randomly choosing a single SNP per RAD tag (−*write-random-snp*). These datasets were analysed with STRUCTURE (same parameters as above) with $K = 3$ and $K = 2$, respectively.

2.2 | Genomic Divergence

As a proxy to genomic divergence, net pairwise sequence divergence between all *Alytes* species/subspecies was computed based on the concatenated RAD tag sequence alignment used in the phylogenomic analyses of Ambu et al. (2023), which comprised 278,267 bp (including 13,764 SNPs) sequenced in 45 non-admixed samples (File S1). The analysis was conducted in MEGA X (Kumar et al. 2018), using the Maximum Composite Likelihood model (Tamura et al. 2004).

2.3 | Bioacoustic Differentiation

The advertisement call of midwife toads consists of single short high-pitched "whistling" notes, emitted at night from the ground or rock/wall crevices. To study the diversity of calls, we combined our own recordings (made with a PCM-A10 Sony recorder) with recordings from online media repositories. Taxonomic assignments follow our phylogeographic results (see Results), and records of unclear origin or from areas of admixture were not included. Recordings were processed and analysed in Raven Pro 1.6.1 (theCornellLab). Specifically, we homogenised sample rates to 44,100 Hz, filtered the calls between 1 to 2 kHz (the broad frequency spectrum of the target species) and created standardised spectrograms (DFT = 1024 samples, overlap = 90%, spectral resolution = 43.1 Hz), following the recommendations of Köhler et al. (2017). Unique individuals were identified, and their notes were measured for four call parameters: the dominant frequency (DF) in kHz; the note duration (ND) in s; the rising time (RT) in s; and the pulse rate (PR) in s^{-1} (for a graphical explanation, see File S1 in Ambu and Dufresnes 2024). The final bioacoustic dataset includes 720 notes from 153 individuals (1–9 notes per individual) representative of all taxa (available in Ambu and Dufresnes 2025). For each parameter, individual averages were used in the statistical analyses.

To visualise call variation, we performed two Principal Component Analyses (PCA) on scaled data using the *R* package *FactoMineR* (Lê et al. 2008): one on the whole genus and the other restricted to subgenus *Alytes*. To evaluate relative call differences between species/subspecies, we computed a matrix of multivariate pairwise Euclidean distances, from which we built a neighbour-joining (NJ) tree (R package *stats*; R Core Team 2023).

2.4 | Morphometric Differentiation

We re-analysed the raw measurements of museum specimens made by Martínez-Gil et al. (2022), reassigned to species and subspecies according to our phylogeographic results. Eight characters were measured (details in Martínez-Gil et al. 2022): snout-vent length (SVL); mouth length (ML); head width (HW); forelimb length (FLL); femur length (FML); tibia length (TBL); foot length (FTL); hindlimb length (HLL). Specimens of unclear origin or originating from areas of admixture were discarded. The final morphometric dataset consists of 211 individuals representing all taxa (available in Ambu and Dufresnes 2025).

To compare body shape without the effect of body size, we applied allometric corrections (Chan and Grismer 2022) with the *R* package *GroupStruct*, using the multispecies method to perform taxon-specific adjustments. As above, variation in body shape was assessed with two PCAs and by computing a matrix and NJ tree of multivariate pairwise Euclidean distances.

2.5 | Environmental Differentiation

We inferred the environmental conditions associated with each species and subspecies based on 35 variables (File S1) in raster format with 30 arc sec resolution (~1 km): 19 bioclimatic layers extracted from the WorldClim 1.4 database (http://www.worldclim.org); 11 land cover layers, including the aridity index (http://www.cgiar-csi.org/data/global-aridity-and-pet-database), the global percent of tree coverage (https://github.com/globalmaps/gm_ve_v1), the global land cover (https://gee-community-catalog.org/projects/globcover_esa/), the spatial homogeneity of global habitat, broadleaf forests, needleleaf forests, mixed forests, shrubs, barren, herbaceous, and cultivated vegetation (https://www.earthenv.org/); five landscape layers, including elevation, hillshade, slope (three nearest cells), the topographic position index, and the terrain ruggedness index.

A total of 7829 high precision (< 1 km) occurrences of *Alytes* were gathered from our own records, museum collections, published data, and publicly available databases (Figure 1; available in Ambu and Dufresnes 2025). Records were assigned to taxa following our phylogeographic results, and those from areas of admixture were subsequently excluded, resulting in 7086 localities. As above, variation among species/subspecies was assessed by two PCAs and a matrix/NJ tree of multivariate pairwise Euclidean distances. To assess the link between environmental differentiation and geography, we also computed pairwise Euclidean spatial distances among the occurrence records of each taxon.

Ecological niche models were reconstructed with MaxEnt 3.4.4 (Phillips et al. 2006), using all variables under the WGS84 projection and a single mask spanning from 31° N to 56° N and 11° W to 15° E, which broadly covers the genus range. For each model, 10 replicates were run, retaining 30% of occurrences for random testing and 20,000 background points. The relative contributions of variables were estimated through a jackknife analysis, and maps of projected distributions were processed with the ClogLog output format (Phillips et al. 2017). Model calibration involved the evaluation of 372 candidate models produced with distinct regularisation multipliers (0.5–6 at intervals of 0.5) and feature classes (resulting from all combinations of linear, quadratic, product, threshold, and hinge response types). The best model was selected considering statistical significance (partial ROC), predictive power (omission rates $E = 5\%$), and complexity level (AICc), computed with the R package *kuenm* (Cobos et al. 2019), and its performance was assessed using the Area Under the Curve (AUC) and the True Skill Statistic (TSS) of the 10 percentile training omission threshold (Allouche et al. 2006). Niche overlap between pairs of lineages was quantified using Schoener's *D* distance (Schoener 1968) computed in ENMTools (Warren et al. 2021).

2.6 | Comparisons Across Datasets

In the absence of environmental barriers to dispersal, cline width (w), which reflects the geographic extent of introgression between two parental genomes across their hybrid zone, offers an *ad hoc* proxy to compare the degree of RI between pairs of lineages with similar dispersal capacities (Dufresnes, Suchan et al. 2021; Dufresnes et al. 2023). To explore which factors best determine RI between lineages of the subgenus *Alytes*, we related the cline width w to the pairwise genomic, bioacoustic, morphological, and environmental divergence computed above. Two comparisons were performed: one on the eight pairs of lineages, and one excluding two pairs of lineages whose transitions appear mediated by geographic barriers (see Results). We further assessed how phenotypic and environmental divergence covaried with genomic divergence, within the subgenus *Alytes*, and across the whole genus. Finally, we examined whether the genetic transitions match environmental transitions for the lineages involved by overlaying, for each transect, the genome average cline with the occurrence probabilities of the sampled populations predicted by the ecological niche models.

3 | Results

3.1 | Lineage Distribution and Hybrid Zone Analyses

In the subgenus *Alytes*, clustering analyses (STRUCTURE) of 314 individuals (163 localities) genotyped at 5111 SNPs suggested up to six genetic groups (File S3), corresponding to the six subspecies *A. a. almogavarii*, *A. a. inigoi*, *A. o. obstetricans*, *A. o. pertinax*, *A. o. boscai*, and *A. o. lusitanicus*. Combined with the ddRAD-seq results from Dufresnes and Martínez-Solano (2020), lineage distributions and geographic transitions were inferred based on 425 individuals from 181 localities, many of which showed intermediate ancestry coefficients, suggestive of admixture (Figure 2).

The cline widths w computed from the average genome ancestries (Q_{pop}) reflect wide (> 50 km: CAN, HUE), intermediate (30–50 km: GAL), narrow (10–30 km: CAT, FRA, PYR) and very narrow (≤ 10 km: POR, MAD) hybrid zones (Table 1, Figure 3). Locus-specific clines yield similar patterns (Table 1, Figure 4); the medians of their w estimates closely matched the genome average Q_{pop} w estimates (File S4). The locus-specific analyses further documented high variation at cline parameters for the wide hybrid zones (CAN, HUE), while the variation is generally reduced for the narrow hybrid zones, where most loci featured clines resembling the genome average (Figure 4).

Two of the narrowest hybrid zones, POR and MAD, featured a distinct pattern, namely very narrow clines for most individual loci ($w < 5$ km), but striking outlier loci with $w > 100$ km (Figure 4). A close inspection of their transitions suggests current geographic isolation. In POR, the predicted center of the hybrid zone corresponds to the Douro River, the highest flow river of the Iberian Peninsula. In our transect area, the Douro River consists of a > 250 m wide brackish estuary surrounded by the Porto urban area, which must effectively separate populations on the northern (*A. o. boscai*) and southern (*A. o. lusitanicus*) shores. In MAD, the predicted center of the hybrid zone falls within a ~ 75 km sampling gap between the Sierra de Gredos (*A. o. lusitanicus*) and the Sierra de Guadarrama (*A. o. pertinax*), which substantially reduces the interpretation of cline width estimates—the confidence interval of the genome average w accordingly spans over more than 100 km (Table 1, Figure 3). This gap seems to represent a real absence of *A. obstetricans*, since only *A. cisternasii* was found in the area (Figure 1). Therefore, while these pairs of lineages probably experienced secondary contacts, as seen from the faint traces of admixture (e.g., the few wide clines, Figure 4), they are now probably allopatric. In contrast, the other six transitions span areas where midwife toads could be continuously sampled (Figure 1), and even in the narrowest hybrid zones, the shared ancestries in populations close to the centers support ongoing gene flow and thus population connectivity between contacting lineages (Figure 2).

Finally, STRUCTURE did not suggest ongoing gene flow between the subgenus *Alytes* and species from other subgenera in areas of sympatry/parapatry (Figure 5). Analyses of *A. cisternasii*, *A. o. lusitanicus*, and *A. o. pertinax* ($K = 3$), and of *A. o. pertinax* and *A. dickhilleni* ($K = 2$), perfectly discriminate each taxon without any mixed ancestry (Figure 5).

3.2 | Genomic Divergence

Based on 278,267 bp (including 13,764 SNPs), net pairwise divergence ranged 1.4‰–9.1‰ across the whole genus, including 1.4‰–4.1‰ among *A. obstetricans* and *A. almogavarii* (subgenus *Alytes*), 3.7‰–5.7‰ among *A. maurus*, *A. muletensis*, *A. dickhilleni* (subgenus *Baleaphryne*), and > 3.9‰ between *A. cisternasii* (subgenus *Ammoryctis*) and other taxa (File S5).

3.3 | Bioacoustic Differentiation

Call parameters, summarised in four variables measured in 153 individuals, generally differ between the subgenus *Alytes* and species from other subgenera (Figure 6A, File S6), except the Moroccan *A. maurus*, which is, however, represented by a single individual. The PCA based on all species partly distinguishes

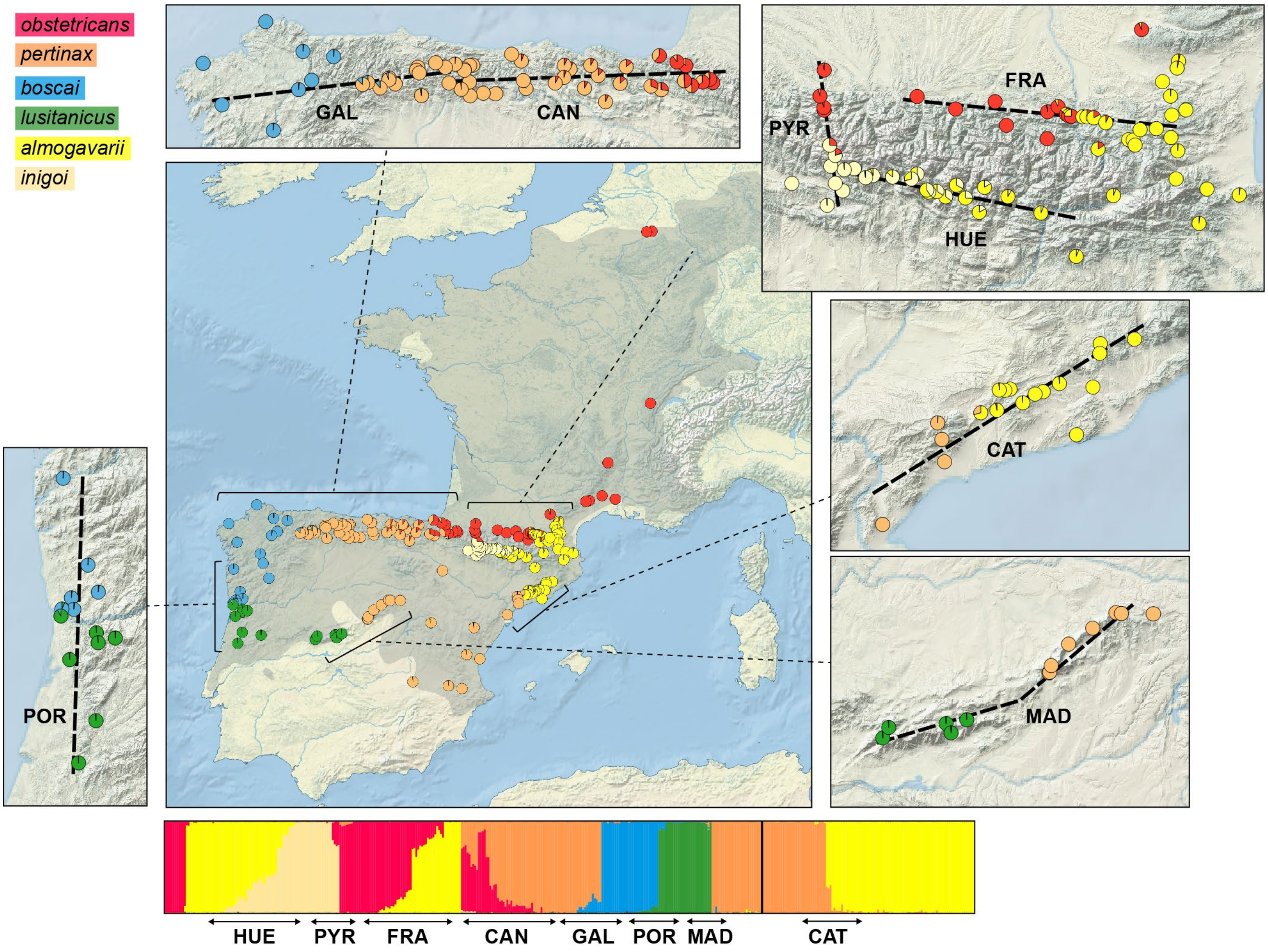

FIGURE 2 | Genetic structure and admixture in the subgenus *Alytes*. Barplots show individual ancestry as inferred by the clustering analysis (STRUCTURE) of 5111 genomic SNPs in six groups (left), or of 433 genomic SNPs in two groups (right; adapted from Dufresnes and Martínez-Solano (2020). Average ancestries are represented on the maps, with insets zooming in on parapatric ranges. On the main map, the known distribution is shown in shaded grey (Figure 1). On the insets, the transects used in the cline analyses (Figures 3 and 4) are shown in dashed lines.

TABLE 1 | Summary statistics for the cline analyses in the eight hybrid zones surveyed; *w*: Width, *c*: Center; CI: 95% confidence interval; sd: Standard deviation. Raw estimates for each diagnostic SNP can be found in the data associated with this study (Ambu and Dufresnes 2025).

			Genome average			Diagnostic SNPs		
	Lineage 1	**Lineage 2**	**loci**	***w* (CI)**	***c* (CI)**	**Loci**	***w* (sd)**	***c* (sd)**
CAN	*obstetricans*	*pertinax*	4042	124.6 (64.8–202.1)	205.5 (174.4–229.1)	279	149 (77.8)	213.9 (43.6)
HUE	*almogavarii*	*inigoi*	2958	57.2 (33.4–116.1)	58.1 (41.2–66.1)	744	53.7 (33.1)	53.1 (17.7)
POR	*boscai*	*lusitanicus*	4967	4.1 (2.3–12.6)	102.6 (100.3–105.1)	2108	4.1 (19.7)	103 (5.7)
MAD	*lusitanicus*	*pertinax*	2127	10.1 (2.4–143.6)	114.7 (64.5–164.4)	2035	2 (17.9)	120.3 (19.8)
CAT	*almogavarii*	*pertinax*	433	15.4 (11.4–26.8)	89.7 (83.9–95.4)	105	16.6 (16.3)	89.6 (4.9)
GAL	*boscai*	*pertinax*	5134	38.1 (17.8–85.6)	186.2 (168.9–198.8)	1176	32.4 (24.2)	189.9 (8.6)
FRA	*almogavarii*	*obstetricans*	3364	21.8 (17.5–43.4)	106.8 (101.4–111.3)	4092	21.2 (9.5)	106.9 (4)
PYR	*obstetricans*	*inigoi*	4042	16.6 (7.4–52.8)	46.8 (33.5–54.9)	8073	8.4 (12.7)	48.3 (6.6)

the Majorcan *A. muletensis* by axis 1, which was influenced by variables DF and PR, and the Iberian *A. cisternasii* and *A. dickhilleni* by axis 2, which was influenced by variables RT and ND.

In contrast, the six lineages of the subgenus *Alytes* share broadly similar call characteristics and are grouped together in the NJ tree of multivariate Euclidean distances (Figure 6A).

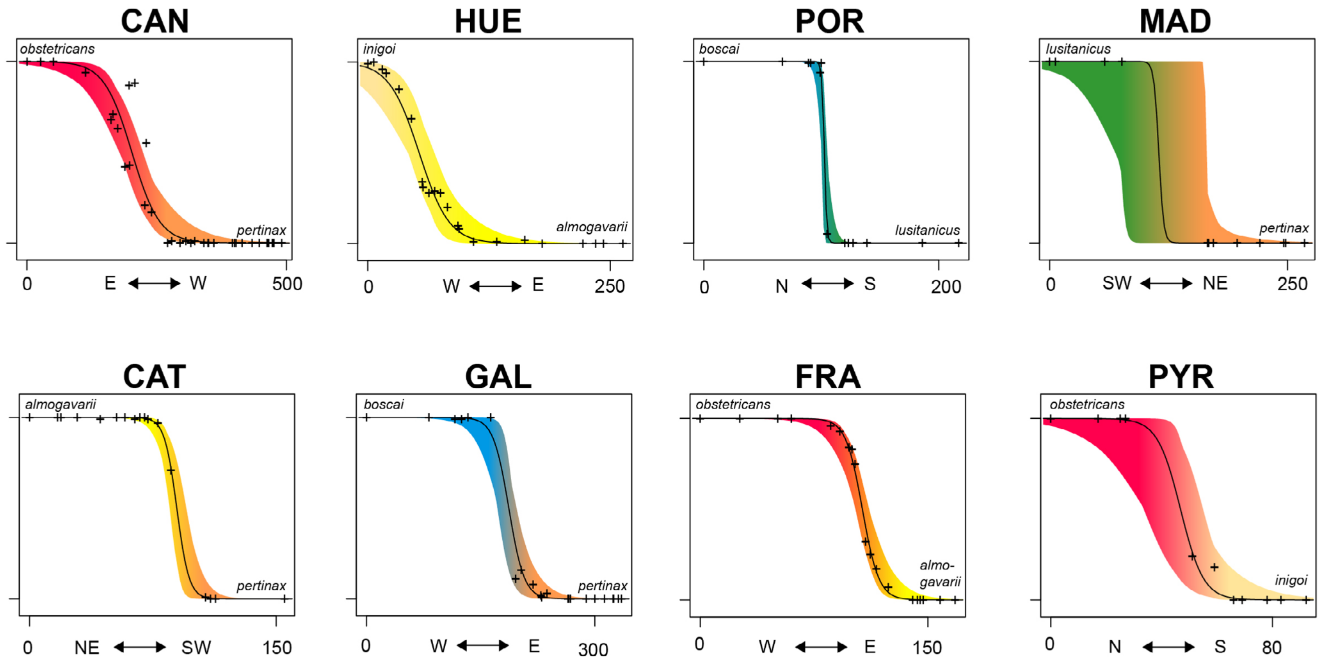

FIGURE 3 | Geographic clines of genome-average ancestries fitted along transects for eight pairs of lineages of the subgenus *Alytes*. Lines show the average clines, and coloured areas show their 95% confidence intervals. Colour gradients illustrate the lineages involved (see Figure 1). Distances are in km. Contact zones are arranged according to the phylogenetic distances of the lineages involved.

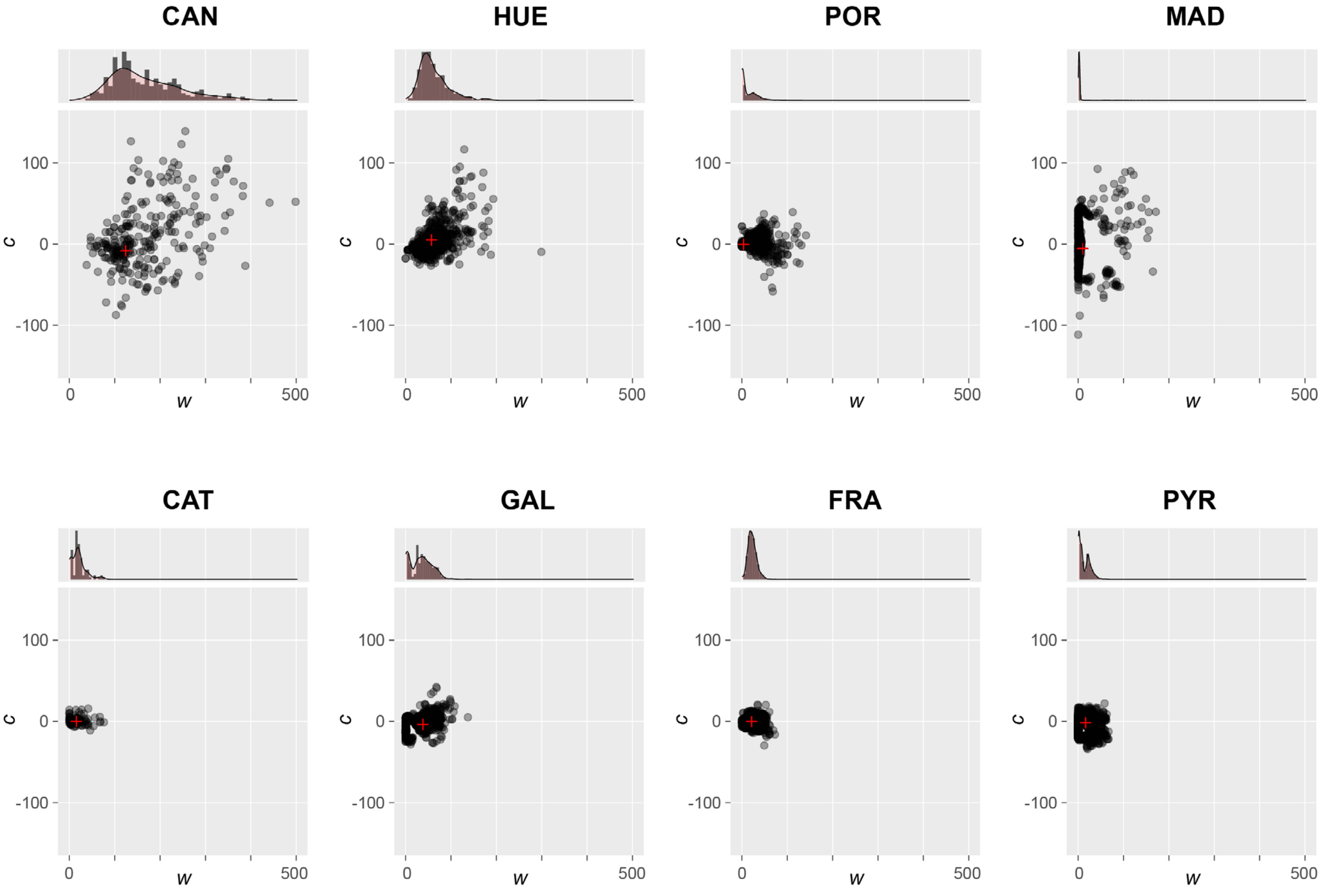

FIGURE 4 | Cline widths (w) and center (c) computed separately for each diagnostic SNP along the eight transects analysed in the subgenus *Alytes*. Center estimates correspond to deviation from the genome average center. Red crosses show the mitochondrial clines. Histograms show estimate distributions (red overlay: Density) for w. All units are in km. Contact zones are arranged according to the phylogenetic distances of the lineages involved.

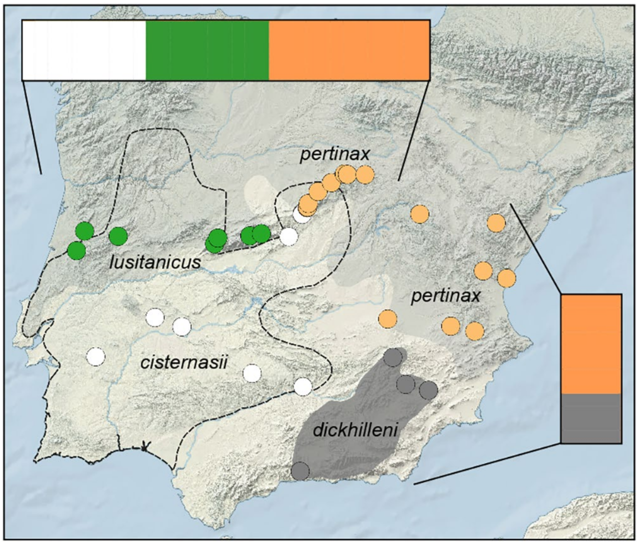

FIGURE 5 | Admixture analyses between species from distinct clades (attributed to distinct subgenera) in their areas of sympatry/near-parapatry. The map shows sample locations, and the barplots show individual ancestry from two separate analyses: (1) between *A. cisternasii* (subgenus *Ammoryctis*) and *A. obstetricans* from central (*pertinax*) and western Iberia (*lusitanicus*) (subgenus *Alytes*); (2) between *A. dickhilleni* (subgenus *Baleaphryne*) and *A. obstetricans* (*pertinax*) from southeastern Iberia (subgenus *Alytes*).

3.4 | Morphometric Differentiation

Body shape, measured from seven variables (adjusted by body size) in 211 individuals, also suggested fewer differences within subgenus *Alytes* than between other species (Figure 6B, File S7). The PCA based on all species distinguishes most individuals of *A. cisternasii*, *A. muletensis*, *A. maurus* and *A. dickhilleni* from those of subgenus *Alytes* (Figure 6B). Within the subgenus *Alytes*, the widespread *A. o. obstetricans* was the most variable, *A. o. boscai* and *A. o. lusitanicus* cluster together but with little overlap, and *A. a. almogavarii* and *A. a. inigoi* share the same morphospace (Figure 6B).

3.5 | Environmental Differentiation

The environmental conditions of 7087 localities, summarised by 35 variables, are the most diverse for the subgenus *Alytes*, which largely overlaps with species from other subgenera, except *A. maurus* (Figure 6C). The PCAs broadly distinguish two climatic spaces, one corresponding to the localities from W-Iberia (*A. cisternasii* and *A. o. boscai*/*lusitanicus*), and the other corresponding to the rest of the ranges (Figure 6C).

The projected distributions of each *Alytes* species and subspecies based on the ecological niche models broadly correspond to their present-day distributions (Figure 7). The average AUC and TSS evaluations spanned 0.81–1.00 and 0.50–0.99, which accordingly indicate high predictive power. Performance metrics for parameter settings are provided in File S2. Several bioclimatic (Bio2, Bio4, Bio6, Bio9, Bio11, Bio14, Bio15, Bio18, Bio19), landcover (barren land, global land cover, % of mixed forest, % of shrubs), and topographic (elevation and terrain ruggedness index) variables had relatively high contributions (> 10%) in all models. The models generally show low niche overlap according to Schoener's D ($D<0.25$, File S8), with the exception of the three W-Iberian taxa *A. o. boscai*, *A. o. lusitanicus*, and *A. cisternasii* ($D=0.35$–0.47).

The pairwise Euclidean distances of environmental conditions (File S9) increase with the niche dissimilarities computed from the reconstructed models (as 1-D; File S10). Environmental and spatial Euclidean distances were significantly correlated (File S11), suggesting spatial autocorrelation of the environmental variables, as expected among phylogeographic lineages (Journé et al. 2020).

3.6 | Comparisons Across Datasets

Genomic, bioacoustic, morphological, or environmental differentiation does not significantly predict the cline width w when all eight transitions are considered (linear regression on log-transformed data; Table 2, Figure 8). When excluding the two potentially allopatric pairs of lineages (POR and MAD), a negative relationship between genomic divergence and cline width becomes evident, with as much as 84% of variance explained (linear regression, Table 2, Figure 8). Morphological differences also increase as cline widths decrease, but in a weaker and non-significant fashion (Table 2, Figure 8). Bioacoustic and environmental differences also show no significant effect on cline width, and even tend to increase for the shallowest hybrid zones (Table 2, Figure 8).

Bioacoustic, morphological, and environmental differentiation generally increase with genomic divergence (Figure 9). At the level of subgenus *Alytes*, however, no significant co-variation was retrieved (Mantel tests on matrices of log-transformed pairwise distances, Table 2, Figure 9). At the level of the whole genus, all three phenotypic variables correlate with genomic divergence (Table 2, Figure 9).

The genetic transitions do not generally correspond to abrupt environmental transitions for the lineages involved (File S12). The occurrence probabilities obtained with the ecological niche models either remain similar on both sides of the hybrid zones, or progressively decrease along the transects from one lineage to the other, but without closely tracking the genetic transitions (File S12), except in only one case—*A. a. inigoi* in PYR, where genomic ancestry and environmental suitability decrease in concert.

4 | Discussion

4.1 | Genetics Precedes Phenetics in the Grey Zone of Speciation

Our results frame the grey zone of speciation of midwife toads within the clades presently delimited as subgenera, as here illustrated by the admixing phylogeographic lineages of our focal subgenus *Alytes*. In this clade, the extent of admixture between the lineages that are currently in contact appears most closely related to their phylogenomic divergence, suggesting a role for genome-wide intrinsic factors (post-zygotic hybrid incompatibilities) in initiating RI in the early stages

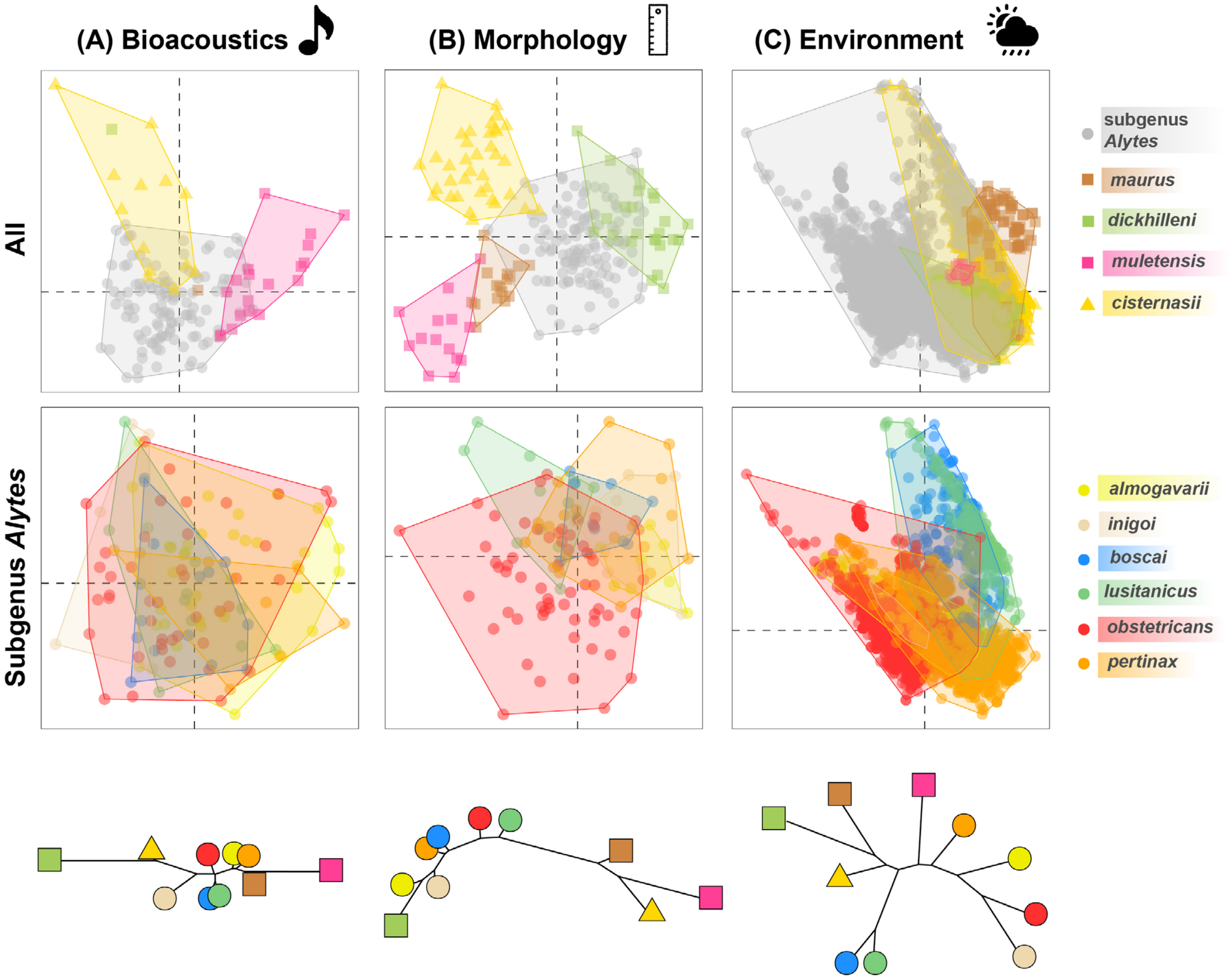

FIGURE 6 | Phenotypic variation in midwife toads. First dimensions of the PCAs built on bioacoustic (A), morphological (B), and environmental variables (C), distinguishing species from the three main clades (top panels) and the six lineages of the subgenus *Alytes* (middle panels), and NJ trees of Euclidean distances (bottom). The dots on the PCAs represent individuals (A, B) or localities (C).

of species formation. In the widest hybrid zones (involving the least diverged lineages), the whole genome continues to introgress freely, but in the narrowest hybrid zones (involving the most diverged lineages), some loci do not admix at all and may represent barrier loci, i.e., loci linked to the genes causing reproductive barriers. These observations are consistent with the mass-of-genes model for the buildup of RI (Dufresnes, Brelsford et al. 2021), which predicts that nascent species become incompatible due to the accumulation of multiple small-effect alleles that together reduce intrinsic hybrid fitness, such as DMIs (Orr 1995; Orr and Turelli 2001; Coyne and Orr 2004). Once a certain degree of divergence is reached, DMIs are numerous enough to substantially affect hybrid viability and fertility (Matute et al. 2010; Moyle and Nakazato 2010), thus reducing gene flow and decreasing the probability of "speciation reversal" through introgressive hybridization and lineage fusion (Kearns et al. 2018).

Conversely, the broad morphological, behavioural, and environmental overlap among the partly isolated *A. obstetricans* and *A. almogavarii* lineages argues against obvious pre-mating or extrinsic post-mating barriers as the initial driver of RI, at least based on the traits assessed here. Besides genetics and distribution, we are not aware of reliable criteria to discriminate these lineages (Ambu et al. 2024), which, however, feature high regional phenotypic variation, e.g., in mating calls (Arntzen and García-París 1995) and coloration (Polo-Cavia et al. 2016). Likewise, more transgressive ecological niches do not associate with more RI, and the genetic transitions of the lineages do not closely track the environmental transitions. Nevertheless, we cannot rule out that emerging phenotypic differences (e.g., consequent to genomic divergence) do contribute to RI alongside intrinsic post-zygotic isolation, especially given that lineages from the narrowest hybrid zones tend to have more dissimilar body shapes (Figure 8C). It would therefore be interesting to gather similar datasets from specimens collected along the transects to test for concordance between morphological and genetic clines. Besides, it is not excluded that local adaptation at range margins resulting in distinct habitat use, breeding phenology, and advertisement calls may contribute some pre-mating isolation, notably via reinforcement (Vences and Wake 2007; Wollenberg Valero et al. 2019).

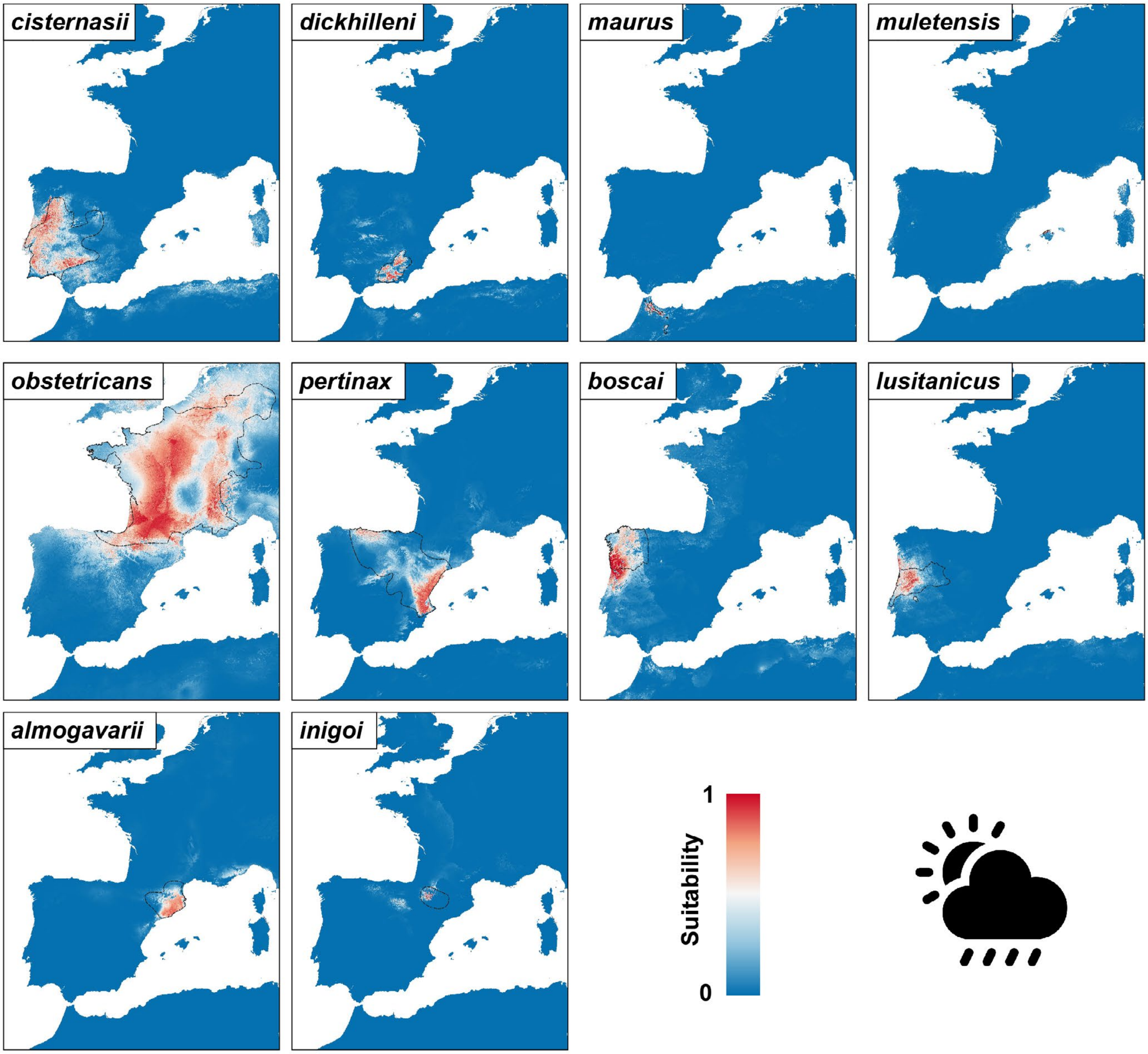

FIGURE 7 | Projected distributions of midwife toad species and subspecies based on the niche models. The same gradient of suitability is used for comparisons. Dashed lines show current distributions (Figure 1).

TABLE 2 | Statistical tests of the relationship between hybrid zone width (*w*) and genomic, bioacoustic, morphometric, and environmental divergence (linear regressions on log-transformed data; R^2: Proportion of variance explained; dof: Degrees of freedom; *F*: *F*-statistic; $p = p$-Value), and between the genomic divergence and the latter three (Mantel tests on log-transformed data; *r*: Correlation coefficient; *p*: *p*-Value). Bold values indicate significance ($p < 0.05$).

	Cline width (*w*)								**Genomic divergence**			
	8 Hybrid zones				**6 Hybrid zones**				**Subgenus *Alytes***		**All**	
	R^2	**dof**	***F***	***p***	R^2	**dof**	***F***	***p***	***r***	***p***	***r***	***p***
Genomic	0.15	6	1.0	0.35	**0.84**	**4**	**21.2**	**0.01**	—	—	—	—
Bioacoustic	0.20	6	1.5	0.26	0.04	4	0.2	0.70	0.20	0.21	**0.49**	**0.02**
Morphometric	0.17	6	1.2	0.31	0.09	4	0.4	0.57	0.25	0.15	**0.56**	**0.02**
Environmental	0.08	6	0.5	0.49	0.28	4	1.5	0.28	−0.37	0.84	**0.38**	**0.01**

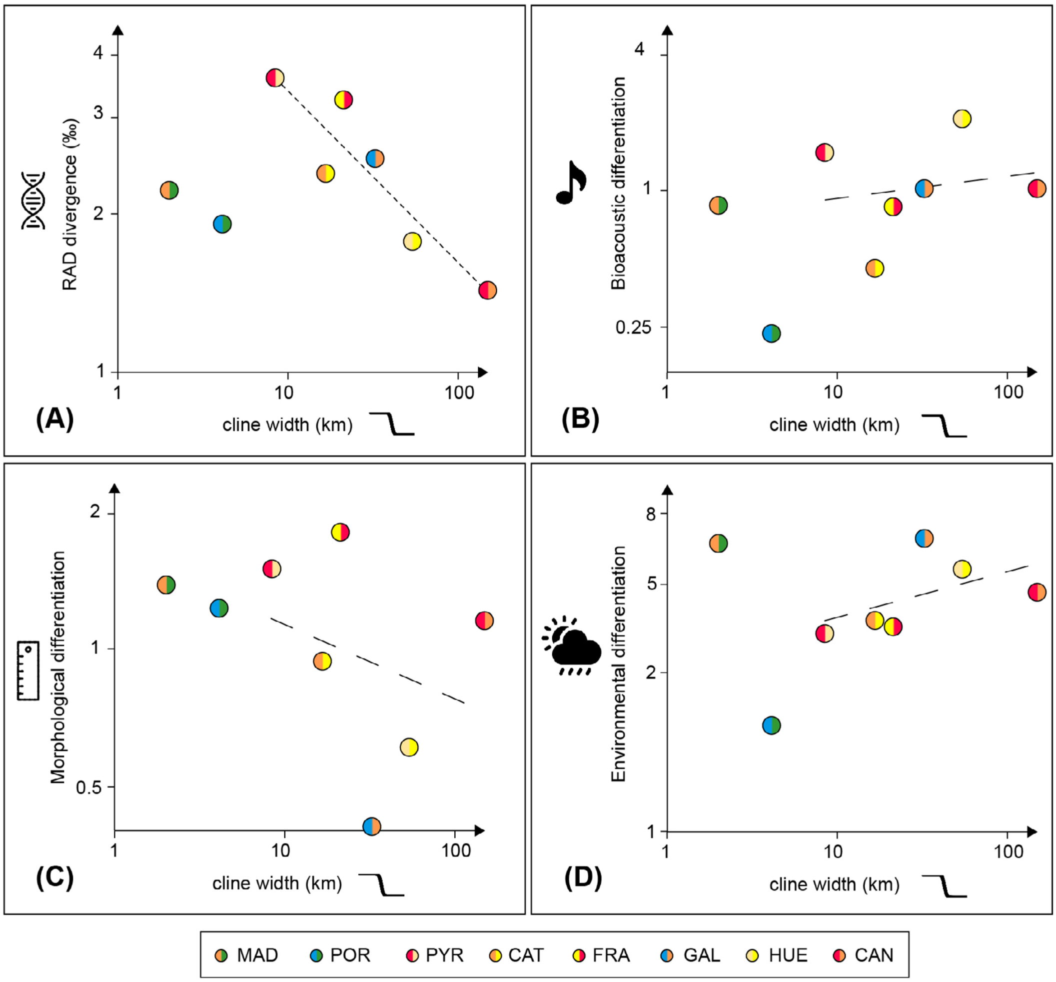

FIGURE 8 | Relationship between cline width and genomic divergence (A), bioacoustic (B), morphological (C), and environmental differentiation (D) in the subgenus *Alytes*. Axes are log-scaled. Dashed lines show linear regressions among the six pairs of lineages currently in contact (tight dashing: Significant regression; loose dashing: Non-significant; Table 2). Colours reflect the lineages involved in the corresponding contact zones.

Reinforcement was actually suspected in FRA, based on local variation in mating calls (Ambu and Dufresnes 2024). Additional traits such as olfaction and physiology, which can potentially play a role in amphibian speciation (Wollenberg Valero et al. 2019), are yet to be examined.

Our sharpest hybrid zones still remain more permeable than classical hybrid zones such as those between the fire-bellied toads *Bombina bombina* and *B. variegata*, which show abrupt (stepped) clines (Szymura and Barton 1991; Dufresnes, Suchan et al. 2021), potentially because they are evolutionarily older (<4 My in *Alytes* vs. >6 My in *Bombina*; Pabijan et al. 2013). Accordingly, in midwife toads, completely isolated species are characterised by deeper phylogenetic divergence, as well as more substantial differences in mating calls, body shape, and environmental envelopes, as seen from the genus-wide correlations (Figure 9). The significant linear link between bioacoustic and genomic divergence is noteworthy, as it supports that anuran vocalisations can contain phylogenetic information at the macroevolutionary scale (Robillard et al. 2006; Goicoechea et al. 2010). In contrast, environmental differences are not necessarily expected to covary with phylogenetics, and the relationship rather reflects the geographic (and thus climatic) partitioning of the main clades, with the southern subgenera *Ammoryctis* and *Baleaphryne* inhabiting essentially Mediterranean environments, while the northern subgenus *Alytes* diversified across more temperate climates (Rodríguez-Rodríguez et al. 2020; Donaire-Barroso et al. 2022; IUCN Red List 2024). As for body shape, besides the evolutionary divergence of species, the interspecific variation could parallel adaptations to soil substrates, with notably different dwelling behaviours between subgenera (Arntzen and García-París 1995).

The generally higher phenotypic differentiation between phylogenetically more distant species of the genus, notably at key reproductive cues (e.g., mating calls), may be relevant in the context of sympatry. The co-existence of related taxa implies efficient pre-mating barriers to limit unfruitful hybridization and avoid wasting reproductive efforts on the one hand, and ecological differences to reduce niche overlap and escape competitive exclusion on the other hand. If these attributes are associated with higher phylogenetic divergence, then sympatry should only be possible for deeply diverged

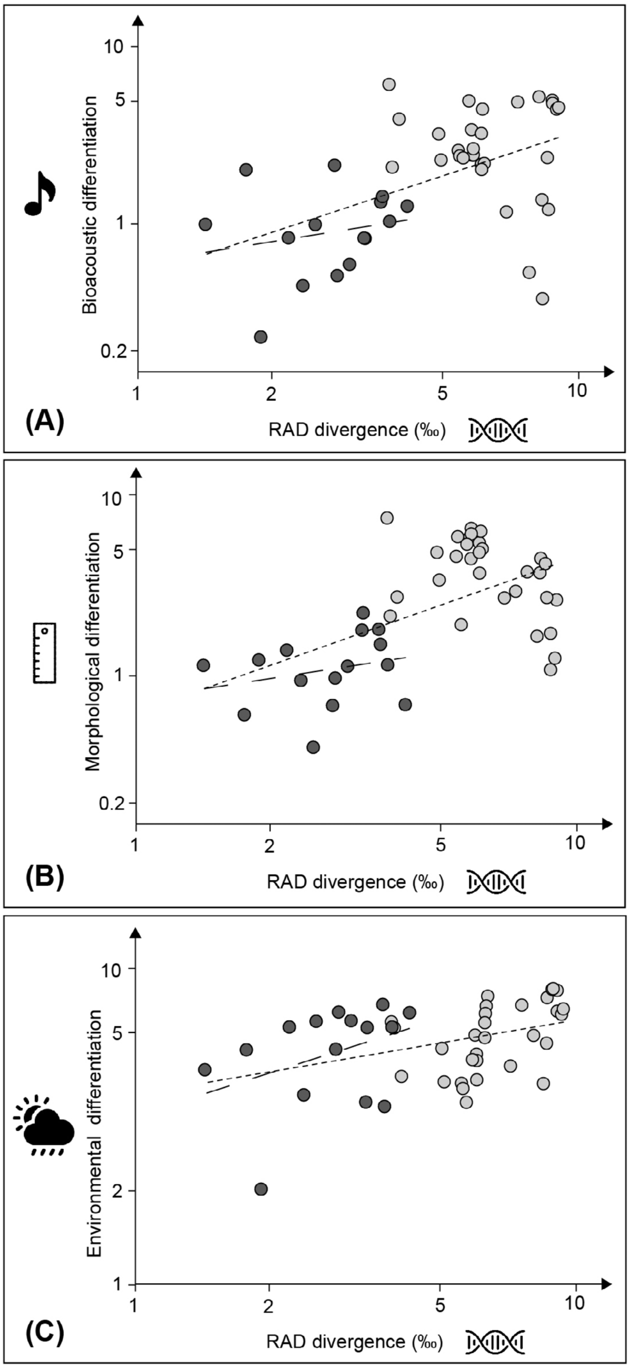

FIGURE 9 | Relationship between genomic divergence and bioacoustic (A), morphological (B), and environmental differentiation (C). Dash lines represent lineage regressions (tight dashing: Significant correlation based on Mantel tests; loose dashing: Non-significant; Table 2) conducted on the subgenus *Alytes* (dark dots) or on the whole data (light dots: Other comparisons).

species. Accordingly, the only two partially sympatric midwife toad species (*A. cisternasii* and *A. obstetricans*) show the oldest divergence (≥13 My; Ambu et al. 2023), which is of the same order of magnitude as the divergence estimated for sympatric species of Palearctic anurans of the same genus (e.g., 20 My in *Hyla* tree frogs, Dufresnes et al. 2020; 16 My in *Bufotes* green toads, Dufresnes, Mazepa et al. 2019; 13 My in *Pelophylax* water frogs; Dufresnes et al. 2024). Sympatry may further boost phenotypic divergence by a combination of divergent selection and selection for assortative mating (de Solan et al. 2022).

As exemplified by classical crossing experiments in anurans (Malone and Fontenot 2008), as well as comparative studies of hybrid zones in vertebrates (e.g., Singhal and Moritz 2013; Dufresnes Strachinis et al. 2019; Dufresnes, Brelsford et al. 2021; Pulido-Santacruz et al. 2020), a gradual relationship between the degree of RI and genetic divergence probably exists across many animal diversifications, as expected if speciation follows the mass-of-genes model. Yet the relationship remains inherently difficult to establish when comparing datasets spanning different organisms, and where the proxy used to infer RI (here, the geographic extent of admixture summarised by the cline width *w*) is influenced by external factors. For instance, a global meta-analysis emphasised the predominant effect of dispersal on *w* (McEntee et al. 2020), which is expected if both vagile and slow-moving species are included. In turn, this reduces the informativeness of hybrid zones to quantify and compare the strength of RI. By focusing only on organisms of globally similar dispersal rates, the effect of divergence time on *w* becomes clearer (e.g., anuran amphibians; Dufresnes, Brelsford et al. 2021). Here, the focus on a single species complex (where all lineages a priori share the same dispersal abilities), the exclusion of currently disconnected transitions (where *w* reflects geographic rather than reproductive barriers), and the estimation of genome-wide divergence rather than indirect estimates (e.g., divergence time, genetic distances from single genes), might all have contributed to the neat relationship retrieved. For instance, a genus-specific assessment in Australian skinks also reported remarkably strong correlations between lineage divergence and the amount of gene flow at range margins (Singhal and Moritz 2013).

In summary, genetic factors potentially precede phenetic factors in the buildup of RI across the *Alytes* grey zone of speciation. This chain of events may be common across the animal kingdom, as suggested by empirical observations of young "cryptic" species (Bickford et al. 2006)—implying that speciation often does not require external differences (at least noticeable by the human eye)—and of well-distinctive sympatric species at the very end of the speciation continuum (e.g., Rasolonjatovo et al. 2020)—implying that strong pre-mating barriers and habitat partitioning appear after speciation has already occurred, potentially in allopatry. Of course, the tempo of speciation could be drastically different in clades where speciation relies on adaptation to different niches (ecological speciation such as some fish species flocks; Seehausen 2006) or on divergent signals involved in mate choice (some birds; Price 2007). What predisposes the mode and tempo of speciation could be addressed by comparing the drivers of RI across species groups that differ in their demographic history, evolutionary age, and potential for ecological diversification, for instance based on their diversity of developmental trajectories (Bright et al. 2016) and environmental niches (Price et al. 2014; Ezard and Purvis 2016).

4.2 | Species Delimitation With and Without Hybrid Zones

As they can inform on the existence of reproductive barriers, patterns of admixture in hybrid zones are useful for delimiting and naming phylogeographic lineages under the ranked classification system—such as the one implemented in the International Code for Zoological Nomenclature—without contributing to taxonomic inflation (Dufresnes, Brelsford et al. 2021; Dufresnes et al. 2023; Chambers et al. 2023; Vences et al. 2024). In European anurans, Dufresnes, Brelsford et al. (2021) proposed two criteria to distinguish species and subspecies based on hybrid-zone analyses: (1) the genome average cline width w, noting that species lineages usually admix over less than 30 km; (2) the heterogeneity of introgression across the genome, noting that species lineages usually feature loci with very steep clines (barrier loci). This second criterion corresponds to the definition of semi-isolated species by Roux et al. (2016), where speciation is interpreted as having started but being incomplete, with some but not all loci being affected by barriers to gene flow. Applying the hybrid-zone criteria necessitates proper transect sampling, and because w is determined by the balance between dispersal and selection against introgression (Barton and Gale 1993), species with smaller dispersal distances than the average European anurans will exhibit steeper clines for the same strength of RI.

Our results emphasise the limits of the approach with irregular sampling and when barriers to dispersal arise in the middle of the hybrid zones. In the POR contact zone (*A. o. boscai*/*lusitanicus*), the Douro estuary likely prevents gene flow; thus, the low w does not necessarily indicate reproductive isolation. Accordingly, the river also mediates the distribution of intraspecific lineages in the newt *Triturus marmoratus* (Kazilas et al. 2024), as do other riverine systems in other Iberian amphibians like fire salamanders (Figueiredo-Vázquez et al. 2021). In MAD (*A. o. lusitanicus*/*pertinax*), the few wide clines testify to admixture, and what is now a distributional gap probably used to be a hybrid zone. In such situations, the clines of loci that never introgressed beyond the edges of the gap are inferred with widths tending towards zero, thus mimicking barrier loci—even though these loci may have admixed within the gap. The issue is also characteristic of GAL (*A. o. boscai*/*pertinax*), where the two delimitation criteria mentioned above provide contradictory outcomes: the average extent of admixture ($w \sim 38$ km) is wide given the number of very steep clines. This can be explained by the ~35 km sampling gap in the hybrid-zone center of GAL, where clines as wide as 15 km would theoretically fit despite negligible admixture in the closest sampled populations (computed from the sigmoid formula; Barton and Gale 1993). Hence, the current sampling of GAL does not truly allow for assessing the presence of barrier loci, and thus to conclude on a species rank between the interacting lineages under the hybrid-zone approach.

In the six other hybrid zones, the neat inverse relationship between genetic divergence and introgression indirectly implies that extrinsic features, such as regional landscape barriers, had little influence on the observed patterns of gene flow. The environmental transitions are accordingly much smoother than the genomic clines. That said, we cannot rule out that the topographic and hydrographic complexity of the study areas locally affected dispersal at the transitions (Velo-Antón et al. 2021). For instance, the hybrid-zone centers of FRA (*A. o. obstetricans*/*A. a. almogavarii*) and PYR (*A. o. obstetricans*/*A. a. inigoi*) broadly match the Ariège river and the Portalet mountain pass. Nevertheless, the retrieved admixture supports connectivity between these lineages, and more generally, comparatively large rivers and high mountain ranges characterise vast areas of introgression (e.g., CAN, *A. o. pertinax*/*obstetricans*), and are found across the distribution of single genetic clusters within subspecies (Lucati et al. 2022), testifying to their broad ecological, notably altitudinal tolerance.

Considering these points, we can provisionally delimit species and subspecies for the six lineages of the subgenus *Alytes* in taxonomic accounts. *Alytes obstetricans* and *A. almogavarii* always feature narrow hybrid zones with putative barrier loci, namely between *A. a. almogavarii* and *A. o. pertinax* (CAT; Dufresnes and Martínez-Solano 2020), *A. a. almogavarii* and *A. o. obstetricans* (FRA; Ambu and Dufresnes 2024), and *A. a. inigoi* and *A. o. obstetricans* (PYR; this study). Patterns of admixture may lack concordance between different hybrid zones involving the same incipient species (Harrison and Larson 2016), depending on the intraspecific lineages involved, the age since the first contact (e.g., Dufresnes et al. 2020), the dispersal capacities (e.g., McEntee et al. 2020), the environmental heterogeneity (Carling and Thomassen 2012), or the demographic dynamics (van Riemsdijk et al. 2023). Narrow transitions across replicate transects are thus the hallmark of stable reproductive barriers (Harrison and Larson 2016). Our study therefore corroborates the species status of *A. almogavarii*, as currently acknowledged (Speybroeck et al. 2020; Sánchez-Vialas et al. 2024) but still disputed (e.g., Lucati et al. 2022). Reciprocally, the wide hybrid zones between the shallowest lineages confirm the subspecies status of *A. a. inigoi* and *A. o. pertinax*. Finally, *A. o. boscai* and *A. o. lusitanicus* are more complicated to rank with the present evidence. We here continue to treat them as subspecies of *A. obstetricans*, given that the genome average cline of the *A. o. boscai*/*pertinax* hybrid zone is overall slightly wider (~38 km) than the 30 km threshold, but these assessments may be revised in the future pending new hybrid-zone analyses based on denser sampling.

When hybrid zones do not exist (or cannot be sampled), genetic or phenotypic divergence may help to rank evolutionary lineages in groups where RI correlates with such divergence, affording species status to lineages that are as divergent as valid species in the same groups (Schweizer et al. 2023). Given their widespread use in phylogenetic and taxonomic studies, sequence divergence at mitochondrial genes or divergence time estimated from those genes may serve as proxies to define operational thresholds for species delimitation when RI associates with such metrics (Dufresnes, Brelsford et al. 2021; Dufresnes and Litvinchuk 2022). As an example, the hybrid-zone validated species *A. obstetricans* and *A. almogavarii* originated ~3.9 Mya, while the hybrid-zone validated subspecies *A. a. inigoi* and *A. o. pertinax* originated <2.5 Mya (Ambu et al. 2023). This temporal window can then assist the ranking of older or younger *Alytes* lineages left to discover as new species or subspecies, respectively. However, rather than estimates derived from clonal mtDNA sequences, raw genomic divergence, which should covary with the average number of alleles involved in RI under the mass-of-genes model, would be more relevant, especially when taxa diversify under reticulate rather than bifurcating processes only—here the subspecies *A. o. pertinax* exhibits ancestry from both *A. o. obstetricans* and *A. almogavarii* (Ambu et al. 2023). In this respect, we found similar levels of inter-specific sequence divergence at ddRAD-seq loci as in our previous analyses on other anuran genera (Dufresnes, Brelsford et al. 2021), namely ~3‰ as a mid-point threshold between species and subspecies. Such comparability is not necessarily expected given that these analyses rely on different sets of markers, on which polymorphism depends on methodological aspects such as bioinformatic stringency and sample scheme. Future investigations should explore the universality of divergence thresholds applicable across genomic datasets and species groups, in the framework of integrating next-generation sequencing approaches in taxonomy (Vences et al. 2024).

Author Contributions

J.A., Í.M.-S. and C.D. designed the research; J.A., S.N.L., C.C.-D., A.N., G.V.-A., H.G., F.M.-F., H.M.-G., J.F.B., D.D.-B., A.H., T.S., P.-A.C., Í.M.-S. and C.D. performed the research; J.A. and S.N.L. analysed the data; J.A. and C.D. wrote the paper, which was improved by S.N.L., C.C.-D., A.N., G.V.-A., H.G., F.M.-F., H.M.-G., J.F.B., D.D.-B., A.H., T.S., P.-A.C. and Í.M.-S.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Raw ddRAD sequence reads are deposited in NCBI SRA (BioProject PRJNA949685) under the accessions listed in File S1; datasets and scripts are deposited on Zenodo (Ambu and Dufresnes 2025; https://doi.org/10.5281/zenodo.14899463).

Benefit-Sharing Statement

The research is relevant to a priority concern (the conservation of the organisms being studied), and the generated data and results have been shared with the broader scientific community on public databases as described above. A research collaboration was developed between scientists from several countries, and all collaborators are included as co-authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.