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The effect of hybrids on phylogenomics and subspecies delimitation in *Salamandra*, a highly diversified amphibian genus

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Abstract. Traditional methods of phylogenetic reconstruction and species delimitation may be impeded by frequent hybridization among lineages. In this study, we conducted phylogenetic and clustering analyses of ddRAD genomic data on the entire genus *Salamandra*, which includes six species and over 25 subspecies of terrestrial salamanders. We expanded previous datasets to include missing subspecies and incorporated new samples, with an emphasis on secondary contact zones. Results obtained from a full dataset of 392 individuals (356,874 bp; 24,192 SNPs) were compared with those obtained after excluding substantially admixed individuals (n = 95; 835,467 bp; 51,557 SNPs) to explore the consequences of introgression on phylogenetic inference and taxonomic arrangement of subspecies. We found conflicting phylogenetic placements for taxa represented by many admixed individuals (identified by clustering ancestries). In contrast, a time-calibrated tree constructed without hybrids largely agrees with previous phylogenetic hypotheses. Within *S. atra*, we found paraphyly of *S. atra atra*, suggesting an additional candidate subspecies. Within *S. infraimmaculata*, two lineages are assignable to known subspecies and we additionally identified a third, deeply diverged lineage sampled near the Turkish/Syrian border. In *S. algira*, we found limited admixture between the subspecies *S. a. tingitana* and *S. a. splendens* despite their geographic proximity. Finally, within *S. salamandra*, we detected significant levels of hybridization between subspecies, which blurred their phylogenetic relationships, although the removal of admixed samples in subset analyses clarified the situation in most cases. Monophyly was recovered for subspecies that were previously found paraphyletic, including *S. s. salamandra*, *S. s. gallaica*,

and *S. s. fastuosa*. *Salamandra s. "alfredschmidti"* was confirmed to be a junior synonym of *S. s. bernardezi*. Previously disputed subspecies, like *S. s. "molleri"* and *S. s. "hispanica"*, correspond to separated lineages but are affected by admixture with other lineages.. Further newly identified candidate subspecies in *S. salamandra* included a southern lineage within *S. s. werneri* and a western lineage within *S. s. bernardezi*. Finally, we re-evaluate the status of recognized subspecies in *Salamandra*, based on evidence from multiple delimitation criteria. Given that the evolutionary history could not be resolved for all subspecies, we highlight taxa within *Salamandra* that warrant further molecular examination and taxonomic revision, notably within the *S. s. gallaica/"molleri"/bejarae* complex. This study illustrates the impact of hybridization in phylogenetic analyses and its downstream effects in the identification of conservation units and their naming in the Linnean classification.

Key words. Amphibia, Caudata, Salamandridae, subspecies, molecular phylogeny, phylogeography, heterozygosity, population genetics, timetree, STRUCTURE, ddRADseq.

Introduction

Hybridization is the interbreeding of groups with distinct gene pools, such as species, subspecies, or populations (Arnold 1997). When hybrids are fertile, hybridization can lead to gene flow – the exchange and introgression of alleles between these gene pools (Gompert & Buerkle 2016). Both hybridization and introgression can have profound consequences for the genetic diversity of populations throughout the course of their evolution (Mallet et al. 2016). The outcome of hybridization between parental species is largely affected by reproductive barriers that are typically stronger with increased evolutionary divergence, and therefore, hybridization occurs more often between closely related organisms than between distant relatives (Arnold et al. 1999). In nature, hybridization and introgression between parapatric lineages typically lead to the formation of hybrid zones, which are narrow geographical areas where the distributions of two lineages and their hybrids overlap (Barton & Hewitt 1985, Gompert et al. 2017). Intraspecific hybridization – hybridization between lineages of the same species – implies that neither hybridization nor gene flow are affected by reproductive barriers (Coates et al. 2018), and introgression can expand over a wide geographic range without dispersal barriers (Hillis et al. 2021, Chambers et al. 2022).

Advances in sequencing methods have made it possible to genotype thousands of loci to detect and quantify subtle amounts of gene flow, which has resulted in a surge of studies reporting on hybridization in reptiles and amphibians at various geographic and phylogenetic scales (e.g., Singhal & Moritz 2013, Zinenko et al. 2016, Rancilhac et al. 2021, Pyron et al. 2022, Ambu et al. 2023).

The spectrum of genetic divergence along which populations may merge or remain evolutionarily separated in the face of hybridization has often been referred to as the "gray zone" of speciation (de Queiroz 1998, Roux et al. 2016). Delineating taxa within this zone then utterly depends on the species concepts and criteria applied, which themselves condition the status of lineages in taxonomic lists, and thus their conservation value (de Queiroz 2007, Roux et al. 2016, Chan & Grismer 2019; Dufresnes et al. 2023a). Renewed interest in species delimitation approaches was triggered by a paradigm shift in the definition of species, which are now most often regarded as evolutionarily independent population-level lineages, following the General Lineage Concept (Mayden 1997, de Queiroz 1998, 2007). This concept has often been applied in concert with the Phylogenetic Species Criterion (PSC), and in this context, it has been criticized as it may lead to taxonomic inflation (Agapow et al. 2004; Isaac et al. 2004). The application of a more traditional Biological Species Criterion (BSC) has been advocated in herpetology to define species boundaries (Hillis et al. 2021) and establish standardized taxonomic lists based upon criteria that reflect reproductive isolation, such as the degree of hybridization between candidate taxa (e.g., Speybroeck et al. 2020).

To this end, the hybrid zones between candidate lineages in Western Palearctic anurans have served as a model system to explore the genetic mechanisms behind reproductive isolation (RI) across a continuum of divergence (Dufresnes et al. 2021) and provide some relevant pointers for species delimitation (Sites & Marshall 2003, Camargo & Sites 2013, Flot 2015; Dufresnes et al. 2020, 2021).

In parallel, the identification of infraspecific taxonomic units, such as subspecies, or of Evolutionary Significant Units (ESUs) and Management Units for conservation (MUs) (Moritz 1994) may also be greatly affected by hybridization. Defining conspecific genetic groups has typically relied on phylogenetic or clustering analyses, often based on limited panels of genetic markers (Coates et al. 2018). The advent of genomics has extended these approaches to incorporate information on thousands of markers, allowing researchers to identify shallow phylogeographic lineages that may admix over large areas (Funk et al. 2012, Fischer et al. 2017, Dufresnes et al. 2023b). On the one hand, genuine lineages may be confounded by isolation by distance (IBD, Potter et al. 2016), i.e., the expected spatial trend of genetic differentiation between populations. On the other hand, as foreign alleles freely diffuse across lineage boundaries, populations located far from presumed parapatric areas may still be affected by admixture, and their inclusion in phylogenomic analyses can potentially result in spurious patterns, such as incorrect tree topologies or admixed individuals being retrieved as separate lineages or genetic clusters (Firneno et al. 2020, Ambu et al. 2023). Such issues may have profound consequences for infraspecific taxonomy, especially if it leads to the incorrect classification of populations (e.g., assigning "lineages" to the wrong taxa), which may in turn misallocate valuable conservation resources (Frankham et al. 2012). However, the difficulties arising from genomic

analyses to identify conspecific phylogeographic lineages so they can be acknowledged in biodiversity inventories have so far received little empirical focus. Especially, it is a matter of interest (i) to explore the conflicting outputs of phylogenetic vs. clustering methods applied to genomic datasets, and (ii) to assess how the inclusion of admixed individuals in the analyses affects infraspecific classification.

The climatic history of Europe over the last million years, characterized by glacial and interglacial phases, has led to numerous instances of population divergence and secondary contact in amphibians (Arntzen et al. 2014, Zieliński et al. 2019, Gaczorek et al. 2023). A textbook example are terrestrial salamanders in the genus *Salamandra*, which includes the species commonly known as fire salamanders and alpine salamanders (Thiesmeier 2004). The classification of *Salamandra* species and subspecies has traditionally relied on patterns of coloration (Kammerer 1904, Boulenger 1911, Mertens & Müller 1940, Eiselt 1958, Bosch & López-Bueis 1994). The use of molecular approaches led to the identification and description of new species and subspecies, but also revealed incongruences with the former color-based taxonomy (Nascetti et al. 1988, Steinfartz et al. 2000, Beukema et al. 2013; 2016). Also, different sets of molecular markers (e.g., mitochondrial vs. nuclear) produced conflicting tree topologies, precluding full resolution of phylogenetic relationships within and among species (Weisrock et al. 2006, Vences et al. 2014, Bonato et al. 2018). Recent studies using genome-wide approaches and an increased number of markers have provided more robust phylogenies, recognizing six species and about twenty-five subspecies of *Salamandra* (Rodríguez et al. 2017, Dinis et al. 2019, Burgon et al. 2021; Mulder et al. 2022).

The oldest species, *S. infraimmaculata*, is composed of several isolated populations distributed in the Near East, including the Levant region, as well as adjacent Turkey and Iran (Bozkurt et al. 2015, Rodríguez et al. 2017). Three subspecies have been described: *S. i. infraimmaculata*, *S. i. orientalis*, and *S. i. semenovi*. For the latter two, the morphological differentiation does not correspond to their proposed geographic distributions (Böhme et al. 2013, Candan 2022); we hereinafter consider them as a single taxon, using the older name *semenovi*.

Salamandra corsica is found exclusively in Corsica and is most closely related to the alpine salamanders *S. lanzai* and *S. atra* (Steinfartz et al. 2000, Escoriza & Hernandez 2019). The uniformly black *S. lanzai* is a stenoendemic species occurring in the Cottian Alps, while *S. atra* is distributed over most of the northern and eastern Alps and along the Dinaric Alps. Within the latter species, most of the uniformly black populations in the Alps are traditionally assigned to *S. a. atra*, while the partially yellow-colored subspecies *S. a. aurorae* and *S. a. pasubiensis* are stenoendemic to the Venetian Prealps, and the black *S. a. prenjensis* is distributed in the Dinaric Alps (Bonato & Steinfartz 2005, Razpet et al. 2016, Bonato et al. 2018, Šunje et al. 2021).

Salamandra salamandra has the broadest distribution range, from the Iberian to the Balkan Peninsula, and comprises most of the subspecies (about 13) recognized in the genus, with nine in the Iberian Peninsula alone (Burgon et al. 2021). Previous studies using a limited number of markers, and especially relying on mitochondrial DNA, recovered *S. s. longirostris* as sister to a clade including all other subspecies (García-París et al. 2003, Vences et al. 2014), and some authors have even considered this taxon to represent a separate species (García-París et al. 1998, Dubois & Raffaëlli 2009). However, recent genomic studies (Burgon et al. 2021; Mulder et al. 2022) provided a different picture, with two main clades within *S. salamandra.* The first clade includes *S. s. bernardezi* from the central and western Cantabrian Mountains (Northern Spain), *S. s. fastuosa* from the eastern Cantabrian Mountains and western Pyrenees, and *S. s. gigliolii* from the Apennine Peninsula. Within this clade, recent findings have unveiled that *S. s. fastuosa* may be paraphyletic relative to *S. s. gigliolii* (Burgon et al. 2021). Moreover, salamanders restricted to a narrow geographic area within the *S. s. bernardezi* range exhibit a remarkable diversity of background coloration and color patterns, which led to their description as a distinct subspecies (*S. s. alfredschmidti*; Köhler & Steinfartz 2006). However, this subspecies is nested within *S. s. bernardezi* and has been subsequently synonymized (Beukema et al. 2016).

The second clade containing all other *S. salamandra* subspecies comprises *S. s. almanzoris* from the Sistema Central Mountains in Central Spain, recovered as sister to all other subspecies, which formed another clade (Burgon et al. 2021). Within the latter, *S. s. longirostris* is sister to a clade formed by *S. s. morenica* from southern Spain north of the Guadalquivir River and *S. s. crespoi* from Algarve (Burgon et al. 2021, Mulder et al. 2022). In addition, two rather widely distributed subspecies in the Iberian Peninsula are *S. s. bejarae* and *S. s. gallaica*, which exhibit complex patterns of phenotypic and genetic variation and include populations to which the name *S. s. "molleri"* may be applicable (Mertens & Müller 1928, Reis et al. 2011, Burgon et al. 2021). Most of Central Europe is inhabited by two subspecies, the western *S. s. terrestris* (ranging from the north-eastern Pyrenees to West Germany) and the eastern *S. s. salamandra* (from the Balkan Peninsula to East Germany), with a wide contact zone in Germany (Veith 1992, Steinfartz et al. 2000). More locally restricted subspecies are *S. s. werneri* in Greece, *S. s. beschkovi* in Bulgaria, and a lineage from the Montseny Massif in Catalonia, north-eastern Spain, synonymized with *S. s. terrestris* by many authors but sometimes referred to as *S. s. "hispanica"* (Mertens & Wermuth 1960, Grillitsch & Grillitsch 1991, Steinfartz et al. 2000, Burgon et al. 2021). Burgon et al. (2021) also detected paraphyly in *S. s. salamandra* and *S. s. werneri,* raising further doubts about the current subspecies-level classification.

Salamandra algira, the sister species of *S. salamandra,* is distributed in Morocco and Algeria (Rodríguez et al.

2017). Five subspecies are recognized: *S. a. algira* from Algeria, the partially pueriparous *S. a. tingitana* (Dinis & Velo-Antón, 2017) in the northwest Moroccan Rif, *S. a. splendens* in the southwest Moroccan Rif (both subspecies forming a contact zone), *S. a. atlantica* from the Middle Atlas, and *S. a. spelaea* restricted to the Beni Snassen Massif (Escoriza & Comas 2007, Merabet et al. 2016, Hernandez & Escoriza 2019, Dinis et al. 2019).

As aforementioned, the exact number of *Salamandra* infraspecific lineages and their distinction as subspecies in some cases continue to be matters of discussion, and such taxonomic instability affects effective conservation policies (Bonato et al. 2018, Dinis et al. 2019, Burgon et al. 2021, Rancilhac et al. 2021). One of the primary reasons for the uncertainty might be the substantial level of admixture between lineages, as illustrated by the existence of several contact zones (Table 1). However, previous studies have not explicitly investigated range-wide admixture among phylogeographic samples, which could affect the patterns of genetic diversity and evolutionary relationships retrieved, as well as their interpretation for the formulation of taxonomic hypotheses.

In this study, we tested the effect of individual hybrid ancestry in phylogenetic analyses of *Salamandra*, with an explicit focus on the taxonomic implications. We built upon the previous double digested restriction-site associated DNA sequencing (ddRADseq) dataset of Burgon et al. (2021), substantially expanded with 300 new individuals spanning unsampled subspecies, geographical gaps, and secondary contact zones. We designed datasets with and without samples admixed between pre-identified lineages and compared the phylogeographic patterns retrieved in terms of tree topology and genetic diversity (heterozygosity and genetic structure).

Material and methods

Sampling

Our dataset included 196 individual samples previously genotyped by Burgon et al. (2021) (data from Sequence Read Archive Bioproject PRJNA686117), covering all six *Salamandra* species through most of their geographic ranges (plus two *Lyciasalamandra* species as outgroups), and 300 newly sampled individuals, including two previously unsampled subspecies (i.e., *S. atra aurorae* and *S. a. prenjensis*) and an increased geographic sampling density of putative and known contact zones (e.g., *S. salamandra gallaica*/*bernardezi* in North-Western Spain, *S. salamandra salamandra*/*terrestris* in Germany, *S. algira tingitana*/*splendens* in northern Morocco). We used newly collected tissue samples from recent sampling campaigns as well as samples collected specifically for this study in 2021 and 2022. The samples were preserved in 100% ethanol.

All individuals were assigned to currently recognized subspecies according to known diagnostic phenotypic characters (especially coloration) and/or their geographic provenance (see Table S1).

Genomic DNA extraction, library preparation, and data processing

The Macherey-Nagel Nucleo-Spin® Tissue kit or XS Tissue kit were used to extract genomic DNA, following manufacturer protocols. Four new ddRADseq libraries were prepared following the modified protocol of Recknagel et al. (2015) described in Burgon et al. (2021). Libraries included three technically replicated samples as controls. Digestion of DNA was performed using the restriction enzymes PstI-HF® and AclI (New England Biolabs, Ipswich). Unique combinations of paired barcodes combined with Illumina adapters were attached to the digested DNA, resulting in individually tagged fragments for each sample. Size selection of multiplexed samples (tight selection 383 bp, range 345–421 bp) was done using a PippinPrep (Sage Science, Beverly). In a final step, each library was amplified in an enrichment PCR using forward and reverse RAD primers. The libraries were sequenced on four runs of the Illumina NextSeq™ 500 platform at Glasgow Polyomics, resulting in approximately 2743.4 million paired-end reads of 75 nucleotides in length. The new data is available in the NCBI Sequence Read Archive under BioProject PRJNA686117. Alignments and other analysis files are available from the Zenodo repository: https://doi.org/10.5281/zenodo.10844241.

Newly sequenced products, as well as the five raw libraries of Burgon et al. (2021), were processed using STACKS v2.6.1 (Catchen et al. 2013). Initially, individually tagged samples were demultiplexed, and barcodes were removed using the "process_radtags" along with the "--paired" and "--inline_inline" barcode options. As one of our applied restriction enzymes (AclI) was not included in the list of supported enzymes in STACKS, it was manually added to the renz.cc file. Reads were trimmed to a length of 60 nt, and low-quality sequences (Phred score < 10) were removed. Barcodes and RAD tags were rescued using the -r flag. Retained reads from newly sequenced libraries ranged from 470.8 M to 717.9 M, while those from the re-demultiplexed libraries of Burgon et al. (2021) ranged from 601.3 M to 752 M. We chose to include the low-quality reverse reads that were discarded in Burgon et al. (2021), resulting in different numbers of retained reads between both analyses.

RAD tags were built up into a catalog using the denovo_map.pl pipeline (ustacks, cstacks, sstacks and tsv2bam). Assembly, alignment, SNP calling, and phasing were conducted with the gstacks pipeline, which was also implemented in the "denovo_map.pl" pipeline of STACKS. Mismatches allowed between stacks within individuals as well as between individuals were set to 2. Other options were set to default. Genetic datasets used in downstream analyses were obtained using the module populations of STACKS.

To assess the impact of missing data in preliminary runs, we varied the number of individuals for which a locus must have been genotyped by modifying the parameter -p (using p as the number of individuals) and fixing parameter -r to 1. These preliminary runs allowed us to flag individuals

with a high proportion of missing data, which were subsequently discarded. In addition, we compared the technical replicates and kept those replicates with less missing data.

Identification of admixed individuals

In order to detect admixed individuals between clades, we conducted two exploratory analyses of the entire dataset, which includes all individuals passing the previous filtering steps (n = 392, Table S1).

First, we created a concatenated supermatrix (356,874 bp; 24,192 SNPs; p = 370; r = 1; Table S2) of the entire dataset by using the -phylip-var-all output option in STACKS. From the output file, a maximum-likelihood phylogeny was inferred using IQTree v. 1.6.12 (Nguyen et al. 2015). The substitution model was determined with ModelFinder (Kalyaanamoorthy et al. 2017). We calculated 100 non-parametric bootstrap replicates. Other options were set at default. The tree was visualized using the ggtree package in R (Yu et al. 2017).

In a second exploratory analysis, we used STRUCTURE (Pritchard et al. 2000) to identify population ancestry patterns. When generating a genotype matrix for STRUCTURE, we choose the parameter --write-random-snp to avoid physically linked loci, set -p to 370 and -r to 1 (resulting in 2,490 SNPs; Table S3). In STRUCTURE, we conducted 10 replicate runs for each number of assumed populations (K = 20) by applying the admixture model without prior assignment of localities (LOCPRIOR) and correlated allele frequencies among populations. To process several runs simultaneously in different threads and thereby increase computational efficiency, we used Structure_threader (Pina-Martins et al. 2017). For all runs, the number of MCMC iterations was set to 100,000 with a burnin of 20,000. In large genomic datasets with uneven sample sizes, STRUCTURE often does not converge towards optimal solutions, and some of the clusters retrieved do not actually represent any genetic variation in the dataset, called "ghost clusters" (Guillot et al. 2005) or "spurious clusters" (Puechmaille 2016, Meirmans 2019). Therefore, we examined STRUCTURE results by using StructureSelector (Li & Liu 2018) and selected the highest K that did not include such ghost clusters (threshold 0.8). We favored the best replicate run according to the highest estimated Ln (probability of the data).

Additionally, to explore patterns of genetic structure and admixture within *S. salamandra* and *S. algira* based on more informative loci, especially in the parapatric ranges of identified lineages, five new subsets were obtained by filtering the loci present in all individuals of the subset (in an effort to reduce the amount of missing data; Table S3). For each subset, a separate STRUCTURE analysis was performed using the same settings as above, except for the maximum K (K< 10). The population ancestry coefficients retrieved from the STRUCTURE analyses were plotted as pie charts on maps created with QGIS v. 3.22.3.

Based on the exploratory analyses, we created a reduced dataset of 95 non-admixed ingroup individuals, selected based on the following criteria: (i) representing – in balanced sample sizes – all major lineages identified from the analysis of the full dataset; (ii) representing all currently accepted subspecies; (iii) with the least missing data; (iv) with no signs of admixture (i.e., as suggested by intermediate ancestry coefficients and intermediate phylogenetic positions).

Time-calibrated phylogenetic reconstruction

Based on the reduced dataset (n = 95 plus two *Lyciasalamandra* samples used as outgroup, Table S1), a second sequence supermatrix was obtained using STACKS (835,467 bp; 51,557 SNPs; -p = 85; -r = 1; Table S2). A time-calibrated Bayesian inference (BI) analysis was performed in BEAST 2.6 (Bouckaert et al. 2019), using a birth-death tree model, an optimized relaxed molecular clock (Douglas et al. 2021), and a GTR+ G + I model of sequence evolution. We used the divergence time estimates and 95% confidence intervals (CI) of the secondary lineage-level calibrated tree from Ehl et al. (2019) to set a normally distributed prior for the crown diversification of *Salamandra* (i.e., the first split within the genus) (9.4 Ma [CI: 6.15 Ma, 12.76 Ma]). An initial MCMC chain was run for 10,000,000 iterations, sampling every 10,000 iterations. By applying the performance suggestions provided by the software, we set up a second run, this time with 20,000,000 iterations and sampling every 10,000 iterations. This two-step approach was applied to increase the effective sample size (ESS) of estimated parameters. For both runs, Tracer 1.7 (Rambaut et al. 2018) was used to monitor parameters and ensure that likelihood ESS was large (> 200) and stationary. Finally, a maximum clade credibility tree was produced in the BEAST module TreeAnnotator applying a burnin threshold of 10%. The timetree was visualized using FigTree v1.4.4 (Rambaut & Drummond 2018). For comparison, a maximum-likelihood tree based on the reduced dataset was reconstructed with 100 non-parametric bootstrap replicates using IQTree v. 1.6.12 and ModelFinder (Nguyen et al. 2015, Kalyaanamoorthy et al. 2017).

Heterozygosity

The denovo_map.pl pipeline in STACKS phases alleles cosegregating at the same locus in the same individual (following IUPAC nomenclature). We screened the two phylip input files using R for base ambiguity codes (according to IUPAC nomenclature: R, Y, S, W, K, and M) corresponding to these bi-allelic loci. The proportion of heterozygous positions among all known sites was calculated and plotted on a map using QGIS v. 3.22.3.

Results

Phylogenetic analysis and population clustering: full dataset

The maximum likelihood tree based on the entire dataset (392 plus two *Lyciasalamandra* individuals as outgroup; 356,874 bp; 24,192 SNPs, Table S2), recovered all *Salamandra* species as fully supported clades (100% bootstrap support [BS], Figs 1/S1), noting that *S. lanzai* was represented by a single individual. Moreover, all currently accepted subspecies within *S. infraimmaculata* and *S. algira* are grouped into two independent clades (BS > 70%, Figs 1/S1). The subspecies lineages of *S. atra* were also monophyletic, except for *S. a. atra*, which comprised two distinct lineages that were not sister to each other. In the most widespread species, *S. salamandra*, our sampling contained many individuals sampled in hybrid zones, and the reconstructed topology differed from previous analyses (Burgon et al., 2021). Specifically, the hybrid-inclusive analysis retrieved two sister clades containing (i) the Eastern and Central European subspecies *S. s. terrestris*, *S. s. salamandra*, *S. s. beschkovi*, *S. s. werneri*, and *S. s. "hispanica"*, and (ii) samples assigned to the Iberian and Italian subspecies *S. s. fastuosa*, *S. s. gigliolii*, *S. s. "alfredschmidti"*, *S. s. bernardezi*, and *S. s. gallaica* (Figs 1/S1). In this tree, several taxa were paraphyletic: *S. s. salamandra*, *S. s. fastuosa*, *S. s. bernardezi*, *S. s. gallaica*, *S. s. "molleri"*, and *S. s. werneri* (Figs 1/S1).

The highest number of realistically clustering genetic groups we could retrieve with STRUCTURE for the entire dataset (392 individuals; 2,490 SNPs, Table S3) was K = 11 (Figs 1/S1/2), which accordingly corresponded to runs with the highest likelihood estimates. The analysis retrieved *S. infraimmaculata*, *S. corsica*, *S. atra,* and *S. algira* as four distinct clusters, with the remaining seven clusters corresponding to *S. salamandra*. The last species, *S. lanzai*, was not reliably assigned to any cluster (a single individual was analyzed). Within *S. salamandra*, the identified clusters correspond to the following sets of subspecies: 1) *S. s. longirostris* and *S. s. almanzoris* (marked yellow in the corresponding figures); 2) *S. s. crespoi*, *S. s. morenica*, *S. s. "molleri"*, and *S. s. bejarae* (orange); 3) *S. s. gallaica* (pink); 4) *S. s. bernardezi* and *S. s. "alfredschmidti"* (limegreen); 5) *S. s. gigliolii* and *S. s. fastuosa* (green); 6) *S. s. salamandra* (red); and 7) *S. s. terrestris* (maroon). Three sample groups, attributed to *S. s. beschkovi*, *S. s. "hispanica"*, and *S. s. werneri*, display different mixes of ancestries from three clusters (pink, red, and maroon); Fig. 2.

Admixture patterns in *Salamandra* contact zones

The clustering analyses conducted within *S. salamandra* and *S. algira* revealed additional genetic structure and informed on admixture across the parapatric ranges of contacting lineages. In Galicia, the extent of introgression across the *S. s. gallaica*/*bernardezi* hybrid zone (69 individuals; 1,877 SNPs, K = 2, Table S3) differed between the two areas sampled (Fig. 3A). Admixture extends over a greater geographic distance in populations from A Coruña province than in populations from Lugo province, which are only separated by a few kilometers. In Western Iberia, individuals assigned to *S. s. gallaica*, *S. s. "molleri"*, and *S. s. bejarae* were distinguished in up to five clusters (26 individuals; 2,732 SNPs; K = 5; Table S3; Fig. 3B). One corresponds to *S. s. gallaica*, two correspond to *S. s. "molleri"* (hereinafter named North and South), and two additional clusters correspond to *S. s. bejarae* (West and East). In the

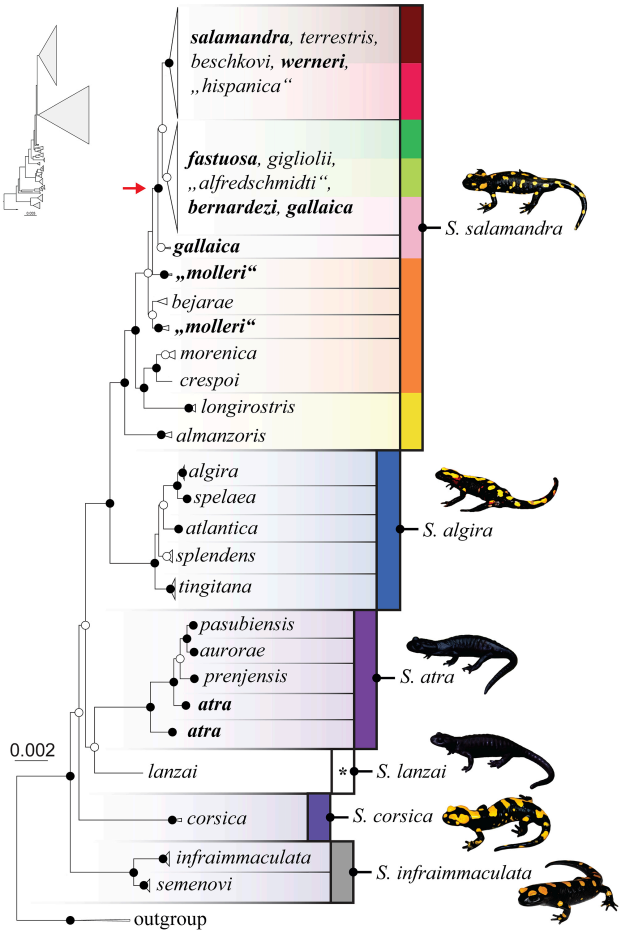

Figure 1. Maximum-likelihood tree of the genus *Salamandra* based on the entire ddRADseq dataset with admixed individuals (n = 392; 356,874 bp; 24,192 SNPs, Table S2), illustrating possible phylogenetic artefacts arising from their inclusion. The arrow points to the highly supported node placing the *fastuosa* clade nested with *S. salamandra* due to the presence of individuals admixed with *gallaica* (compare Fig. 4). Branch support values higher than 70%, based on 100 non-parametric replicates, are indicated by white dots, and fully supported branches (100%) by black dots. For presentation purposes, clades are non-proportionally condensed; a depiction in proportion is shown in the top left corner. An uncondensed version of the tree with all sample codes and localities is provided in the supplements (Fig. S1). The colors represent simplified STRUCTURE ancestry assignments (K = 11; 2,490 SNPs), also shown in detail Fig. S1. A mix of multiple ancestries (not shown here) was recovered in *S. lanzai* (asterisk). Non-monophyletic taxa are indicated by bold names. Disputed subspecies names are in quotation marks.

Pyrenees, two clusters were retrieved corresponding to *S. s. fastuosa* and *S. s. terrestris*; the latter also included samples assigned to *S. s. "hispanica"* (30 individuals; 2,786 SNPs; K = 2; Table S3; Fig. 3C). Admixture between these clusters was detected in the Central Pyrenees (Val d'Aran and Bagnères-de-Bigorre) and in one population at La Bastide-Clairence, close to the French Atlantic coast (Fig. 3C). Further east, the subset analysis encompassing *S. s. terrestris* and *S. s. salamandra* (117 individuals, 567 SNPs, K = 3, Table S3, Fig. 3D) retrieved these subspecies as distinct clusters, with wide admixture in Northern Germany, and a third cluster further distinguishes the southern vs. northern populations of *S. s. salamandra* (North and South). Finally, within *S. algira* four clusters were retrieved, each corresponding to a distinct subspecies, except for *S. algira spelaea* which clustered together with *S. algira algira* (42 individuals, 1,463 SNPs, K = 4, Table S3, Fig. 3E). The analysis revealed admixture between *S. algira tingitana* and *S. algira splendens*, which was regionally restricted to a hybrid zone along the Oued Laou in the Rif Mountains (Morocco, Fig. 3E).

Time-calibrated phylogenetic analysis: reduced dataset

Based on selected samples, the time-calibrated Bayesian tree and the maximum-likelihood tree (6,392 RAD tags, 835,467 bp; 51,557 SNPs; Table S2; Fig. 4) supported the species-level topology of the exploratory phylogenetic analysis (Figs 1/S1).

Phylogenetic relationships within *S. salamandra*, however, differ from the full-dataset analysis. A first two major clade contained *S. s. bernardezi, S. s. "alfredschmidti", S. s. fastuosa,* and *S. s. gigliolii* (clade B in Fig. 4), and a second one contained all other subspecies (clade A in Fig. 4), a pattern more consistent with the recent literature (Burgon et al., 2021). The divergence between these two clades was estimated at 5.55 million years (Ma, 95% confidence interval of 8.14–2.59 Ma). Both clades (A and B) were fully supported (1.0 PP and 100% BS) but their phylogenetic position as sister clades was not. Within *S. salamandra*, most subspecies form either fully or highly supported clades (PP > 0.95, BS > 70), except for *S. s. "molleri"* and *S. s. werneri*, which were retrieved as paraphyletic as their respective phylogeographic lineages do not branch together. Moreover, the analysis confirmed the phylogenetic position of *S. s. longirostris* as the sister lineage of *S. s. morenica* and *S. s. crespoi*, with an estimated divergence time of 3.07 Ma (4.71–1.43 Ma). We further note that the Italian *S. s. gigliolii* branches with the Iberian lineages of clade B, and its divergence from *S. s. fastuosa* was estimated to be 1.19 Ma (0.48–1.8 Ma).

Within *S. algira,* the crown divergence was estimated at 6.26 Ma (9.08–2.83 Ma). All subspecies of *S. algira* were robustly supported in the phylogeny, noting that the split between the most closely related *S. a. algira* and *S. a. spelaea* was dated to only 0.62 Ma (0.97–0.24 Ma). With-

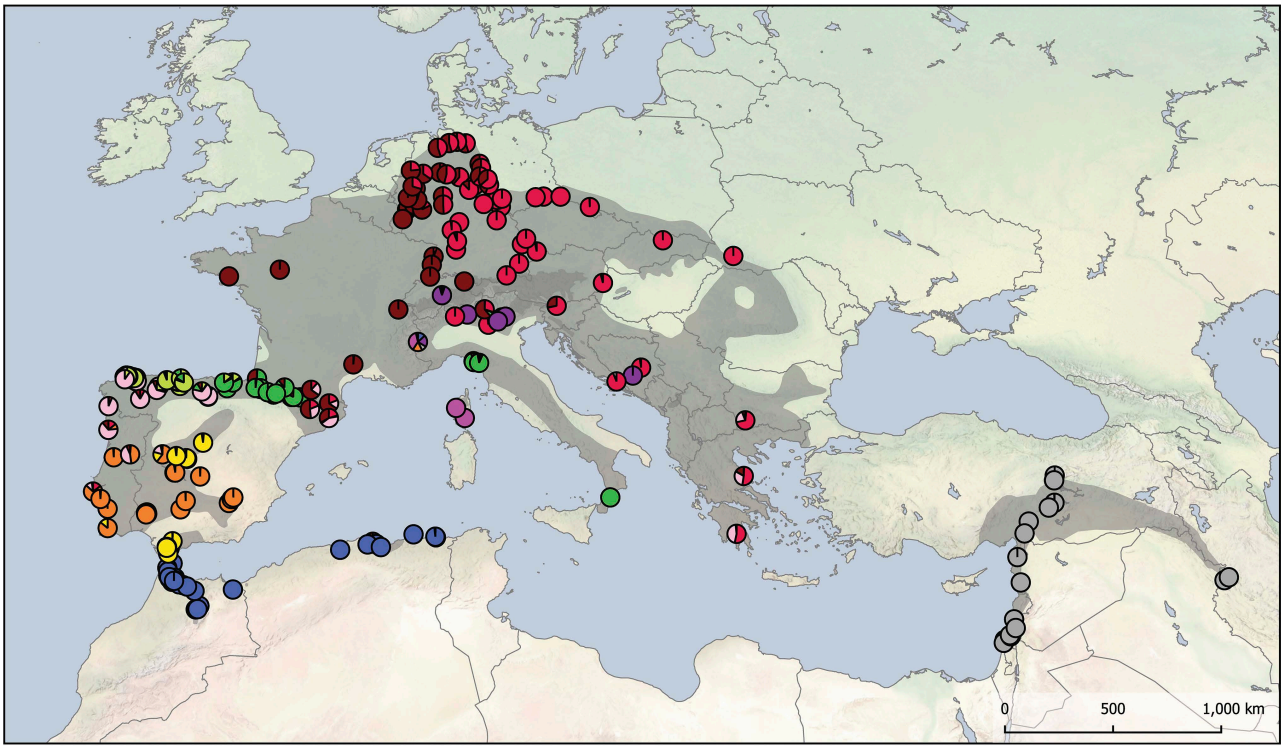

Figure 2. Map showing averaged STRUCTURE Q ancestries (K = 11) of all sampled populations based on ddRADseq data (see Table S1 for locality information of all samples). Approximate distribution ranges of the six *Salamandra* species (IUCN 2009, 2021a, b, c, Bonato et al. 2018, Candan 2022) are represented by grey shadow shapes on the map. The color scheme used is consistent with Figs 1/S1.

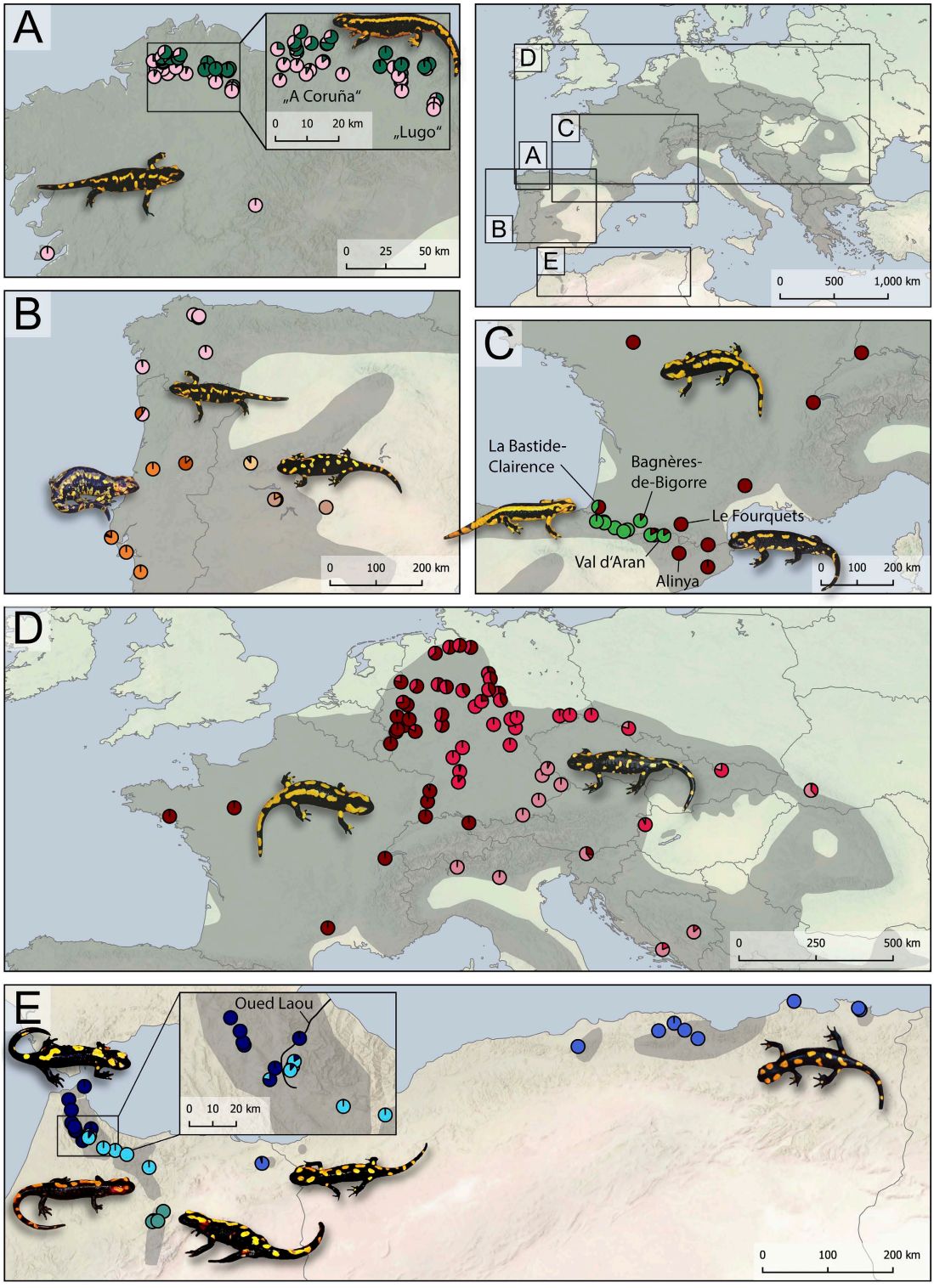

Figure 3. Admixture patterns in four contact zones of *Salamandra salamandra* (A–D) and in *S. algira* (E). Pie charts depict Q ancestries from five independent STRUCTURE analyses based on separately filtered subsets (see Table S3 for details). Approximate distribution ranges of the two *Salamandra* species (IUCN 2009, 2021a, b, c) are represented by grey shadow shapes on the map. A, *S. s. gallaica* [pink] and *S. s. bernardezi* (West) [dark green]; B, *S. s. gallaica* [pink], *S. s. "molleri"* (North) [rust orange], *S. s. "molleri"* (South) [orange]; *S. s. bejarae* (West) [beige], and *S. s. bejarae* (East) [light brown], C, *S. s. fastuosa* [green] and *S. s. terrestris* (top), *S. s. "hispanica"* (bottom) [maroon]; D, *S. s. terrestris* [maroon], *S. s. salamandra* (North) [red], and *S. s. salamandra* (South) [greyish pink]; E, *S. a. tingitana* [navy], *S. a. splendens* [cyan], *S. a. atlantica* [teal], and *S. a. spelaea* [isolated population in Morocco] + *S. a. algira* [blue].

Table 1. Contact zones of *Salamandra* (sub-)species analyzed genetically in previous studies with references to gene flow.

(Sub)species in contact	Contact zone	Gene flow/admixture	Number of samples/ localities	Type of markers	Number of loci
Salamandra s. crespoi and *S. s. gallaica*	Sesimbra and Alcoutim, Portugal	"*S. s. crespoi* displays **signs** of gene flow among the sampled locations whereas *S. s. gallaica* shows evidence of **some restriction** to gene flow" (Reis et al. 2011)	168/12 (Reis et al. 2011)	cytB [mtDNA] (Reis et al. 2011)	1 (Reis et al. 2011)
S. s. gallaica and *S. s. bernardezi*	Galicia/Asturias, Spain	"**limited** gene flow at the nuclear level" indicated by brick bars in Fig. 5 (García-París et al. 2003); "**widespread presence** of admixed phenotypes in this contact zone.", Fig. 2 (Velo-Antón et al. 2021)	?/? (García-París et al. 2003), 129/86 (Velo-Antón et al. 2021)	allozyme [nuDNA] (García-París et al. 2003), µsat [nuDNA] (Velo-Antón et al. 2021)	33 (García-París et al. 2003), 15 (Velo-Antón et al. 2021)
S. s. bernardezi and *S. s. fastuosa*	Asturias/Cantabria, Spain	"we also found a **high proportion** of admixed individuals", Fig. 4 (Velo-Antón et al. 2021)	123/58 (Velo-Antón et al. 2021)	µsat [nuDNA] (Velo-Antón et al. 2021)	15 (Velo-Antón et al. 2021)
S. s. fastuosa and *S. s. terrestris*	Navarra, Spain	"**limited** gene flow at the nuclear level" indicated by brick bars in Fig. 5 (García-París et al. 2003)	?/? (García-París et al. 2003)	allozyme [nuDNA] (García-París et al. 2003)	33 (García-París et al. 2003)
S. s. bernardezi and *S. s. bejarae* (*S. s. gallaica* according to our findings).	Cantabrian Mountains, Spain	"**negligible signs** of admixture and hybridization between the larviparous and large *S. s. bejarae* and the pueriparous and small *S. s. bernardezi*", Fig. 3 (Velo-Antón et al. 2021)	73/38 (Velo-Antón et al. 2021)	µsat [nuDNA] (Velo-Antón et al. 2021)	15 (Velo-Antón et al. 2021)
S. s. bejarae and *S. s. gallaica* (likely *S. s. "molleri"* [North] according to our findings).	Iberian Central System	"**significant** gene flow across all contact zones", Fig. 4 (Pereira et al. 2016)	10/4 (Pereira et al. 2016)	coding gene [nuDNA]	5 (Pereira et al. 2016)
S. s. bejarae and *S. s. almanzoris*	Iberian Central System	"**significant** gene flow across all contact zones", Fig. 4 (Pereira et al. 2016)	37/14 (Pereira et al. 2016)	coding gene [nuDNA]	5 (Pereira et al. 2016)
S. s. terrestris and *S. s. salamandra*	Germany	"The existence of two clines at the loci Ck-1 and Pgm document **introgression** between *S. s. salamandra* and *S. s. terrestris*." (Veith 1992); Fig. 1 (Weitere et al. 2004)	2183/48 (Veith 1992)	allozyme [nuDNA] (Veith 1992)	14 (Veith 1992)
S. salamandra and *S. atra*	Central Switzerland	"**no signs** of hybridization were observed" (Vences et al. 2014)	45/1 (Vences et al. 2014)	coding gene [nuDNA] (Vences et al. 2014)	2 (Vences et al. 2014)
S. algira tingitana and *S. a. splendens*	Moroccan Rif, Morocco	"**continuous** gene flow between *S. a. tingitana* and *S. a. splendens* in the Rif Mountains" (Dinis et al. 2019)	?/6 (Dinis et al. 2019)	µsat [nuDNA] (Dinis et al. 2019)	14 (Dinis et al. 2019)

Table 2. Evidence of species-level distinction between all pairs of *Salamandra* species. [1] Rodriguez et al. (2017); [2] Fig. 4 (this study); Rodriguez et al. (2017), Burgon et al. (2021); [3] calculated from sequences published in Rodriguez et al. (2017); [4] based on Fig. 4; [5] Thiesmeier & Grossenbacher (2004), Nascetti et al. (1988); Degani (1986); Sparreboom (2014); [6] Mulder et al. (2022); [7] Vences et al. (2014).

Pairwise species comparisons	Reciprocally monophyletic (mtDNA[1]/phylogenomics[2])	uncorrected pairwise distance (16S)[3]	Estimated age in million years[4]	Diagnostic morphological differences[5]	Distinct reproductive mode[6]	Syntopy without admixture[7]
S. atra Laurenti, 1768 vs. *S. corsica* Savi, 1838	yes/yes	3.5%	8.09	yes	yes	no
S. atra Laurenti, 1768 vs. *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988	yes/yes	3.8%	6.61	yes	no	no
S. infraimmaculata Martens, 1885 vs. *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988	yes/yes	4.0%	8.89	yes	yes	no
S. salamandra (Linnaeus, 1758) vs. *S. infraimmaculata* Martens, 1885	yes/yes	4.0%	8.89	yes	partially	no
S. salamandra (Linnaeus, 1758) vs. *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988	yes/yes	4.0%	7.51	yes	partially	no
S. algira Bedriaga, 1883 vs. *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988	yes/yes	4.1%	7.51	yes	partially	no
S. algira Bedriaga, 1883 vs. *S. infraimmaculata* Martens, 1885	yes/yes	4.2%	8.89	yes	partially	no
S. salamandra (Linnaeus, 1758) vs. *S. atra* Laurenti, 1768	yes/yes	4.2%	7.51	yes	partially	yes
S. salamandra (Linnaeus, 1758) vs. *S. algira* Bedriaga, 1883	yes/yes	4.4%	6.26	yes	partially	no
S. atra Laurenti, 1768 vs. *S. infraimmaculata* Martens, 1885	yes/yes	4.4%	8.89	yes	yes	no
S. corsica Savi, 1838 vs. *S. lanzai* Nascetti, Andreone, Capula & Bullini, 1988	yes/yes	4.4%	8.09	yes	yes	no
S. corsica Savi, 1838 vs. *S. infraimmaculata* Martens, 1885	yes/yes	4.5%	8.89	yes	no	no
S. salamandra (Linnaeus, 1758) vs. *S. corsica* Savi, 1838	yes/yes	4.7%	8.09	yes	partially	no
S. atra Laurenti, 1768 vs. *S. algira* Bedriaga, 1883	yes/yes	5.3%	7.51	yes	partially	no
S. corsica Savi, 1838 vs. *S. algira* Bedriaga, 1883	yes/yes	5.4%	8.09	yes	partially	no

in *S. atra,* the crown divergence was estimated at 2.85 Ma (4.56–1.26 Ma), with the youngest lineages being *S. a. aurorae* and *S. a. pasubiensis* (0.5 Ma, CI: 0.83–0.21 Ma). Finally, the oriental *S. infraimmaculata* was retrieved as the sister group of all other *Salamandra* species, with a crown divergence estimated at 2.93 Ma (4.55–1.21 Ma, Fig. 4G).

Heterozygosity

In the alignment of 2,730 RAD tags (356.874 bp; 24,192 SNPs; Table S2), the percentage of heterozygous sites varied from 0.01% to 0.32% between individuals. The proportion of heterozygous sites was the highest in *S. salamandra*, with a median of 0.14% (0.144% mean, 0.062% standard deviation [sd], Fig. 5). Conversely, the single *S. lanzai* individual had the lowest proportion of heterozygous sites (0.029%), followed by *S. corsica* (0.033% median, 0.034% mean, 0.009% sd), *S. atra* (0.035% median, 0.032% mean, 0.01% sd), *S. algira* (0.067% median, 0.074% mean, 0.033% sd), and *S. infraimmaculata* (0.069% median, 0.063% mean, 0.035% sd, Fig. 5).

Discussion

As illustrated by our case study in genus *Salamandra*, identifying phylogeographic diversity, notably acknowledging this diversity as infraspecific units through taxonomic recognition, or as ESUs or MUs is challenging when lineages hybridize and large parts of population ranges show pervasive admixture (Coates et al. 2018). Infraspecific units, such as subspecies, are usually seen as reflecting patterns of geographic variation emerging from genetic drift or local adaptation. They may display different phenotypes and/or represent different lineages. Therefore, a comprehensive,

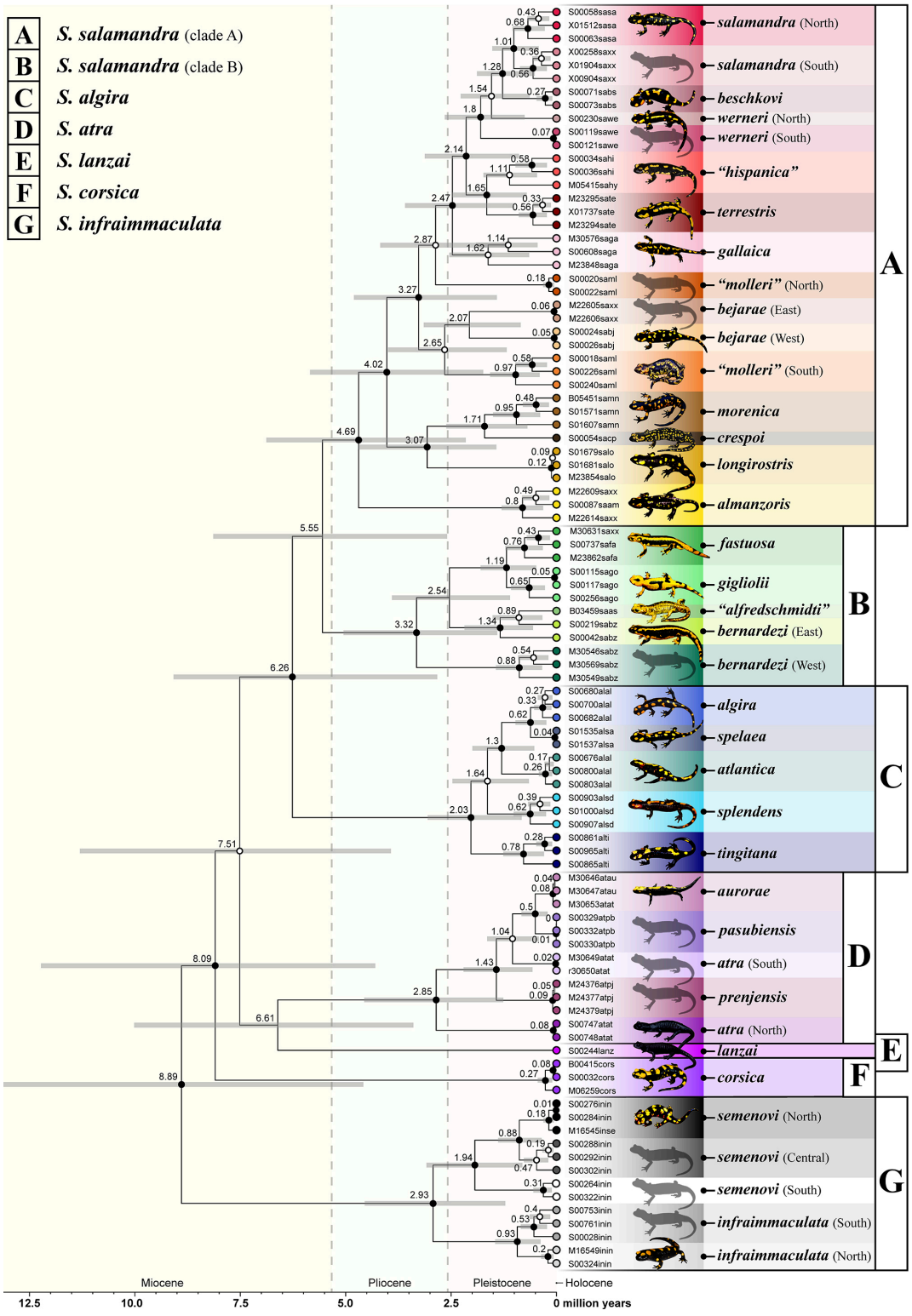

Figure 4. Time-calibrated Bayesian tree of the genus *Salamandra* based on a ddRADseq alignment (n = 95; 835,467 bp; 51,557 SNPs; Table S2) of the reduced dataset with selected, non-admixed individuals representing lineages identified from the entire dataset (Fig. 1). White dots on nodes indicate branch support (> 0.95 posterior probability [PP] and > 70% non-parametric bootstrap [BS]), while black dots denote full branch support (1.0 PP and 100% BS). Transitions between Miocene/Pliocene and Pliocene/Pleistocene are indicated by dotted vertical lines. The outgroups (*Lyciasalamandra*) were removed to improve readability. A map with samples shown here is presented in Fig. S2. Salamanders are not to scale. Disputed subspecies names are in quotation marks.

multifaceted, integrative approach is necessary to delineate subspecies boundaries, notably by taking population history, phenotypic differences, and geographic range into account.

The effect of hybrids on phylogenetic delimitation

Genomes are usually inherited within populations that share a common ancestor, and their evolutionary history can usually be described by a bifurcating tree. However, this is impeded when genes are exchanged between more distantly related populations, for instance, through hybridization and subsequent introgression (Mallet 2005). This can lead to reticulated evolution that is best described by a network rather than a bifurcating tree. Reticulated evolution, as best studied through allopolyploid hybrid species, is frequent in many groups, e.g., plants (polyploidization) or prokaryotes (horizontal gene transfer), and also in some animals, including vertebrates (Grant & Grant 1992, Mallet et al. 2016).

Hybridization and introgression pose challenges to phylogenetic reconstruction and to the taxonomic identification of candidate lineages. Organellar markers (in animals, mitochondrial genes) of matrilinear inheritance are often used as DNA barcodes, both to identify taxa, notably cryptic taxa (Pons et al. 2006), and to delimit them as distinct lineages. These analyses can be severely biased by introgressive hybridization. Historical exchanges can cause mitochondrial trees to depart from species trees, while recent exchanges can lead to the misidentification of specimens used in phylogenetic analyses, and thus skew tree-based taxonomic conclusions (Edwards et al. 2016, Bonnet et al. 2017, Dufresnes & Jablonski 2022). With nuclear DNA, especially with phylogenomic analyses, introgression can have additional consequences for phylogenetic tree reconstruction (Pyron et al. 2022, Ambu et al. 2023). In such cases, the inclusion of hybrids may lead to artificial topologies, such as ladder-like patterns mediated by the relative proportion of ancestry from each parental lineage, combined with the respective placement of these lineages in the phylogeny. Admixed samples between non-sister lineages are the most problematic, as they expectedly form distinct lineages that branch close to neither of their parents, hence mimicking candidate species or subspecies (Dolinay et al. 2021, Chan et al. 2022, Pyron et al. 2022). Both of these artefacts are observable in our exploratory tree based on the full data set (Fig. S1A), where many admixed individuals from the *S. s. salamandra*/*terrestris* contact zone (Fig. 3D, Table S1) form a ladder-like topology intertwined by one *S. s. terrestris* individual from Western France (admixed by *S. s. fastuosa*). In addition, the inclu-

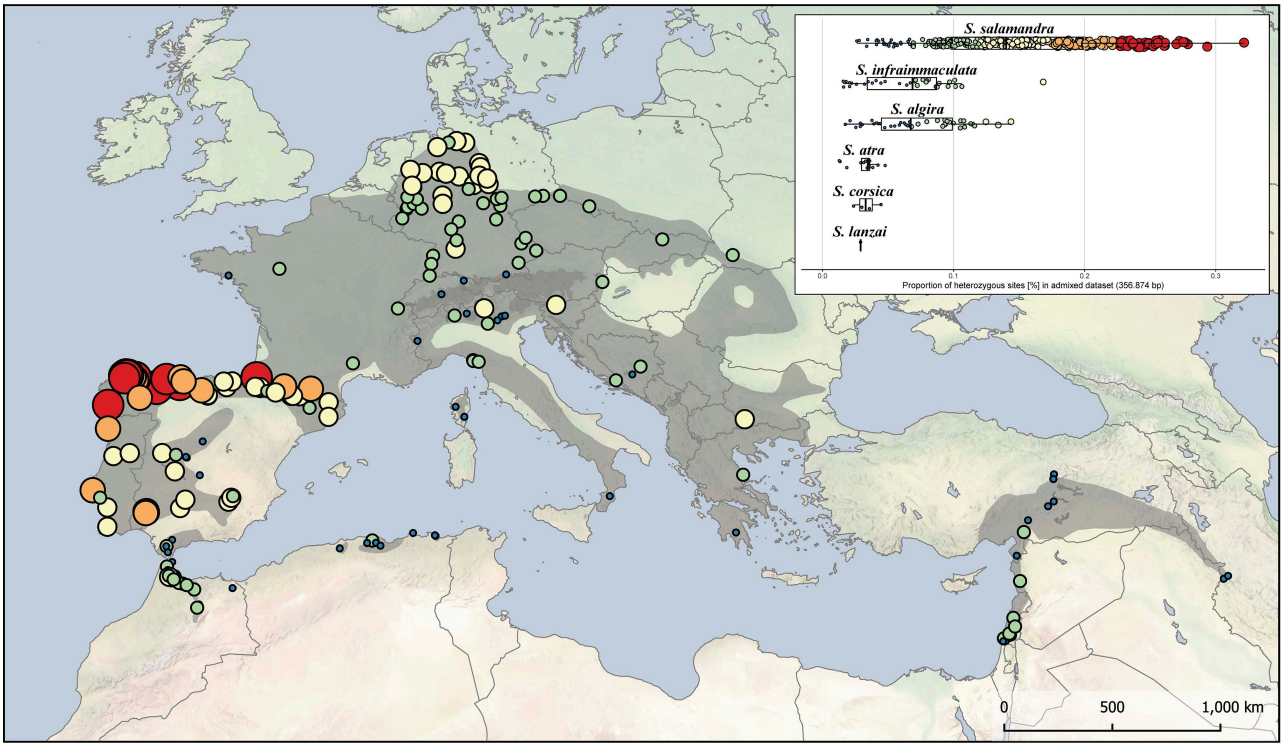

Figure 5. Proportion of heterozygous sites per population in the genus *Salamandra*, calculated from the ddRADseq alignment (356.874 bp) used as input for the maximum-likelihood tree in Fig. 1. The distribution of individuals is presented in the boxplots and grouped by species. Approximate distribution ranges of the six *Salamandra* species (IUCN 2009, 2021a, b, c, Bonato et al. 2018, Candan 2022) are represented by grey shadow shapes on the map. Color scale: > 0.22% red, 0.17–0.22% orange, 0.12–0.17% yellow, 0.07–0.12% green, < 0.07% blue.

Table 3. Evidence from different delimitation criteria for distinctness of *Salamandra* subspecies. NA refers to data unavailable or not consistently analyzed. Color codes: Green – supportive evidence/supported status, red – contradictive statement/unsupported status, yellow – ambiguous evidence/uncertain status, grey – neutral.

Subpecies	Monophyletic in mtDNA tree (literature)	Monophyletic in previous phylogenomic analyses (literature)	Monophyletic in our phylogenomic analysis with admixed individuals (Fig. 1, this study)	Monophyletic in our phylogenomic analysis with reduced dataset (Fig. 4, this study)	Nested within another subspecies with name priority (Fig. 4)	Secondary contact zone identified (Table 1, Fig. 3)	Taxonomic status
S. s. salamandra (Linnaeus, 1758)	NA	**no** (Burgon et al. 2021)	**no**	**yes** (fully supported), two monophyletic lineages (North/South)	**no**	**yes** (with *S. s. terrestris*)	**supported**
S. s beschkovi Obst, 1981	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	NA	**supported** (denser sampling in South-Western Bulgaria and in-depth delimitation from *S. s. salamandra* needed)
S. s. werneri Sochurek & Gayda, 1941	NA	**no** (Burgon et al. 2021)	**no**	**no**, two paraphyletic lineages (North/South)	**no**	NA	**supported** (split between Northern and Southern lineage required to recover monophyly)
S. s. "hispanica" Wolterstorff, 1937	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (supported)	**no**	NA	**supported** (denser sampling in North-Eastern Spain and in-depth delimitation from *S. s. terrestris* needed)
S. s. terrestris Lacépède, 1788	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	**yes** (with *S. s. salamandra*)	**supported**
S. s. gallaica López-Seoane, 1885	NA	**no** (Burgon et al. 2021)	**no**	**yes** (supported)	**no**	**yes** (with *S. s. bernardezi* and *S. s. "molleri"*)	**supported**
S. s. "molleri" Bedriaga, 1889	NA	**no** (Burgon et al. 2021)	**no**	**no**, two paraphyletic lineages (North/South)	**no**	**yes** (with *S. s. gallaica* and *S. s. bejarae*)	**uncertain** (denser sampling in North-Eastern Portugal and in-depth delimitation between *S. s. "molleri"* [North], *S. s. gallaica*, and *S. s. "molleri"* [South] required, split may be needed to recover monophyly)
S. s. bejarae Wolterstorff, 1934	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (unsupported), two monophyletic lineages (West/East)	**no**	**yes** (with *S. s. almanzoris*)	**supported** (denser sampling in Central Spain and in-depth delimitation from *S. s. "molleri"* [North] needed)
S. s. morenica Joger & Steinfartz, 1994	**yes** (Vences et al. 2014)	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	NA	**supported** (denser sampling in Southern Spain/ Portugal and in-depth delimitation from *S. s. crespoi* needed)
S. s. crespoi Malkmus, 1983	NA	**yes** (Burgon et al. 2021)	NA (only one individual included)	NA (only one individual included)	**no**	**yes** (with *S. s. gallaica*)	**supported**
S. s. longirostris Joger & Steinfartz, 1994	**yes** (Vences et al. 2014)	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	**no**	**supported**
S. s. almanzoris Müller & Hellmich, 1935	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	**yes** (with *S. s. bejarae*)	**supported**
S. s. fastuosa Schreiber, 1912	NA	**no** (Burgon et al. 2021)	**no**	**yes** (fully supported)	**no**	**yes** (with *S. s. gallaica* and *S. s. terrestris*)	**supported**

Table 3 continued

Subpecies	Monophyletic in mtDNA tree (literature)	Monophyletic in previous phylogenomic analyses (literature)	Monophyletic in our phylogenomic analysis with admixed individuals (Fig. 1, this study)	Monophyletic in our phylogenomic analysis with reduced dataset (Fig. 4, this study)	Nested within another subspecies with name priority (Fig. 4)	Secondary contact zone identified (Table 1, Fig. 3)	Taxonomic status
S. s. gigliolii Eiselt & Lanza, 1956	NA	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**no**	NA	**supported**
S. s. "alfredschmidti" Köhler & Steinfartz, 2006	**no** (Beukema et al. 2016), **yes** (Vences et al. 2014)	**yes** (Burgon et al. 2021)	**yes**	NA (only one individual included)	**yes** (*S. s. bernardezi* [East])	NA	**unsupported** (synonym of *S. s. bernardezi*, in agreement with Beukema et al. 2016)
S. s. bernardezi Wolterstorff, 1928	**no** (Beukema et al. 2016)	**no** (Burgon et al. 2021)	**no**	**no**, two paraphyletic lineages (East/West)	**no**	**yes** (with *S. s. gallaica* and *S. s. fastuosa*)	**supported** (denser sampling in Northern Spain and in-depth delimitation between *S. s. bernardezi* (West), *S. s. bernardezi* (East), and *S. s. fastuosa* required, split needed to recover monophyly
S. algira algira Bedriaga, 1883	**no** (Dinis et al. 2019), **yes** (Merabet et al. 2016)	**yes** (Burgon et al. 2021), **yes** (Dinis et al. 2019)	**yes**	**yes** (fully supported)	**no**	**no**	**supported**
S. algira spelaea Escoriza & Comas, 2007	**yes** (Dinis et al. 2019), **yes** (Merabet et al. 2016)	**yes** (Burgon et al. 2021), **yes** (Dinis et al. 2019)	**yes**	**yes** (fully supported)	**no**	**no**	**supported**
S. algira atlantica Hernandez & Escoriza, 2019	**yes** (Dinis et al. 2019)	**yes** (Burgon et al. 2021), **yes** (Dinis et al. 2019)	**yes**	**yes** (fully supported)	**no**	**no**	**supported**
S. algira splendens Beukema, de Pous, Donaire-Barroso, Bogaerts, Garcia-Porta, Escoriza, Arribas, El Mouden & Carranza, 2013	**yes** (Dinis et al. 2019), **yes** (Merabet et al. 2016)	**yes** (Burgon et al. 2021), **yes** (Dinis et al. 2019)	**yes**	**yes** (fully supported)	**no**	**yes** (with *S. algira tingitana*)	**supported**
S. algira tingitana Donaire-Barroso & Bogaerts, 2003	**yes** (Dinis et al. 2019), **yes** (Vences et al. 2014), **yes** (Merabet et al. 2016)	**yes** (Burgon et al. 2021), **yes** (Dinis et al. 2019)	**yes**	**yes** (fully supported)	**no**	**yes** (with *S. algira splendens*)	**supported**
S. atra atra Laurenti, 1768	**no** (Šunje et al. 2021)	**yes** (Burgon et al. 2021)	**no**	**no**, two lineages (North/South)	**no**	**no**	**supported** (split between Northern and Southern lineage needed to recover monophyly)
S. atra prenjensis Miksic, 1969	**yes** (Šunje et al. 2021)	**NA**	**yes**	**yes** (fully supported)	**yes** (*S. atra atra*)	**no**	**supported**
S. atra aurorae Trevisan, 1982	**yes** (Vences et al. 2014), **yes** (Šunje et al. 2021)	**NA**	**yes**	**yes** (fully supported)	**yes** (*S. atra atra*)	**no**	**supported**

Table 3 continued

Subspecies	Monophyletic in mtDNA tree (literature)	Monophyletic in previous phylogenomic analyses (literature)	Monophyletic in our phylogenomic analysis with admixed individuals (Fig. 1, this study)	Monophyletic in our phylogenomic analysis with reduced dataset (Fig. 4, this study)	Nested within another subspecies with name priority (Fig. 4)	Secondary contact zone identified (Table 1, Fig. 3)	Taxonomic status
S. atra pasubiensis Bonato & Steinfartz, 2005	**yes** (Šunje et al. 2021)	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported)	**yes** (*S. atra atra*)	**no**	**supported**
S. infraimmaculata infraimmaculata Martens, 1885	**yes** (Vences et al. 2014)	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported), two lineages (North/South)	**no**	**NA**	**supported**
S. infraimmaculata semenovi Nesterov, 1916	**yes** (Steinfartz et al. 2000)	**yes** (Burgon et al. 2021)	**yes**	**yes** (fully supported), three lineages (North/Central/South)	**no**	**NA**	**supported** (in-depth delimitation from conspecific lineages needed, old divergence estimates between Southern and Northern/Central lineages might result in split)
S. infraimmaculata orientalis Wolterstorff, 1925	**yes** (Steinfartz et al. 2000)	**NA**	**NA**	**NA**	**NA**	**NA**	**uncertain** (geographic distribution and morphological differentiation disputed, therefore not assigned to any samples in this study. Might be applicable to *S. i. infraimmaculata* (North), *S. i. semenovi* (South) or *S. i. semenovi* (Central). Denser sampling in southern Turkey and from type locality (Adana) needed for delimitation from conspecific lineages)

sion of *S. s. bernardezi/gallaica* hybrids is likely responsible for twisting the position of clade B within the other *salamandra* subspecies. Hence, the comparison of the two phylogenetic analyses – with and without admixed individuals (Figs 1/4) – illustrates the need to carefully screen for admixture before drawing taxonomic conclusions from phylogenomic patterns (see also Unmack et al. 2022, Ambu et al. 2023).

Species or subspecies? Lineages in the "gray-zone" of species delimitation

According to traditional and long-established taxonomic conventions and within the framework of the International Code for Zoological Nomenclature (ICZN), taxa can be assigned to two hierarchical terminal ranks, i.e., species and subspecies (Ride et al. 1999). All six *Salamandra* species were successfully identified in phylogenetic and exploratory clustering analyses, except *S. lanzai* in the latter. However, the artefactual mix of multiple ancestries retrieved for populations represented by one or a few individuals, as in the case of *S. lanzai* in our data set, is a well-known limitation of STRUCTURE, especially for large datasets with uneven sample sizes (Puechmaille 2016, Meirmans 2019). As discussed above, the correct assignment of infraspecific taxonomic units to distinct lineages was massively impeded when admixed individuals were not excluded beforehand. The exclusion of hybrids alleviated these issues; however, the numbers of genetic clusters retrieved and admixture patterns between them differed between analyses conducted at different phylogenetic scales, which complicates their identification and candidacy for taxonomic recognition (Rancilhac et al. 2023).

While there is general agreement that two populations should be considered different species when they face complete or near-complete reproductive isolation, the classification of taxa in the gray zone of speciation and below remains disputed. Hence, the subspecies rank has been the subject of various definitions, each with its virtues and limitations (Wilson and Brown 1953, Mayr 1982, Frost & Hillis 1990, de Queiroz 2020). In recent years, a growing debate has questioned the usefulness of subspecies in taxonomy, with some authors suggesting that they should be de-emphasized or not used at all (Padial & De la Riva 2021, Burbrink et al. 2022), while others propose the opposite (Dufresnes et al. 2023a, Vences et al. 2024).

According to Hillis (2020), and building upon Frost & Hillis (1990), "the subspecies category could be used theoretically for sublineages not incontrovertibly removed from the possibility of interaction with other sublineages". Further, it is argued that subspecies could be used for formerly isolated "sublineages within one species that are now reproductively interacting" again, rather than being employed to name slices of continuous geographical variation that do not represent lineages (Hillis 2020). Under this definition, the extent of introgression in a hybrid zone, as often quantified by the width of geographic sigmoïd

clines fit on allele frequencies (Barton, 1983) can be used to measure reproductive isolation and thus define species vs. subspecies, with respect to the dispersal capabilities of organisms (Dufresnes et al. 2021, Chambers et al. 2022). Hence, assuming no dispersal barrier, lineages that come into secondary contact and form narrow hybrid zones characterized by sharp clines (suggestive of advanced RI) may be regarded as species, whereas lineages featuring wide hybrid zones with genetic admixture that is seemingly not constrained by natural selection (suggestive of little or no RI) should be regarded as subspecies (Dufresnes et al. 2020, Hillis et al. 2021, Dufresnes et al. 2021, 2023a).

Within the wide distribution of *Salamandra*, no hybrid zones among species-level lineages have been detected so far (Table 1). Despite the syntopy of *S. atra* and *S. salamandra* in the Alps and in the Dinaric Alps, there is no evidence for introgressive hybridization, which supports their distinction as separate species within *Salamandra* (Vences et al. 2014). In contrast, our analyses revealed considerable levels of admixture between parapatric subspecies both within *S. salamandra* and in *S. algira* (Fig. 3), as documented in previous studies (Table 1). By incorporating a large set of new individuals, our genomic study laid the groundwork for future in-depth hybrid zone analyses implementing transect sampling and cline analyses to investigate the actual level of reproductive isolation in *Salamandra* subspecies. This is particularly relevant for the contact zones of *S. a. tingitana*/*S. a. splendens* along the Oued Laou in the Moroccan Rif (Fig. 3E) and *S. s. gallaica*/*S. s. bernardezi* in the Galicia/Asturias border in NW Spain (Fig. 3A), which have been characterized using microsatellite data by Dinis et al. (2019) and Velo-Antón et al. (2021), respectively, for which our ddRADseq data provides preliminary genomic information to be completed by future studies. Additionally, our study narrows down the geographic location of one contact zone between *S. s. fastuosa* and *S. s. terrestris* in the eastern Pyrenees (near Val d'Aran), as previously suspected (García-París et al. 2003) (Fig. 3C). The populations assigned to *S. s. "hispanica"* (Alinya) in the southeastern Pyrenees and *S. s. terrestris* (Le Forquets) in the northeastern Pyrenees showed no admixture by *S. s. fastuosa*, despite being only ~60 km apart from Val d'Aran.

So far, there is no strong evidence to split *S. salamandra* into several species. Clade B, comprising *S. s. fastuosa*, *S. s. bernardezi*, *S. s. gigliolii*, and *S. s. alfredschmidti*, presents several hybrid zones with various degrees of gene flow with the subspecies of clade A (*S. s. salamandra* and others). Some transitions are relatively narrow, as in the Cantabrian Mountains, while others are wider, as in northern Galicia, which is characterized by heterogenous environments. (Table 1; Velo-Antón et al. 2021). Evidence of extensive mitochondrial introgression across the range of *S. s. bernardezi* (Lourenço et al. 2019, Figueiredo-Vázquez et al. 2021) further suggests historical hybridization between these two clades A and B. Our genomic data confirm that gene flow occurs between *S. s. bernardezi* (clade B) and *S. s. gallaica* (clade A) in the Galician contact zone, although we did not statistically quantify its extent. This contact zone is of particular interest since these hybridizing lineages (clades A and B) are divergent both in morphology (e.g., body size, head shape, and color pattern; Alarcón-Ríos et al. 2020) and reproductive strategies (larviparous vs. pueriparous; Velo-Antón et al. 2015).

In parallel, Velo-Antón et al. (2021) analyzed another contact zone between members of the two main *S. salamandra* clades, namely *S. s. bernardezi* (clade B) and populations provisionally assigned to *S. s. bejarae* (clade A) in the Cantabrian Mountains. In our analyses, however, the latter cluster with populations assigned to *S. s. gallaica* and not with populations from the type locality of *S. s. bejarae* (Fig. S1). Unlike the Galician contact zone, only a few admixed individuals were retrieved, which is a priori consistent with reproductive isolation and will warrant further analyses, notably by accounting for local landscape barriers.

Taxonomic implications of *Salamandra* phylogenomics

Through ad hoc procedures, we carefully minimized the effects of admixed individuals on phylogenomic tree reconstruction, noting that we did not account for historical gene flow, which often requires cumbersome analyses (e.g., Durand et al. 2011). Our two phylogenetic trees, which differed in the inclusion vs. exclusion of hybrids (Fig. 1 vs. Fig. 4), did not reveal species-level topological discordances. The major disagreements were rather found within the species clades, especially regarding several subspecies that were retrieved as paraphyletic and in different phylogenetic positions in the tree including hybrids (*S. s. fastuosa* and *S. s. gallaica*, Figs 1/S1). In addition, some closely related subspecies, such as *S. s. morenica*, *S. s. crespoi*, and *S. s. longirostris*, were not retrieved as distinct genetic groups in the clustering analyses, although these appear as distinct lineages in both phylogenetic trees (Figs 1/S1/4). One potential reason for this discrepancy is the difficulty in obtaining single datasets that contain loci conserved enough between deeply diverged species but evolved enough polymorphism to also distinguish their young phylogeographic lineages, even more so with uneven sample sizes (Puechmaille 2016).

Our timetree, which encompassed all species and their subspecies, provides new insights on the evolutionary history of *Salamandra* with respect to previous studies (e.g., Vences et al. 2014). Nevertheless, the divergence times presented in Fig. 4 should be interpreted with caution, as confidence intervals, particularly for older ages, are wide, which is expected since we used a single calibration point, and adequate fossil calibrations are not available for the genus *Salamandra*. Moreover, given the many examples of present-day contact zones (Table 1), lineages may have already been in contact following past range expansions during the interglacial phases of the Pleistocene (Schmitt 2007). Hence, historical gene flow may affect our divergence time estimations in the same way it could

have skewed our phylogeny, particularly for the hybridizing *S. salamandra* lineages (see above).

In the following paragraphs, we discuss the genomic evidence for infraspecific structure, phylogeography, and taxonomy for each species separately. Tables 2 and 3 summarize the available evidence for taxon delimitation and distinctiveness among *Salamandra* species and subspecies.

For *S. infraimmaculata*, our results agreed with the previously published phylogeny of Burgon et al. (2021) that also retrieved two deeply diverged lineages, here associated to the subspecies *S. i. infraimmaculata* and *S. i. semenovi*. These subspecies may come into contact in northern Syria, where *S. i. semenovi* has further diversified (Figs S1/S2). Determining their genetic interactions and, subsequently, their taxonomic status, especially the distinction of *S. s. semenovi* and *S. s. orientalis*, will require future sampling and analyses.

For *S. atra*, our study is the first genomic assessment of the subspecies *S. a. aurorae* and *S. a. prenjensis*. We confirmed the monophyly of the species but also retrieved the nominate subspecies *S. a. atra* as paraphyletic, based on its accepted distribution. Specifically, *S. a. atra* corresponds to two lineages, a southern one found in the southern Prealps (Orobie, Italy), which branch together with the young taxa *S. a. aurorae, S. a. pasubiensis* (Venetian Alps), and *S. a. prenjensis* (Dinaric Alps), and a northern one, here represented by two individuals from Central Switzerland. The southern lineage from Orobie was discussed as a potential candidate subspecies (Bonato et al. 2018, Šunje et al. 2021), but previous research assumed relatedness to the populations of *S. a. atra* found across its main, northern range. The distribution range of *S. atra* in the southern Prealps is fragmented and consists of isolated patches of populations that exhibit high genetic differentiation associated with geographic barriers such as deep glacial valleys (Bonato et al. 2018). Although the fragmented populations found in the Dinarides, associated with *S. a. prenjensis,* are not as strongly differentiated compared to the southern Alp populations (Šunje et al. 2021), it would be important to extend genomic analyses to additional isolates to complete our understanding of the evolutionary history of this subspecies. In contrast, the Swiss populations appear genetically homogenous across hundreds of kilometers, which implies that all these populations correspond to the northern lineage of *S. a. atra* (Dufresnes et al. 2022). In light of our results, investigations should extend to additional populations previously attributed to *S. a. atra*, which will be necessary to propose taxonomic revisions.

For *S. lanzai* and *S. corsica,* our datasets only included a small set of individuals (N = 1 and N = 4, respectively). Using a phylotranscriptomic dataset, Rodriguez et al. (2017) found the nodes connecting *S. atra, S. corsica*, and *S. lanzai* to be insufficiently resolved due to conflicting gene tree topologies that may reflect past hybridization. Conflicting results were also obtained by Mulder et al. (2022) using exome capture data and a multi-species coalescent approach: 66% of all topologies support *S. lanzai* and *S. corsica* as sister species, noting that these differ in their reproductive strategies (larviparous vs. pueriparous). In our trees, *S. lanzai* and *S. atra* form either a moderately supported (Fig. 1) or an unsupported clade (Fig. 4), and *S. corsica* is placed as the sister lineage of a clade grouping *S. lanzai*, *S. atra*, *S. algira*, and *S. salamandra*, however, with moderate support perhaps reflecting past introgression between *S. lanzai*, *S. corsica*, and *S. atra* (Mulder et al. 2022). Here, the low values of heterozygosity found in these three species (Fig. 5) do not provide an indication of hybridization. Alternatively, the separation of these three species may have occurred almost simultaneously in geological time, reflecting a "hard" polytomy, hence the difficulty in retrieving a single, robust topology (see, e.g., Ambu et al. 2023). Whatever their evolutionary history, the species status of both *S. corsica* and *S. lanzai* is evident due to their high molecular, morphological, behavioral, and ecological distinctness (Table 2).

Our findings on *S. algira*, based on extensive sampling, align with previous studies by Dinis et al. (2019) and Burgon et al. (2021): all five recognized subspecies are retrieved in the phylogenomic trees, and with high statistical support in the entire dataset (Fig. 1), despite the inclusion of samples admixed between *S. a. tingitana* and *S. a. splendens* (Fig. 3E). As in Burgon et al. (2021), we recovered *S. a. tingitana* as sister to a clade including all other *S. algira* subspecies, but contrary to the ddRADseq tree of Dinis et al. (2019), where *S. a. splendens* and *S. a. tingitana* are sister lineages. Our results also differ from the microsatellite inferences of Dinis et al. (2019), by not separating *S. a. algira* and *S. a. spelaea* as distinct clusters (Fig. 3E). Nonetheless, *S. a. spelaea* forms a supported, albeit shallow, lineage both in our phylogenomic tree (Fig. 4) and in the mitochondrial trees of Merabet et al. (2016) and Dinis et al. (2019), which may continue to justify its recognition as a valid subspecies. Finally, admixture between *S. a. tingitana* and *S. a. splendens,* despite the presence of a putative geographic barrier (Oued Laou), suggests that considering *S. a. tingitana* as a distinct species is premature (Dubois & Raffaëlli 2009). The identification of the contact zone between these two lineages offers a promising avenue to assess their interactions in a species delimitation framework, but for the time being, we retain the current infraspecific arrangement.

For *S. salamandra,* the deepest intraspecific node in our phylogenomic timetree distinguishes *S. s. fastuosa*, *S. gigliolii*, *S. s. bernardezi*, and *S. s.* "*alfredschmidti*" (clade B) from other lineages/subspecies within this species (clade A), albeit with low support (Fig. 4). In their ddRADseq tree, Burgon et al. (2021) previously retrieved a nested position of *S. s. gigliolii* within *S. s. fastuosa* and of *S. s. "alfredschmidti"* within *S. s. bernardezi*. Because the exclusion of hybrids allowed us to retrieve *S. gigliolii* and *S. s. fastuosa* as separate, fully supported clades, we hypothesize that the inclusion of *S. s. fastuosa*/*bernardezi* hybrids from Basque Country in our exploratory tree caused the paraphyly of *S. s. fastuosa* (Fig. S1) and might similarly explain the findings of Burgon et al. (2021). In parallel, our only sample of *S. s. "alfredschmidti"* analyzed clusters within two

S. s. bernardezi samples of the same area (Fig. S2), which casts doubt on the validity of the former taxon. The lineage originally described as *S. s. "alfredschmidti"* is known to represent an extreme example of color polymorphism in fire salamanders (Beukema et al. 2016, see also Donaire et al. 2016). However, no strict association between genomic differences and color differences has been found in previous studies (Burgon et al. 2020). In addition, populations assigned to *S. s. "alfredschmidti"* based on color traits do not occupy different habitats compared to neighboring *S. s. bernardezi* populations, suggesting little ecological divergence (Beukema et al. 2016). Therefore, based on our results, we follow the perspective of Beukema et al. (2016) and consider *S. s. "alfredschmidti"* as a junior synonym of *S. s. bernardezi*. Within the latter, we retrieve several lineages (Fig. 4), although without a robust topology, perhaps due to the sampling scheme and admixture-driven artefacts. Based on mtDNA, García-París et al. (2003) hypothesized the presence of at least two separate lineages in *S. s. bernardezi* in Asturias, which is consistent with the increasing evidence for phylogeographic diversification in amphibians from this region (Recuero & García-París 2011, Ambu et al. 2023, Dufresnes et al. 2023c). A broader, range-wide sampling of *S. s. bernardezi* thus appears timely to study its potential diversification and improve taxonomic arrangements.

Salamandra s. longirostris is a geographically isolated lineage found in the Penibetic region of Southern Spain, separated from other salamander populations by the Guadalquivir River Basin, which presumably promoted its divergence since the Pliocene (García-París et al. 1998, Antunes et al. 2018). Our timetree supports this hypothesis (Fig. 4). It has previously been found to be the sister clade to all other *S. salamandra* lineages based on mtDNA, but nuclear DNA analyses grouped it with the geographically proximate lineages *S. s. crespoi* and *S. s. morenica* (Vences et al. 2014, Burgon et al. 2021). In our clustering analysis, *S. s. longirostris* corresponds to the same cluster as *S. s. almanzoris*, even though both form separate lineages in our trees – *S. s. longirostris* branches as the sister of a lineage comprising *S. s. crespoi* and *S. s. morenica* (Figs 1/4). Thus, different approaches yield different impressions of the relative degree of differentiation of *S. s. longirostris*. Its mtDNA may indicate an early divergence, that was subsequently attenuated in the nuclear genome through introgression from other *S. salamandra* lineages (Burgon et al. 2021). Because *S. s. longirostris* is presently well isolated from other *S. salamandra* lineages, we cannot directly assess their amount of RI, e.g., based on hybrid zone analyses. However, given the relative placements of *S. s. longirostris* in respect to other, putatively more diverged yet genetically compatible lineages, e.g., *S. s. almanzoris* and *S. s. bejarae*, where admixture is extensive (Martínez-Solano et al. 2005, Pereira et al. 2016, Antunes et al. 2021), a species status for any of these taxa is not warranted. Finally, we confirmed the previously reported sister relationship between *S. s. crespoi* and *S. s. morenica* (Reis et al. 2011, Burgon et al. 2021).

Our analyses confirmed at least five genetic groups in North-Western Iberia, which correspond to *S. s. bejarae, S. s. gallaica*, and *S. s. "molleri"* (Figs 1/4). Accordingly, two of these groups were identified among populations previously assigned to the widely distributed *S. s. gallaica* as considered in previous studies (García-París et al. 2003, Pereira et al. 2016). The southern group can be assigned to *S. s. "molleri"* (South), while the group found in the Serra da Estrela in Central Portugal is presently unnamed (*"molleri"* North). However, the intermediate phylogenetic and geographic position of the latter between *S. s. "molleri"* (South) and *S. s. gallaica* calls to investigate its origin, as these signals could reflect hybridization or isolation by distance. Such investigations will require dedicated sampling, fine-scale analyses of genetic structure and admixture, as well as statistical frameworks to disentangle among hypotheses (e.g., Approximate Bayesian Computation), combined with the genetic analysis of type localities.

Outside the Iberian Peninsula, *S. s. salamandra* and *S. s. terrestris* display widespread admixture across Germany (Fig. 3D), following secondary contact after their separation and divergence in eastern vs. western European refugial zones during the Pleistocene (Steinfartz et al. 2000). This is quite in line with the phylogeographic subspecies criteria proposed by Hillis (2020; see also Dufresnes et al. 2023, Vences et al. 2024). *Salamandra s. salamandra* was further divided into two genetic groups (Fig. 3D), one present both in Germany and northern Italy, and one present in the Balkans. The paraphyly of the Greek subspecies *S. s. werneri*, already reported by Burgon et al. (2021), was confirmed in our phylogenetic analyses (Figs 1/4). The northern lineage of *S. s. werneri* from the type locality of Mount Pelion branches with *S. s. beschkovi* and *S. s. salamandra*, while the Peloponnese population, also traditionally referred to as *S. s. werneri*, corresponds to an unrelated lineage that might constitute a new candidate subspecies. This region is a hotspot of endemism for subspecies or even species of otherwise widespread amphibians and reptiles (Dufresnes et al. 2019, Thanou et al. 2021, 2023). We note, however, that samples from the Balkans are underrepresented in our dataset (Fig. 3). Hence, the Peloponnese lineage could also result from demographic processes (e.g., isolation by distance). Again, taxonomic revisions should await more spatially comprehensive sampling in the easternmost range. Similar caution applies to formally distinguishing *S. s. "hispanica"* from *S. s. terrestris*, whose robust divergence (comparable to other recognized subspecies) a priori suggests a valid status.

Conclusions

This study revisits the phylogeny of *Salamandra* by augmenting previous sampling to additional localities and taxa, as well as extending analyses to genomic clustering, time tree inferences, and a critical appraisal of tree topologies with respect to identified hybrid individuals. Our re-

sults confirm the differentiation between all six species and illustrate the challenges of identifying meaningful infraspecific units when genetic diversity is influenced by introgressive hybridization. This is best seen in the incongruent tree topologies when samples of admixed ancestries are included, as frequently encountered across the ranges of *Salamandra*. Our spatially explicit analyses also confirmed the existence of many hybrid zones (e.g., between *S. salamandra* subspecies across Germany), some involving more than two lineages and thus acting as "melting pots" of diversity. Moreover, the combination of phylogenetic and population genetic methods has newly revealed or corroborated the existence of undescribed lineages of potential taxonomic and conservation interest (e.g., *S. s. werneri* [South], *S. s. bernardezi* [West], or *S. a. atra* [South]), which should encourage additional investigations to assess both their evolutionary origin and their taxonomic status.

While defining every single shallow lineage as a unit of biodiversity (taxon or ESU) would dilute conservation and taxonomic resources (Vences et al. 2024), considering deep infraspecific lineages as subspecies, even when these lack obvious phenotypic differentiation, may warrant appropriate attention (e.g., Kindler & Fritz 2018, Scherz et al. 2022, Dufresnes et al. 2023a). Importantly, one pragmatic criterion might be to keep the prevalent taxonomic tradition in the target group. In a species where several ESUs but no subspecies have been defined in the past, naming a new ESU as a subspecies while leaving other ESUs without a Linnean trinomen will create inconsistency and confusion and therefore should be avoided. A similar situation would arise in a genus like *Salamandra*, with a long tradition of subspecies taxonomy, if some of these were instead considered ESUs, for instance, based on a lack of obvious phenotypic differentiation following the criteria of Coates et al. (2018), or simply following the original criteria of Moritz (1994). As formulated by Fitzpatrick (2010), any genuine standardization of a subspecies concept is precluded by the diversity of evolutionary phenomena characterizing these units, and *Salamandra* provides good examples: some subspecies are well-defined and phenotypically distinct mtDNA lineages recovered also in phylogenomic and clustering analyses, are in some but not all cases phenotypically distinct (e.g., *S. a. aurorae, S. a. pasubiensis, S. s. longirostris*), while some other subspecies are lineages with a shallow genomic and phenotypic divergence (*S. s. beschkovi, S. a. spelaea*), or lineages found to be massively admixed by other lineages in phylogenomic analyses (*S. s. gallaica*).

Our study underscores the importance of careful evaluation of the potential extent and impact of hybridization, in conjunction with other lines of evidence, and lays the groundwork for future taxonomic revisions of *Salamandra*.

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Supplementary data

The following data are available online:

Supplementary Table S1. Sampling information for all individuals used in this study.

Supplementary Table S2. Composition and filtering option in STACKS for generating the alignments for the two different datasets.

Supplementary Table S3. Composition of different datasets/subsets and their STACKS filtering option.

Supplementary Figure S1. Maximum-likelihood tree of all sampled individuals of the genus *Salamandra*, with *Lyciasalamandra* used as outgroup.

Supplementary Figure S2. Map showing localities of selected non-admixed samples in the reduced dataset.