



Molecular biogeography and niche climatic diversification of sand lizards (*Lacerta agilis*) with special emphasis on the history of the Pyrenean populations

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Abstract. Pleistocene climate oscillations had a dramatic impact on the distribution of temperate ectothermic organisms. These range fluctuations plausibly have left a footprint on the species' genetic structure which is linked with modelled paleoclimatic conditions, allowing us to infer which environmental factors shaped the evolutionary history of Sand lizards. In this study, we evaluated the patterns of niche diversification of *Lacerta agilis* in Eurasia, based on mitochondrial DNA analyses and ecological niche models. The lineage of Sand lizards evolved 3.8–1.8 Mya, being the most basal in the east-Caucasian subclade, with eight mitochondrial subclades separated into two groups. These groups represent two independent waves of expansion from the ancestral region constituted by the Caucasus and the adjacent northern plains to the Palearctic. Paleoclimatic models suggested a high instability of the range of this lineage in the last 3 Mya, with niche contractions during the colder glacial periods and expansions following the glacier retreat. This suggested an allopatric diversification process, with subspecies boundaries upon secondary contact maintained by competitive interactions, at least between closely related pairs. The great mountain systems of the Mediterranean Peninsulas constituted stable refugia during the Pliocene–Pleistocene cycles, favouring the evolution of endemic subclades. These montane subclades have higher mitochondrial diversity than those that occur in the plains. However, the Pyrenean endemic *L. agilis garzoni* is exception possibly due to the occupation of a very small refugium during the recent glacial phases.

Key words. Glacial oscillations, Lacertidae, niche conservatism, paleoclimate, phylogeography.

Introduction

Recent glacial and interglacial cycles have played a crucial role in biotic diversification in the temperate regions of the northern hemisphere (HEWITT 2000). Ectothermic organisms such as reptiles are suitable models to assess the effects of Pleistocene climatic oscillations because of their entire dependence on external sources to thermoregulate and lower ability to disperse compared to mammals and birds (PAULO et al. 2001, 2008, URSENBACHER et al. 2008). Recent studies locate centres of higher lineage diversification of Palearctic reptiles in the great mountain ranges of southern Europe, the Caucasus, Carpathians, Dinaric Alps, Pindus, Apennines, and Pyrenees (JÖGER et al. 2007). This suggests that these mountain ranges were stable climate refugia through the Pleistocene.

The Sand lizard (*Lacerta agilis*) is one of the more widely distributed reptiles of Eurasia, occurring from Mongolia through Central Asia to the western European Atlantic plains, including the British Isles (BISCHOFF 1988). This

species could have an intricate biogeographic history, as indicated by previous phylogenetic studies (KALYABINA et al. 2001, ANDRES 2014). This genetic structure is also associated with some differences in the external morphology that has led to the description of several subspecies based on the geographical variation of these traits (BISCHOFF 1988).

One of these subspecies is *L. agilis garzoni*, a micro endemism confined to the eastern Pyrenees (AMAT et al. 2003). *Lacerta a. garzoni* is distributed allopatrically with *L. agilis agilis*, which is widespread across the western European Atlantic Plain, reaching north to south of the Scandinavian Peninsula (BISCHOFF 1988). This latter subspecies intergrades across a narrow zone with the *L. a. argus* along central Europe, which is replaced in the eastern margins of the European Atlantic Plain by *L. a. chersonensis*. The mountains of the Balkans are inhabited by another endemic subspecies, *L. a. bosnica*. *Lacerta a. exigua* contacts *L. a. chersonensis* along the plains of Ukraine, Bielorrussia, and Russia, extending further to the east, central Asia, and reaching Mongolia. The mountains around the Black Sea

are occupied by other endemic subspecies: *L. agilis tauridica* (Crimea, Ukraine) and *L. a. boemica* (eastern Caucasus, KUKUSHKIN et al. 2020).

Based on morphological characters and molecular data, previous studies indicated that this species originated on the Caucasus-Black Sea coast during the late Miocene to early Pliocene (YABLOKOV 1981, KALYABINA et al. 2001, JOGER et al. 2007). The current species range and the complex lineage genetic structure and morphological variability could be influenced by tectonics and paleoclimate conditions (KALYABINA et al. 2001, JOGER et al. 2007). These studies suggested an early split between western and eastern basal lineages during the late Pliocene, followed by several waves of expansion from the Caucasus region to western Europe. The Balkans and Carpathian mountains were the glacial refugia from which the species reached central Europe not earlier than 100,000 years with a more recent (Holocene) colonization of the Pyrenees (KALYABINA et al. 2001).

This complex diversification process could be governed by niche conservatism, as indicated by the relict presence of several montane subclades in the Mediterranean peninsulas. Niche conservatism has been invoked to explain patterns of speciation and niche diversification in several lineages of temperate reptiles, possibly driven by the Pliocene-Pleistocene climatic instability (SAGONAS et al. 2014, JABLONSKI et al. 2019). According to this hypothesis, the species would have expanded geographically along favourable environmental corridors, whose extension fluctuated following the alternation of warm-cold periods. In this scenario, separated subclades only occupy climatically analogous regions throughout the species' vast area of distribution. The range boundaries between parapatric populations do not follow any environmental discontinuity and are better explained by competitive interactions (FREEMAN 2015). A second testable hypothesis is that this vast geographic range could be explained by niche shifts. In this scenario, some subclades, perhaps those geographically distant or those with more deep genetic divergence, have adapted to use new environments (PYRON & BURBRINK 2009). In this case, the separation between parapatric taxa or lineages clearly follows an environmental discontinuity. In addition, this process of niche diversification would generate a weaker phylogenetic signal in the environmental niche than the previous one.

The goal of our research is to evaluate the following questions: a) determine whether the *L. agilis* mitochondrial genetic structure is associated with past environmental cycles; b) assess the possible diversification mechanisms of this lineage: conservatism versus niche shifts and which variables are shaping niche diversification; c) describe the evolution of the niche of *L. a. garzoni*, including the environmental factors which may have determined its micro-endemic status.

Material and methods

Phylogenetic inference and biogeography

We used DNA samples of 35 individuals from nine localities along the Pyrenean range of *L. agilis* and three samples

from Germany, Hungary, and the United Kingdom stored in the Institut de Biologia Evolutiva (CSIC-UPF) (Supplementary document S1). Molecular sampling was performed using the mitochondrial gene cytochrome b (428 nuc) following the standard sequencing procedures (ŠMÍD et al. 2013). We added to our dataset and aligned using BioEdit (HALL 1999) 251 cytochrome b sequences available in GenBank (Supplementary document I) to increase the taxonomic and geographic scope. Our final phylogeny covered most of the geographic range of the Sand lizards except for the Caucasian *L. a. ioriensis* and the recently described *L. a. mzymtensis*. However, both forms are genetically indistinguishable from *L. a. boemica* and *L. a. exigua* respectively (DORONINA 2021). To date the divergences within the *L. agilis* lineage we built a second dataset by assembling sequences of mitochondrial 12S rRNA and cytochrome b from Gene Bank (Supplementary document I) of lacertid lizards including all the representatives of the genus *Lacerta* and several outgroups. We included three combined sequences of the eastern (*L. a. agilis*) and western (*L. a. exigua*) lineages as well as one *L. a. garzoni*. Then, we used some dated divergences between the outgroup species as points of calibration to date the divergences among these three *L. agilis* subspecies. The divergence between the Eremiinae lacertids *Mesalina* and *Acanthodactylus* was placed at 16.0–13.1 Mya using a log-normal distribution ($\mu = 2.7$, $\sigma = 0.07$, offset = 0). Maximum divergence was established that matches the beginning of the aridification of the Saharo-Arabian region (FLOWER & KENNETT 1994), while the lower limit was established based on the estimated age of the first fossil record of *Mesalina* (RAGE 1976). Although this fossil was previously described as *Eremias*, this genus included at the time of the description *Mesalina*, geographically restricted to Asia and temperate eastern Europe, making unlikely other assignment than this genus. The split between the two Iberian Peninsula *Timon* species was assumed to have occurred at 5.3–1.9 Mya based on their common divergence from the north African *Timon* species after the closure of the Strait of Gibraltar at the end of the Messinian salinity crisis (GAUTIER et al. 1994) and the oldest known fossil of *Timon lepidus* (Pleistocene, AGUSTÍ et al. 2010). This window of divergence time was accounted for by a log-normal model setting $\mu = 1.12$, $\sigma = 0.24$ with an offset = 0. The last point of the calibration was the speciation event involving *Lacerta trilineata* and its sister lineage of green lizards that occurred 18.1–7.0 Mya, based on the first fossil record certainly assigned to this species and this group (CERNANSKY & SYROMYATNIKOVA 2019). In this case, the log-model was fixed to $\mu = 2.4$, $\sigma = 0.23$, offset=0. The 95% confidence interval of the three estimated divergences among sand lizard lineages was used to calibrate a phylogenetic tree of *L. agilis* based on cytochrome b sequences by setting log-normal distributions to account for the maximum and minimum values of the intervals. Bayesian phylogenetic analyses were carried out by Beast 2.6.2 (BOUCKAERT et al. 2019) under relaxed molecular clocks using the calibration schemes previously defined. We selected the GTR and HKY models with gamma for the analysis of cytochrome b plus 12S rRNA

of *Lacerta* and outgroups after finding the best model for each gene and dataset utilizing jModeltest 2.1.10. (DARRIBA et al. 2012), and GTR for the cytochrome b of Sand lizards datasets. We ran three chains of 10^8 iterations taking samples every 10^4 iterations for both analyses, checking for convergence and effective sample sizes using Tracer 1.7.1. (RAMBAUT et al. 2018) and compiled the 100 last trees of each run to compute the maximum credibility tree in each analysis. We also analysed the genetic variability of each mitochondrial lineage by computing the haplotype and nucleotide diversities using DnaSP 6.12 (ROZAS et al. 2017). Biogeographic inferences were made by reconstructing the evolution of ancestral ranges of the mitochondrial lineages of Sand lizards on the maximum credibility tree through BioGeoBears implemented in Rasp 4.2 (MATSKE 2014). Thus, we compared the dispersal–extinction cladogenesis (DEC), Bayesian analysis of biogeography (BAYAREALIKE) and the dispersal–vicariance (DIVA) models using Akaike Information Criterion corrected for small sample sizes (AICc, CAVANAUGH et al. 2019). The analysis was constrained to reconstruct ancestral areas formed by a maximum of two biogeographic units and those that are geographically adjacent, assigning each mitochondrial lineage these geographic units: Western Europe, Pyrenees, Carpathians, Balkans, South Crimea, Eastern Europe, and Caucasus.

Species niche models

We gathered data on the distribution of *Lacerta agilis* across its range from open databases and scientific papers (Supplementary document II). We also used our data from the Pyrenean populations based on fieldwork done by the authors (AMAT et al. 2003). We evaluated the niche divergence in *L. agilis* of the main mitochondrial lineages found by the phylogenetic analysis. We calibrated statistical models using current relationships between lineage distribution and climate conditions. These models were then used to infer niche suitability under fluctuating climatic scenarios, assuming that species responses are stable over time (MARTÍNEZ-MEYER et al. 2004, HOSNER et al. 2014, AMAT & ESCORIZA 2022).

To build the best ecological niche models, we first generated a smaller subset of the locations available in the databases, after removing those separated less than 10 km. This allows us to reduce spatial data autocorrelation and potential model overfitting (YACKULIC et al. 2013). This process has been carried out using spThin routines (AIELLO-LAMMENS et al. 2015) in R (R Core Development Team 2023). Niche models were built using Maxent 3.4.4 (PHILIPS et al. 2006), a method suitable for generating projections based solely on presence data (WEST et al. 2016). Several models were built iteratively, testing successive combinations of feature classes (L, linear; Q, quadratic; H, hinge; P, product; T, threshold) and regularization multipliers and selecting the optimal candidate using the modified Akaike criterion for finite sample sizes. The predictive capability of the models was evaluated using the area under the receiver

operating characteristic curve (AUC) and the continuous Boyce Index (CBI) (DI COLA et al. 2017). CBI provides a better estimate of the predictive capacity of models based only on presence data; this index ranges between -1 and 1 , with negative values indicating a wrong model and a value close to 1 indicating almost perfect predictive accuracy (HIRZEL et al. 2006). These analyses were performed using ENMval functions (MUSCARELLA et al. 2014) in the R programming environment.

Climate data

We used 19 bioclimatic variables and a digital terrain model available in WorldClim 2 (FICK & HIJMANS 2017) for niche modelling. Variable redundancy was estimated with the variable variance inflation factor (VIF) using a model of increasing complexity (CRANEY & SURLS 2002). To calculate the VIF, we defined buffer polygons of 200 km around the species' occurrences and generated 1000 random points within these polygons. The climate data obtained for the random points and the species' occurrences were included in the logistic regression. We started with a minimal logistic model that included only BIO10 (mean temperature of the warmest quarter), BIO11 (mean temperature of the coldest quarter), BIO12 (annual precipitation), and elevation. These variables were initially chosen due to their putative relevance in the life cycles of mesophilic temperate lizards, i.e. including summer and winter temperatures (egg embryonic development and hibernation) and a proxy for the amount of environmental humidity (VAN NULAND & STRIJBOSCH 1981, LI et al. 2013, ESCORIZA & AMAT 2021). Elevation has also been included because the species' southern populations are entirely confined to mountain habitats (NETTMANN et al. 1992, AMAT et al. 2003). Variables that showed a $VIF > 10$ have been excluded from subsequent analyses (SCHROEDER et al. 1990).

To assess niche suitability in the past, we selected the following simulations: Pliocene, Piacenzian stage, (i) 3.3 Mya (cold) and (ii) 3.2 Mya (warm); Pleistocene, Calabrian stage (787 kya); Pleistocene, Chibanian stage (last interglacial, 130 kya); Pleistocene, Last Glacial Maximum (21 kya); Pleistocene, Bølling–Allerød interstadial (14.7–12.9 kya); Pleistocene, Younger Dryas Stadial (12.9–11.7 kya); Holocene, Northgrippian (8.3–4.2 kya) (OTTO-BLIESNER et al. 2006, DOLAN et al. 2015, HILL 2015, FORDHAM et al. 2017, BROWN et al. 2018, KARGER et al. 2021). These stages encompassed successive warm–cold oscillations, with peak-warm periods (global temperatures 3°C higher than the current ones, DE LA VEGA et al. 2020) and cold-peak periods (global temperatures 5°C lower than current ones, KARGER et al. 2021). These projections were conducted using the whole set of the *L. agilis* species occurrences because realized niches could be limited by the presence of parapatric taxa and not only for their physiological responses (PANZACHI et al. 2015). Only in the case of the pair *garzoni* – *agilis*, we assessed their dynamics of niche evolution separately, because these contiguous taxa are completely allopatric.

Niche divergence and phylogenetic signal

We also assessed the amount of niche divergence between pairs of *L. agilis* mitochondrial lineages. The relative niche position of each lineage was first visualized through principal component analysis (PCA) of the normalized environmental variables. This analysis was carried out in R (R Core Team 2023).

Niche comparisons were conducted for all parapatric or contiguous lineages and allopatric and non-contiguous lineages only if they showed broad geographical distributions. Niche divergence was assessed using several tests comparing the D index, which ranges between 0 (no overlap) to 1 (full overlap) (SCHOENER 1970). Range-breaking tests determine sharp changes in environmental conditions at mitochondrial lineages' boundaries. The linear test assumes that this limit between parapatric or contiguous lineages is linear, whereas the blob test assumes that it could be geometrically irregular, being more suitable when the lineage occupies geographical areas differing in extent (GLOR & WARREN 2011). Background tests assess whether the niches of the two species were more similar or dissimilar to each other than to the available conditions (WARREN et al. 2008). The statistical significance of these has been

obtained by comparing the observed values of taxa overlap with those simulated with 500 replicates, using the package ENMTools (WARREN et al. 2021).

The test of phylogenetic effects over niche occupancy was evaluated using BLOMBERG'S K (BLOMBERG et al. 2003). BLOMBERG'S K values range between 0 and ∞ , where the values of $K < 1$ indicate a less phylogenetic signal than that expected under Brownian motion (BLOMBERG et al. 2003). BLOMBERG'S K provides acceptable estimations even with small phylogenies, although it is susceptible to false positives (MÜNKEMÜLLER et al. 2012). The value of K was calculated after 10,000 resamplings of the climate matrix, since it has been recommended not to use average niche values in the estimation of the phylogenetic signal (HARMON & LOSOS 2005). These calculations were conducted using the phytools package (REVELL 2012) in R.

Results

Our phylogenetic analysis placed sand lizards as a well-supported sister clade of that formed by *Lacerta media*, *Lacerta pamphylica*, and *Lacerta trilineata* (Fig. 1), estimating the divergence between the two clades at 8.7–5.0 Mya.

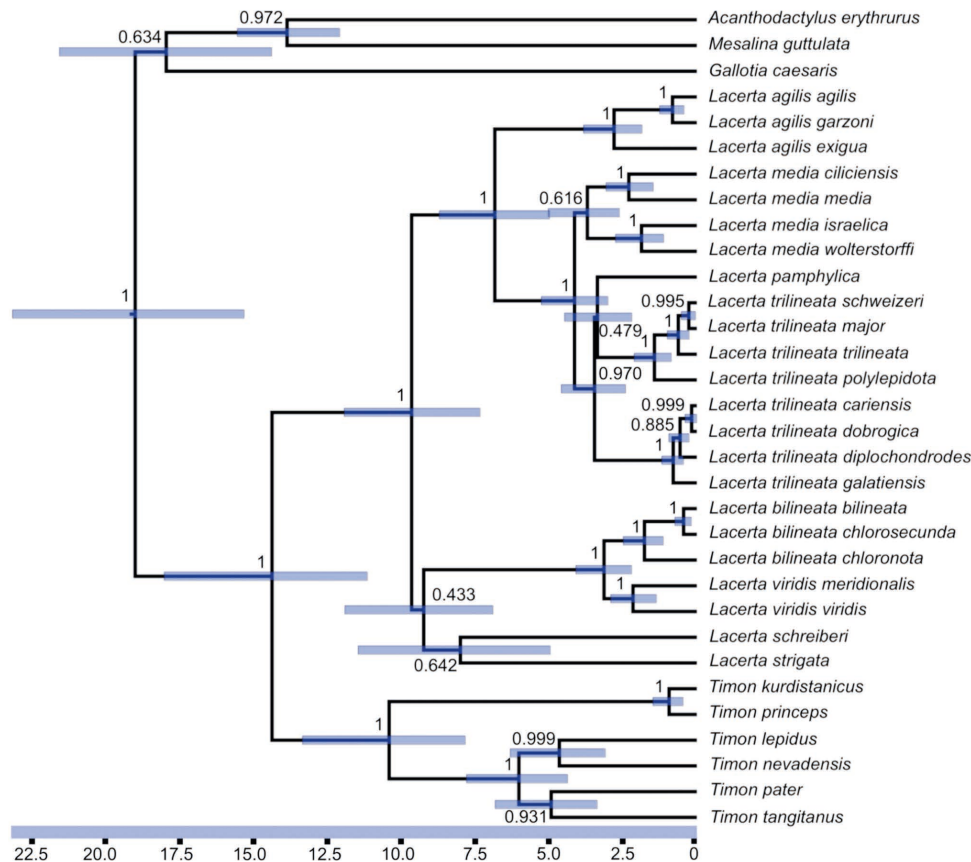


Figure 1. Bayesian analysis of phylogenetic relationships of green lizards (*Lacerta*) based on a concatenated dataset of mitochondrial 12s RNA and cytochrome b. Values on the nodes are the posterior probabilities. Divergences between taxa estimated using a relaxed molecular clock are shown in bars (95% confidence intervals).

Our results revealed that the origin of Sand lizards can be placed during the late Miocene-Pliocene within a time window of 3.8–1.8 Mya. Using the divergence dates among the three lineages of Sand lizards in this phylogenetic tree and the full dataset of cytochrome b sequences, we identified several mitochondrial lineages with high support (Fig. 2). The most basal subclade (south Caucasian *L. a. boemica*) split at 2.9–1.7 Mya from the other *L. agilis* mitochondrial subclades. The other group also split shortly after (2.9–1.6 Mya): an eastern group constituted by the subspecies *L. a. bosnica*, *L. a. exigua* and *L. a. tauridica*, and a western group formed by *L. a. agilis*, *L. a. chersonensis*, *L. a. argus*, *L. a. garzoni* and an unnamed subclade confined to the Carpathian mountains. Within the eastern group, the Balkanic lineage diverged early (2.6–1.3 Mya) and became separated into three well-supported subclades, in the Pindos mountains, Bosnia, and the rest of the Balkans, with a minimum interclade divergence estimated between 930–680 kya years ago. The other two lineages, the south Crimean *L. a. tauridica*, which was formed by two well-supported clades diverging at 1.2–0.4 Mya, and *exigua*, became mitochondrially distinct at 1.8–0.8 Mya. The western group began to diversify at 1.9–0.9 Mya originating the *chersonensis* lineage, and a group of lineages ranged from

the easternmost, located in the Carpathians, to the westernmost, which included *garzoni*, *agilis*, and *argus* subspecies diverging at 1.5–0.8 Mya. Following our results, the origin of Pyrenean endemic *garzoni* was older than previously stated, being at 1.2–0.7 Mya in its divergence from the common ancestor of the *agilis* plus *argus* subspecies, which diverged most recently (1.0–0.4 Mya).

Sand lizard lineages differed in the amount of genetic diversity (Table 1). Therefore, despite sampling thirty-five individuals of the Pyrenean *L. a. garzoni*, this subspecies was uninformative for cytochrome b in contrast to southern lineages, *L. a. boemica*, *L. a. bosnica*, and *L. a. tauridica* that showed higher haplotype and nucleotide diversity. The other lineages had low levels of mitochondrial diversity even in the case of *L. a. argus*, *L. a. agilis*, and *L. a. exigua* with very extensive geographic ranges.

The reconstruction of the past biogeography of *Lacerta agilis* was based on the DIVA model, which performs better than the others (AICc weight = 0.670). We found the area formed by the Caucasus plus the adjacent part of eastern Europe as the most likely ancestral range of Sand lizards. Evolving from the common ancestor, one lineage, *L. a. boemica*, remained confined to the eastern Caucasus, and another become separated into two groups and experienced

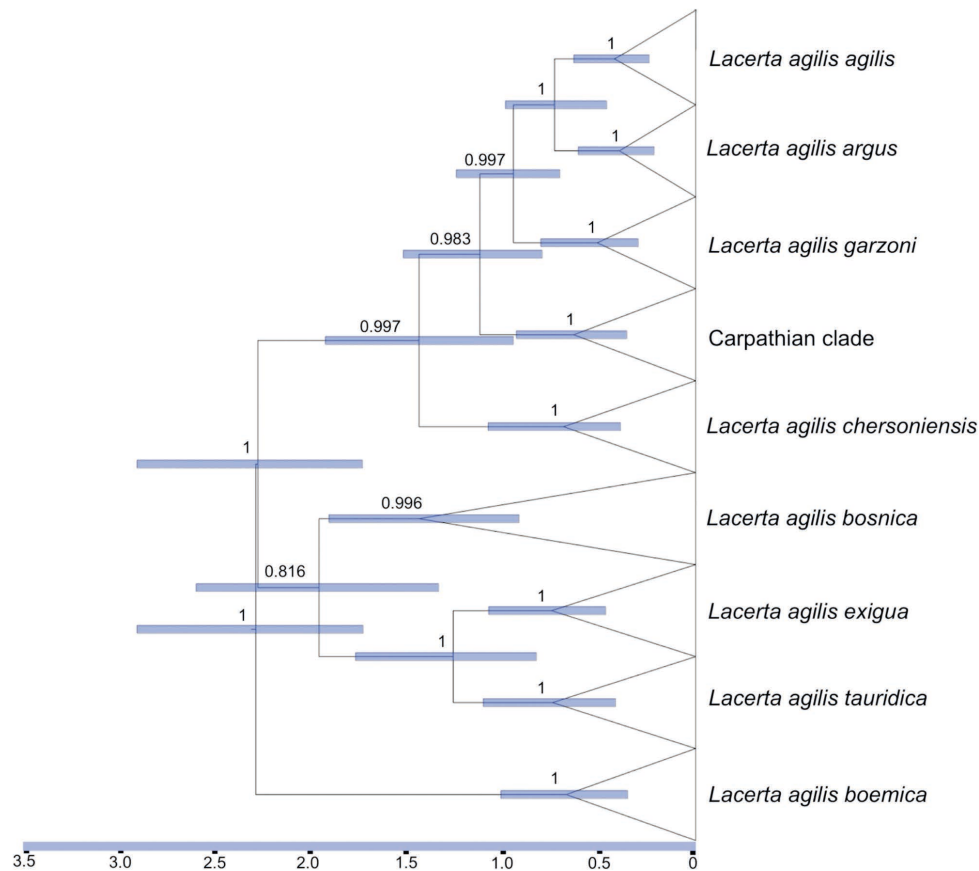


Figure 2. Bayesian analysis of cytochrome b sequences of 286 sand lizards. For simplicity, samples were collapsed into the nine main identified clades, most of them assignable to the currently recognised subspecies. The values of the nodes are the posterior probabilities. The bars represent the 95% confidence intervals of the divergence time among clades based on a relaxed molecular clock.

Table 1. Genetic variation of the main lineages of sand lizards based on mitochondrial cytochrome b.

Lineage	n	Number of haplotypes	Haplotype diversity	Nucleotide diversity
<i>argus</i>	28	2	0.138±0.084	0.0006
<i>agilis</i>	13	6	0.718±0.128	0.0055
<i>boemica</i>	7	7	1.000±0.076	0.0082
<i>bosnica</i>	19	10	0.825±0.084	0.0019
<i>carpathians</i>	9	8	0.972±0.064	0.0085
<i>chersonensis</i>	35	7	0.659±0.057	0.0036
<i>exigua</i>	94	10	0.356±0.062	0.0023
<i>garzoni</i>	36	1	0.000±0.000	0.0000
<i>tauridica</i>	45	23	0.945±0.017	0.0071

two independent step by step expansions to the rest of Europe (Fig. 3). The westernmost group colonized independently the Pyrenees and Carpathians, from plain-dwelling ancestors, that also derived into the *L. a. agilis*, *L. a. argus*, and *L. a. chersonensis* subspecies. Another group colonized the Balkans and southern Crimea from a plain-dweller ancestor that also evolved into *L. a. exigua*. The latter subspecies spread through the steppes towards Central Asia.

The PCA showed the position of each lineage locality depending on climate variations (Fig. 4). The first axis (explained variance = 41.02%) separated the lineages' sites depending on the temperatures of the driest (BIO9, variable partial contribution [vpc] = 23.9%) and the colder quarter (BIO11, vpc = 22.7%) and the amount of rainfall (BIO12, vpc = 15.7%) and its seasonality (BIO15, vpc = 15.2%) and the second axis (explained variance = 24.59%) represent-

ed the altitudinal gradient (altitude, vpc = 36.7%) and the temperatures of the warmest period (BIO10, vpc = 25.7%) (Fig. 4).

The models' performance was good, according to the AUC and CBI, and the mapped predictions were reliable (Supplementary document III). Under current conditions, the model forecasts showed broad regions climatically suitable distributed sequentially from west to east, extending across the Great European Plain to the West Siberian Plain (*agilis*–*argus*–*chersonensis*–*exigua*) with smaller suitable regions restricted to the Pyrenean, Carpathian, Dinaric Alps, Pindus, Tauric (Crimean), and Caucasus mountain systems, which also sequentially correspond to the endemic montane subspecies (Fig. 5).

Paleoclimatic models showed important fluctuations between the gain-loss of suitable habitats in Europe during the last 3.3 Mya. In the Pliocene, the mapped projections revealed a significant loss of suitability across the Great European Plain and the Mediterranean peninsulas, when at this moment the suitable regions were confined to the great mountainous systems and the north-eastern margins of the Great European Plain (Fig. 6). During colder interstadial periods, the Great European-West Siberian Plains became suitable for this species, but not most of the Mediterranean peninsulas. During extremely cold periods, such as the last glacial maximum (21 kya), there was a major loss of suitable habitats in the northern and eastern distribution, including the West Siberian Plain and most of the Great European Plain, while the Atlantic margins of the Great European Plain and the north of the Mediterranean Peninsulas likely act as refugia (Fig. 6). Interestingly, habitats at the glacier margins could have been suitable for this species in western Europe.



Figure 3. Reconstruction of the historical biogeography of sand lizards based on the dispersal-vicariance model. The image depicts the inferred process of dispersal to western Europe experienced by the lineage comprising the clades assignable to the *garzoni*, *agilis*, *argus*, *chersonensis* subspecies and the unnamed clade inhabiting the Carpathians (in blue) and the formed by *bosnica*, *tauridica*, and *exigua* (in red). The Caucasus and adjacent plains of eastern Europe probably were the ancestral range of *Lacerta agilis*. The *boemica* subspecies (in black) was the first clade to evolve independently to the others and lives in the Eastern Caucasus.

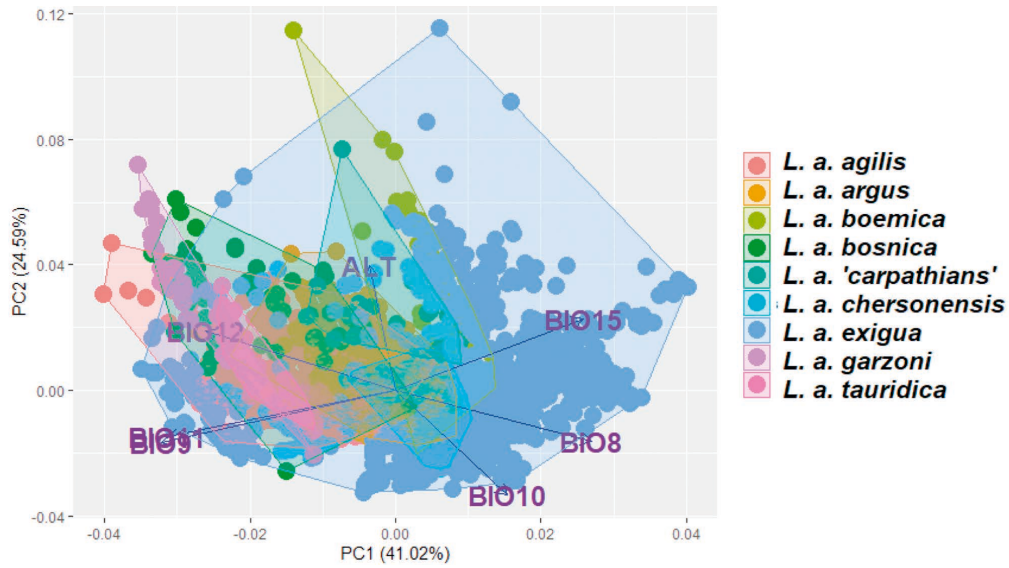


Figure 4. PCA scatter plot representing the relative position of each lineage of *Lacerta agilis* depending on climatic variation. The localities of each species are shown encompassed by a convex hull. ALT, terrain elevation. BIO, climatic variables.

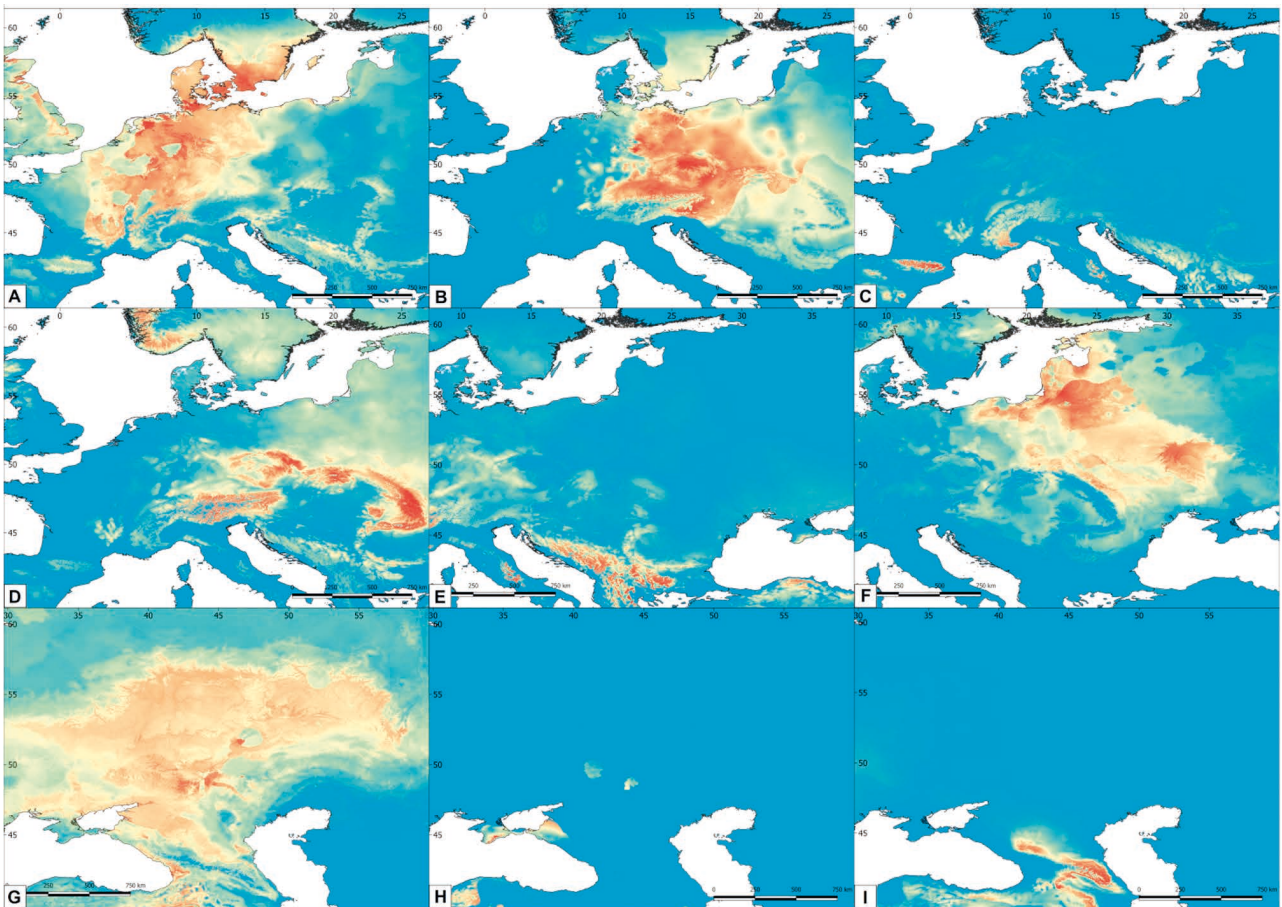


Figure 5. Ecological niche models built with Maxent based on WorldClim 2 variables representing the niche of *Lacerta agilis* lineages, generated from Worldclim bioclimatic variables and terrain elevation. (A) *Lacerta agilis agilis*; (B) *L. a. argus*; (C) *L. a. garzoni*; (D) *L. a. 'Carpathians'*; (E) *L. a. bosniensis*; (F) *L. a. chersonensis*; (G) *L. a. exigua*; (H) *L. a. tauridica*; (I) *L. a. boemica*.

In the case of *L. a. garzoni*, the Pyrenean had suitable habitats for this subspecies since 787 kya. Pleistocene models indicated the persistence of suitable climatic conditions for *L. a. garzoni* on the southeastern edges of the Pyrenees, maintaining geographic isolation from the nominate subspecies (Fig. 7).

The lack of statistical significance for the comparisons of the linear and blob range-breaking tests allows us to reject the hypothesis of sharp environmental variations at the subspecies range boundaries (Table 2). The background tests revealed higher similarity than expected by chance in *L. a. agilis* – *L. a. argus* and *L. a. bosnica* – *L. a.* ‘Carpathians’, and lower similarity than expected by chance in *L. a. agilis* – *L. a. garzoni*, *L. a. agilis* – *L. a. exigua*, *L. a. argus* – *L. a.* ‘carpathians’, and *L. a. argus* and the two eastern plains subspecies (*L. a. chersonensis* and *L. a. exigua*) (Table 3).

The phylogenetic signal (BLOMBERG’S K) varied widely depending on the environmental variable evaluated, but tended to be highest (i.e. more conserved variables) in those describing extreme temperatures: warmest (mean $K = 0.825$) and coldest quarter (mean $K = 0.799$) and pre-

Table 2. Ecological niche hypothesis tests: p-values of the range break tests linear and blob reject. Non-significant values rejected the hypothesis of sharp environmental variations at the species’ range boundaries. AL, allopatric and non-contiguous subspecies.

	Linear	Blob
<i>L. a. agilis</i> – <i>L. a. argus</i>	0.373	0.078
<i>L. a. agilis</i> – <i>L. a. garzoni</i>	0.412	0.129
<i>L. a. agilis</i> – <i>L. a. chersonensis</i>	AL	AL
<i>L. a. agilis</i> – <i>L. a. exigua</i>	AL	AL
<i>L. a. argus</i> – <i>L. a. bosnica</i>	0.392	0.231
<i>L. a. argus</i> – <i>L. a.</i> ‘carpathians’	0.019	0.255
<i>L. a. bosnica</i> – <i>L. a.</i> ‘carpathians’	0.333	0.178
<i>L. a. argus</i> – <i>L. a. chersonensis</i>	0.137	0.337
<i>L. a. argus</i> – <i>L. a. L. a. exigua</i>	AL	AL
<i>L. a.</i> ‘carpathians’ – <i>L. a. chersonensis</i>	0.196	0.406
<i>L. a. exigua</i> – <i>L. a. chersonensis</i>	0.159	0.149
<i>L. a. exigua</i> – <i>L. a. boemica</i>	0.294	0.099
<i>L. a. exigua</i> – <i>L. a. tauridica</i>	0.082	0.079

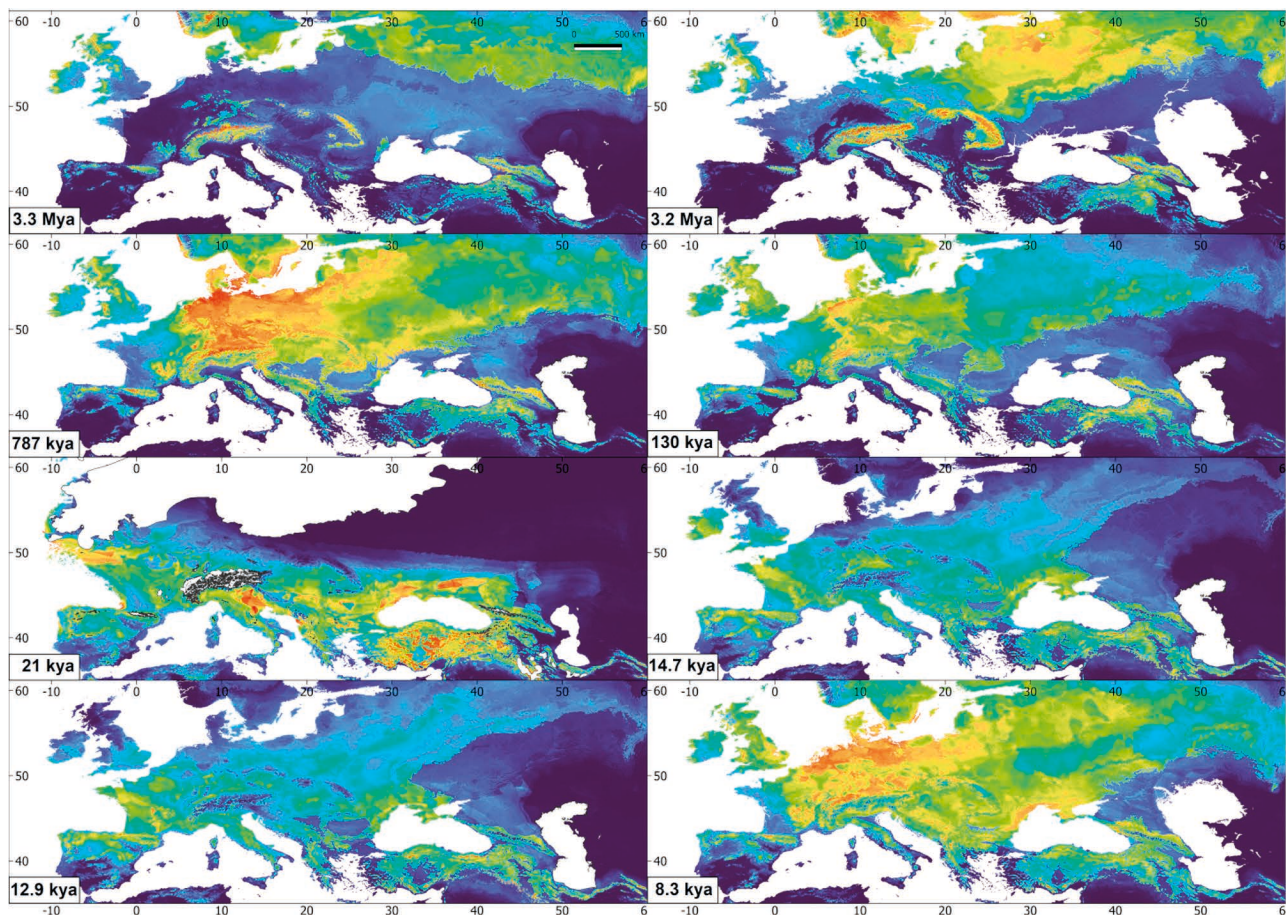


Figure 6. Paleo-climatic projections, based on models statistically calibrated with the current climatic conditions: Pliocene, Piacenzian stage, (i) 3.3 ma (cold) and (ii) 3.2 ma (warm); Pleistocene, Calabrian stage (787 kya); Pleistocene, Chibanian stage (last interglacial, 130 kya); Pleistocene, Last Glacial Maximum (21 kya); Pleistocene, Bolling-Allerød interstadial (14.7–12.9 kya); Pleistocene, Younger Dryas Stadial (12.9–11.7 kya); Holocene, Northgrippian (8.3–4.2 kya).

Table 3. Ecological niche hypothesis tests: Schoener's D and p-values of the background tests. Non-significant (n.s.) values rejected the hypothesis of greater similarity or divergence than would be expected by chance.

		Direction	
<i>L. a. agilis</i> – <i>L. a. argus</i>	Empirical	0.225	Similarity
	Mean simulated	0.165	
	p	0.020	
<i>L. a. agilis</i> – <i>L. a. garzoni</i>	Empirical	0.015	Dissimilarity
	Mean simulated	0.248	
	p	0.019	
<i>L. a. agilis</i> – <i>L. a. chersonensis</i>	Empirical	0.163	n.s.
	Mean simulated	0.185	
	p	0.078	
<i>L. a. agilis</i> – <i>L. a. exigua</i>	Empirical	0.234	Dissimilarity
	Mean simulated	0.254	
	p	0.039	
<i>L. a. argus</i> – <i>L. a. bosnica</i>	Empirical	0.208	n.s.
	Mean simulated	0.201	
	p	0.451	
<i>L. a. argus</i> – <i>L. a. 'carpathians'</i>	Empirical	0.363	Dissimilarity
	Mean simulated	0.723	
	p	0.019	
<i>L. a. bosnica</i> – <i>L. a. 'carpathians'</i>	Empirical	0.450	Similarity
	Mean simulated	0.219	
	p	0.019	
<i>L. a. argus</i> – <i>L. a. chersonensis</i>	Empirical	0.276	Dissimilarity
	Mean simulated	0.629	
	p	0.048	
<i>L. a. argus</i> – <i>L. a. exigua</i>	Empirical	0.183	Dissimilarity
	Mean simulated	0.392	
	p	0.019	
<i>L. a. 'carpathians'</i> – <i>L. a. chersonensis</i>	Empirical	0.083	n.s.
	Mean simulated	0.128	
	p	0.137	
<i>L. a. exigua</i> – <i>L. a. chersonensis</i>	Empirical	0.204	n.s.
	Mean simulated	0.224	
	p	0.157	
<i>L. a. exigua</i> – <i>L. a. boemica</i>	Empirical	0.209	Dissimilarity
	Mean simulated	0.279	
	p	0.019	
<i>L. a. exigua</i> – <i>L. a. tauridica</i>	Empirical	0.067	Dissimilarity
	Mean simulated	0.177	
	p	0.039	

precipitation seasonality (mean $K = 0.792$). However, we found a lower phylogenetic signal ($K \leq 0.707$) in the other variables of the environmental niche (terrain elevation, annual mean precipitations and mean temperatures of the driest and wettest periods of the year) (Table 4).

Discussion

Our study infers a realistic biogeographic scenario to explain how Sand lizards achieved their current huge geographic range, emphasizing the benefits of combining phy-

Table 4. Estimation of the phylogenetic signal (BLOMBERG'S K) in the diversification of the ecological niche in Sand lizards. The mean value and the standard error after 10,000 resamplings of the possible values of the niche for each subspecies are shown.

Code	Name	BLOMBERG'S K
	Elevation	0.707±0.002
BIO8	Temperature wettest quarter	0.671±0.001
BIO9	Temperature driest quarter	0.706±0.001
BIO10	Temperature warmest quarter	0.825±0.002
BIO11	Temperature coldest quarter	0.799±0.001
BIO12	Annual precipitation	0.685±0.001
BIO15	Precipitation seasonality	0.792±0.002

logeographic and paleoclimatic methods (e.g. WIELSTRA et al. 2013).

In general, as expected, climate models indicated that the geographical range of the whole species has not been stable in the last 3 Mya. The models also suggested that extensive secondary contacts between the plain-dwelling subspecies have only occurred during the interglacial-interstadial periods. The absence of abrupt discontinuities in the limits of the subspecies and niche similarity between several pairs of geographically contiguous subspecies suggests allopatric differentiation processes, compatible with the use of separated refugia during prolonged climatically hostile periods.

Our results revealed that this species has spread through much of the cool-temperate regions of Eurasia favoured by a certain plasticity of the climatic niche, particularly in the amount and seasonality of precipitations. This plasticity has allowed these subspecies to colonize habitats exposed to very seasonal hydrological regimes, such as the cold steppes of Central Asia, or not, such as the oceanic climates of the European Atlantic plain (ANANJEVA et al. 2006). On the contrary, the tolerance to temperatures (particularly to the maximum ones) is more conserved, which would have limited the penetration of this species in regions with a Mediterranean climate, despite the numerous potential ways of dispersal available during their long evolutionary history. These findings mirror those of previous studies in the Mediterranean region, including several species and genera, suggesting that maximum temperatures are an important factor that limits the ecological diversification in lacertid lizards (ESCORIZA et al. 2021).

The similarity of the niches when comparing closely related subspecies and the absence of sharp environmental discontinuities, such as *L. a. agilis* and *L. a. argus* and *L. a. chersonensis* and *L. a. exigua*, suggests that range boundaries are likely to be constrained by competitive interactions between conspecific populations. The results showed that niche divergence is high between some subclades with deep genetic divergence, i.e. when *L. a. boemica* is compared to the surrounding plain-dweller subspecies *L. a. exigua*. In this case, niche divergence may favour their separation even if range-breaking tests did not support abrupt changes

in their range boundaries. However, in this case, subspecies replacement can occur at a lower spatial resolution, associated with gradual, fine-scale changes in the landscape (e.g. riparian forest to steppe), not captured in our study.

Based on our results, it is likely that the lineage of *L. agilis* arose in the region comprising the Caucasus and the adjacent plains between the Pleistocene–Pliocene tran-

sition. Therefore, the origin of the species may be more recent than previously stated (Pliocene, YABLOKOV et al. 1980, KALYABINA et al. 2001, JOGER et al. 2006) and is not clear whether it evolved from a plain-dweller or mountain ancestor. In this sense, the basal position of the *boemica* subspecies respect to the other lineages could equally indicate a Caucasian origin of sand lizards or an early coloniza-

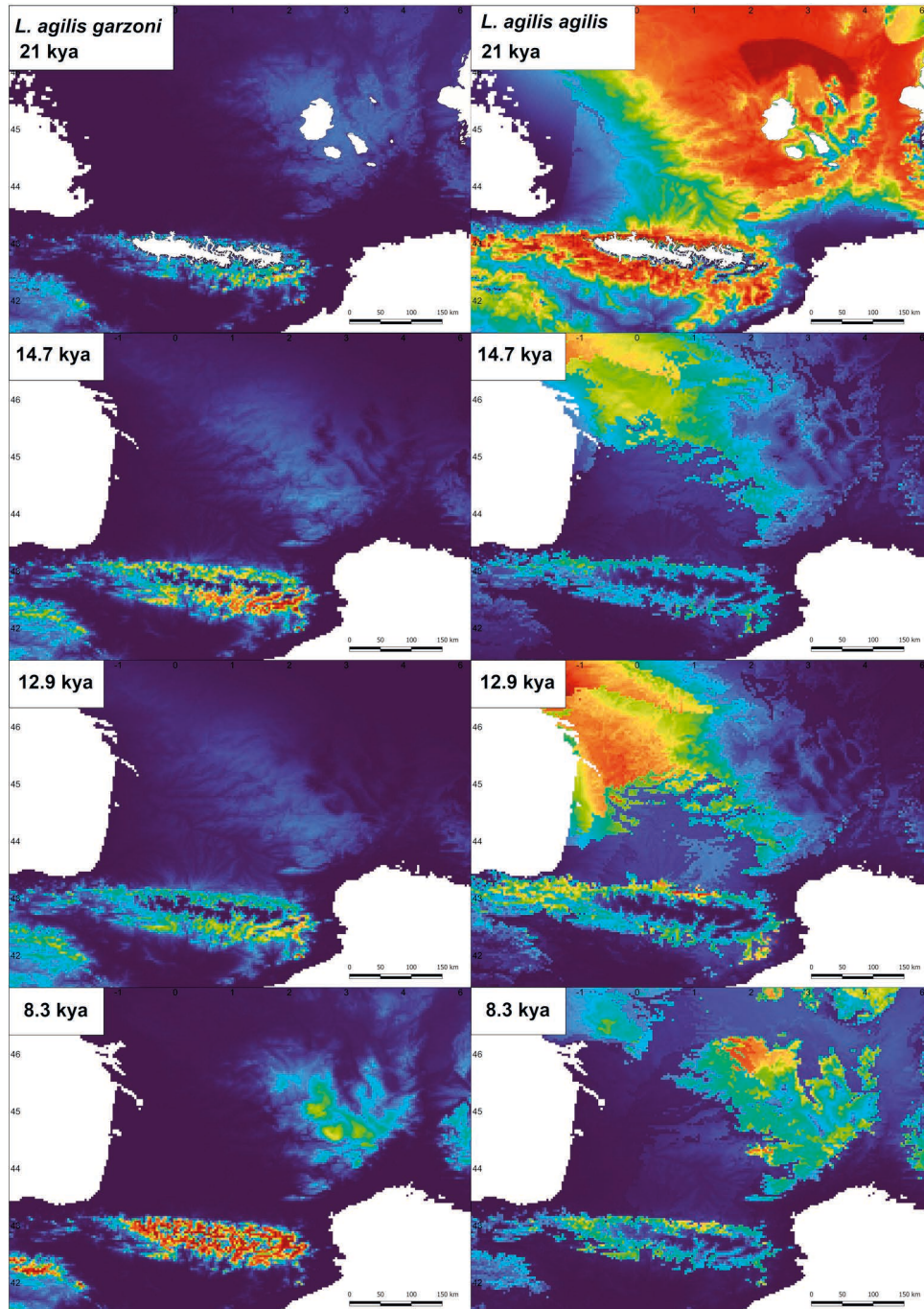


Figure 7. Detailed paleo-climatic projections of the Pyrenees, based on models statistically calibrated with the current climatic conditions specifically for *L. a. agilis* and *L. a. garzoni*: Pleistocene, Last Glacial Maximum (21 kya); Pleistocene, Bølling-Allerød interstadial (14.7–12.9 kya); Pleistocene, Younger Dryas Stadial (12.9–11.7 kya); Holocene, Northgrippian (8.3–4.2 kya).

tion of this mountain range. The split between *boemica* and the common ancestor of the western and eastern lineages is old enough to make this subspecies the candidate to be recognised as a full species (JÖGER et al. 2006). However, the taxonomic revision of Sand lizards will be greatly benefited from an analysis of nuclear DNA markers to quantify the amount of introgression between *boemica* and the other lineages.

Paleoclimatic projections combined with molecular divergence times rejected previous hypotheses that proposed a Holocene origin of the western lineage (KALYABINA et al. 2001). In this sense, paleoclimatic projections indicated extensive habitat suitability across the plains of western Europe at a minimum of 787 kya. This period encompasses the lower bounds of estimated divergence times of the *agilis*, *garzoni*, and *argus* subspecies. Our results rejected a recent colonization of the Pyrenees (PALACIOS & CASTROVIEJO 1975, CARRETERO et al. 2002), reinforcing the sub-specific identity of the populations that live in the massif.

The mapped projection showed larger habitat suitability for the *garzoni* subspecies, than the actual distribution of the species. This incongruence could be caused by several reasons: the use of a very small glacial refugium, which would not have allowed the re-expansion along the rapidly emerging favourable habitats, the presence of impassable barriers, or competition with other congeneric lacertid species (HELTAI et al. 2015, RYAN & GUNDERSON 2021). Paleoclimatic projections indicated that during the Last Glacial Maximum, suitable conditions for this subspecies were restricted to a very small region on the southeastern edge of the Pyrenees. A putative glacial refuge could be located in the valley of the Tec River, thus facilitating the connection of the two main groups of Pyrenean populations (ARRIBAS 1999, AMAT & ROIG 2003, GENIEZ & CHEYLAN 2012). This valley is the only one with an east-east orientation inhabited by the species in the Pyrenees, and this orientation (toward the sea) may favour milder thermal conditions. The existence of only one glacial refugium for Pyrenean populations is congruent with the absence of mitochondrial DNA variation in our sampling, similar to other microendemic lizards (*Iberolacerta aurelioi* and *I. aranica*, CARRANZA et al. 2004). Other Pyrenean species, more widely distributed, however, have a more complex geographic structure (*Zootoca vivipara*, MILÁ et al. 2013 and *Iberolacerta bonnali*, CARRANZA et al. 2004, FERCHAUD et al. 2015), suggesting multiple climate refugia through the Pyrenees. The Last Glacial Maximum projection also predicted large habitat suitability for the *agilis* subspecies along the periphery of the main chain that overlapped with the estimated for *garzoni*. However, the habitat suitability of the intermediate region between the Pyrenees and the Central Massif was low, suggesting a maintained isolation between the populations of both mountain ranges. Therefore, the absence of this species in the central and western Pyrenees could be explained by a slow process of expansion from this refugium (or others located on the southeastern edges of the Pyrenees). Although competition with *Lacerta bilineata* could also be arguable, Sand liz-

ards roughly coexist with other green lizards (*Lacerta viridis*, HELTAI et al. 2015).

Our mapped projections indicated that the great plains of Europe become corridors for an early dispersal, likely from the plains close to the Black Sea to colonize independently Balkan mountain ranges, than was previously hypothesised, but also rejecting the role of the Balkans as the centre of dispersal for the eastern lineage (KALYABINA et al. 2001). The paleoclimatic projections indicated that the expansion towards Central Asian steppes by *L. a. exigua* occurred recently, around 8,300 years (also suggested by KALYABINA et al. 2001).

Noticeably, we found higher mitochondrial diversity in all southern montane clades with the sole exception of the Pyrenean *garzoni* due to the prolonged stable habitat suitability in the great mountain ranges of the Balkans and eastern Europe. In contrast, paleoclimatic projections and lower mitochondrial diversity suggested a smaller extent of the refugia of *exigua*, *chersonensis*, *argus*, and *agilis* subspecies with few populations. This scenario was reversed, after the glacier retreat allowed the expansion to the plain-dweller lineages across the Eurasian plains.

Our study evidenced that Pleistocene glacial cycles have deeply shaped the evolutionary history of *L. agilis*, leading to the isolation of mitochondrial lineages in the southern Europe refugia, in plains, and separated mountain ranges. Subsequent range expansions could be more rapid in the plain-dweller subspecies (*agilis*, *argus*, *chersonensis*, and *exigua*), enabling them to expand over vast geographic areas, while mountain lineages remained more confined. Interestingly, except for the Pyrenean subspecies, southern montane lineages have a higher mitochondrial diversity more broadly distributed than plain-dwelling lineages, suggesting larger glacial refugia in the former or, at least, that the properties of the habitats allowed larger population sizes.

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Supplementary data

The following data are available online:

Supplementary document I. Genbank accession numbers of the 12s and cytochrome b used in molecular analysis.

Supplementary document II. Sources used to compile data on *Lacerta agilis* locations.

Supplementary document III. Best candidate Maxent models.