phylogeographic structure in a lizard

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Abstract

Aim We examined dorsal colouration in and genetic relationships among Iberian populations of the lizard *Psammodromus algirus* to determine the extent to which the current distribution of phenotypic variation is correlated with phylogeographic history or local environmental conditions.

Location Iberian Peninsula, western Palearctic.

Methods We sequenced mitochondrial DNA (ND4 and adjacent tRNAs genes) in 36 populations, and seven microsatellite loci in eight representative populations. In 23 populations, lizards were classified according to the presence and intensity of a dorsal striped pattern, the heritability of which was estimated by means of mother-offspring regressions. To determine whether colour pattern is an adaptation for crypsis, we compared the time taken by humans to detect striped and unstriped lizards in different environments.

Results The analysis of mtDNA revealed an ancient split between a western clade, subdivided into south- and northwestern haplogroups, and an eastern clade with central, southeastern and eastern haplogroups. In contrast, nuclear markers showed a postglacial admixture of central and western haplogroups, with the central haplogroup apparently isolated from the rest of its clade. This was consistent with variation of the dorsal striped pattern, a heritable phenotypic trait: central and western lizards were unstriped, whereas eastern lizards were striped. We then suggest that dorsal colouration promotes crypsis: in eastern locations detection times were longer for striped than for unstriped

lizards, whereas the opposite was true in western and central locations.

Main conclusions Our results indicate that natural selection for crypsis may promote not only divergence within clades, as suggested by the apparent isolation between unstriped central lizards and striped members of eastern haplogroups, but also admixture between them. We conclude that ecologically-driven selection is crucial for understanding the phylogeographic background of phenotypic variation, because recent adaptation to the environment can blur the effects of ancestral isolation.

Key words: Colouration, Cytonuclear disequilibrium, Heritability, Lacertidae, mtDNA polymorphism, Predation, *Psammodromus*.

INTRODUCTION

A central goal of evolutionary biology is to explain the morphological diversity observed within and across species. In fact, intraspecific variation, which is perhaps the most remarkable feature that is shared by all species, provides the raw material both for adaptation and speciation. For Darwin (1859), whose concept of species was largely morphological, the association between speciation and adaptation was straightforward: if speciation is defined by the accumulation of phenotypic differences between populations, then proof of speciation requires that such differences are demonstrably caused by natural selection (see review by Schluter, 2009). However, this link lost importance with the rise of the biological species concept (Mayr, 1942) and its emphasis on reproductive isolation rather than on adaptive differences. Nevertheless, recent decades have witnessed a revival of the idea that the macroevolutionary process

of speciation results from ecologically-based divergent selection, which is the kind of microevolutionary process that takes place at the level of intraspecific variation (Schluter, 2001, 2009; Rundle & Nosil, 2005; Rosenblum & Harmon, 2011). Intraspecific variation may arise for a number of reasons, such as geographic or genetic isolation, drift, mutation, migration, natural or sexual selection, phenotypic plasticity, or a combination of any of these. As a consequence, the consistency between phenotypic differentiation among populations and underlying patterns of genetic variation can range between close match and outright disagreement. Depending on the amount of genetic structure, gene flow, and the intensity of selection, populations of the same species adapted to different environments may cover the entire span between no apparent genetic structure and almost complete speciation (Rosenblum, 2006; Rosenblum & Harmon, 2011).

Here, we focus on the extent to which phenotypic variation is correlated with local environmental conditions *versus* phylogeographic history (Hoekstra *et al.*, 2005). Phylogeography has extended phylogenetic thinking to the intraspecific level (Avise *et al.,* 1987), allowed the geographic determinants of isolation to be tracked (Hewitt, 1996; Godinho *et al.,* 2008), and shed light on how demographic processes have shaped genetic and phenotypic variation at the interface between populations and species (Hickerson *et al.,* 2010). Within this framework, the Iberian Peninsula offers ample opportunities to compare the roles of phylogeographic history and environment in shaping phenotypic variation: it enjoys a variety of Mediterranean and Atlantic climates and has several east-west oriented mountain ranges that allow survival of species in the face of climate changes by altitudinal shifts (Hewitt, 1996). These characteristics, together with its large area, favoured the occurrence of multiple refugia during the Pleistocene glaciations (Gómez & Lunt, 2007). Multiple refugia are especially common

100 for reptiles, whose low mobility and dependence on thermal conditions should have
101 promoted fragmentation of populations in suitable habitats, facilitating the appearance
102 of phylogeographic structure (Gómez & Lunt, 2007).
103 In this study we analyse intraspecific differentiation in the Large
104 Psammodromus *Psammodromus algirus* (Lacertidae; Linnaeus, 1758), a medium-sized
105 (adult snout-vent length 60-90 mm) lizard which is widely distributed in shrub and
106 woodland habitats of the Iberian Peninsula, southeastern France and north-west Africa.
107 This species consists of two reciprocally monophyletic mtDNA clades (Verdú-Ricoy *et*
108 *al*., 2010): an eastern clade confined to Iberia, and a western clade present in both Iberia
109 and North Africa. Both clades diverged approximately between 3.6 and 3 Ma (Carranza
110 *et al.,* 2006; Fitze *et al.,* 2011). We collected genetic and phenotypic data to set the
111 phylogeographic background of phenotypic and current genetic variation. We
112 characterized interpopulation differences in colouration according to the development of
113 a dorsal striped pattern, a trait that has been regarded as an adaptation for crypsis in
114 other taxa (Karpestam *et al.,* 2013). We also performed two experiments to test the
115 effects of colouration on detectability by simulated (human) predators, and we
116 measured habitat features potentially related to visibility for aerial predators. Finally, we
117 estimated the heritability of the dorsal striped pattern by assessing its development in
118 females and in their offspring. We used our data to test the following hypotheses: 1) If
119 history is the only source of phenotypic differentiation, then the geographic distribution
120 of the striped pattern should match that of the mtDNA clades; 2) alternatively, if local
121 adaptation is strong enough to overcome the effects of history and gene flow, then no
122 consistency between the geographic distribution of the striped pattern and the mtDNA
123 clades should be expected; and 3) If both local environmental conditions and
124 phylogeographic history are important determinants of phenotypic variation, then the

distribution of the striped pattern should match that of the mtDNA clades only in part. This latter hypothesis would also imply that recent adaptation to the environment can blur the effects of ancestral isolation, promoting either divergence within clades (if lizards of the same clade show different striped or unstriped patterns) or convergence between them (if lizards from different clades show the same patterns).

MATERIALS AND METHODS

Field sampling

We sampled 36 *P. algirus* populations (Table 1) for DNA extraction between April and June of 2006 and 2008-12; 23 of these populations were also employed for phenotypic characterization of lizards, although not necessarily at the same time or using the same individuals in a given population. Lizards were noosed or captured by hand. When necessary (see below), lizards were transported to the lab and housed under the standard conditions described in Díaz *et al.,* (2012). After laboratory work had finished, all animals were released at their site of capture.

Genetic variation

Mitochondrial DNA variation was studied on a sample of 319 individuals from 36 populations (Table 1). A fragment of the fourth subunit of the NADH dehydrogenase (ND4) and adjacent tRNAs (His, Ser, and Leu) genes was amplified using primers ND4 and Leu (Arévalo *et al.,* 1994). Resultant sequences (length: 849 bp) were used to construct a haplotype network with gene genealogy software TCS version 1.21

(Clement *et al.,* 2000). The resulting network confirmed the existence of five haplogroups separated by more than 10 mutational steps and grouped into western and eastern clades with two (W1 and W2) and three (E1, E2, and E3) haplogroups, respectively (see Results). Haplogroup identity was used as a categorical predictor in subsequent analyses.

In addition, 7 microsatellite loci specifically designed for *P. algirus* (Bloor & Dávila, 2008) were amplified in eight populations (Fig. 1), which were chosen to represent localities either close to the borders of the distribution range or in the putative limit between eastern and western mitochondrial lineages (Verdú-Ricoy *et al*., 2010). Microsatellite data were analysed with Peak Scanner™ v.1.0 (Applied Biosystems). We used Arlequin 3.5.1 (Excoffier *et al.,* 2005) to test for deviations from Hardy-Weinberg equilibrium and for linkage disequilibria and to assess the extent of differentiation among mtDNA clades (estimated as the mean number of nucleotide changes). We used Structure 2.3.3 to detect clusters that minimize Hardy-Weinberg and linkage disequilibria (Pritchard *et al.,* 2000). The number of ancestral clusters (K) was set to 4 after comparing log-likelihood ratios in multiple runs (n = 20 for each value of K between 1 and 12). This was the value that maximized mean log-likelihood ratio (Evanno *et al*., 2005).

Finally, we combined mtDNA and microsatellite data to estimate migration rates between pairs of populations with IMa2, a coalescent-based method that uses loci from two or more populations and Markov chain Monte Carlo simulations of gene genealogies to estimate the posterior density of various parameters that are part of an 'isolation with migration' model (Hey, 2010).

A detailed explanation of all the genetic analyses performed can be found in the online appendix S1.

175

176 **Phenotypic variation and habitat characteristics**

177

178 In a subsample of 23 populations (Table 1), lizards were assigned to a colour category
179 according to the presence, length and intensity of a mid-dorsal dark stripe (online
180 appendix S1). In 14 of these populations, we had a large enough sample size ($n \geq 10$) to
181 estimate the frequency of striped lizards with enough precision to model its dependence
182 on landscape features. To assess vegetation cover at each of these 14 localities we
183 walked a 1-km transect stopping every 10 m. At each stop, we placed a marked stick
184 standing vertically on ground level to assess whether vegetation was contacted at 0, 10,
185 30, 50 or 100 cm height. If so, the type of the plant(s) contacted was noted (grass,
186 deciduous shrub or perennial shrub). We also considered the presence or absence of leaf
187 litter on the ground and of tree canopy over the vertical projection of the stick. Partial
188 Least Squares regression (PLS), an extension of multiple regression explicitly designed
189 to deal with numerous and highly collinear predictors (Carrascal *et al.,* 2009), was
190 employed to model the relationship between the frequency of striped lizards and habitat
191 variables.

192

193 **Crypsis**

194

195 We tested the influence of dorsal colouration on the detection times of lizards by
196 humans, as a proxy for visually oriented natural predators. We chose two eastern (E2)
197 and two western (W2) populations, and we captured three adult males at each of the
198 four sites. All lizards from eastern populations were striped, and all lizards from western
199 populations were unstriped. Pictures of lizards were taken at the four different capture

sites by putting cold-anesthetized lizards in randomly chosen realistic positions. Human detection times were tested by showing the images (N = 48, 3 lizards x 4 populations x 4 pictures) to a group of 12 researchers who were asked to find the location of the animal. Observers were questioned one by one, blindly with respect to others' performance. Upon detection, time was measured with a chronometer to the nearest 0.5 s, up to a maximum searching time of 1 min. Photographs were presented to subjects in a randomized order that was the same for all observers, thus leaving habitat, phenotype, habitat x phenotype interaction, observer (which was the only random factor), and error, as the only possible statistical sources of variation in detection times.

Because lizards belonging to the E3 haplogroup were genetically closer to the eastern striped lizards, but phenotypically closer to the western unstriped ones (see Results), we also compared the detectability of eastern unstriped (E3), western unstriped (W2), and eastern striped (E2) lizards at a single E3 location (Pelahustán) in a second experiment. Our aim was to verify if the colour pattern of E3 lizards would decrease their detectability in their own environment, and if their detection times would be more similar to those of W2 unstriped lizards than to those of striped lizards. We captured three adult males from the nearby populations of Aranjuez (E2, striped), El Pardo (W2, unstriped) and Pelahustán (E3, unstriped). Lizards were photographed, and the photographs examined by 14 researchers, following the aforementioned protocols.

Heritability

During the 2013 breeding season, we captured and transported 26 gravid female lizards to the lab from El Pardo, Pelahustán, and Aranjuez, to assess the mother-to-offspring heritability of the dorsal colouration pattern, which is present from hatching. Female

lizards were scored from 0 to 5 by five independent observers according to the presence, extension and intensity of the dorsal dark stripes (online appendix S1). We checked gravid females daily to detect egg laying. After a female had laid a clutch, the eggs were removed and incubated until hatching. After hatching, juveniles were marked, weighed, measured, and kept in individual terraria. When all hatchlings had emerged from the eggs, they were assigned a dorsal colouration score by the same observers. To assess whether interindividual variation of dorsal colouration is heritable, we tested the significance of the standardized regression coefficient of offspring mean scores on dam mean scores (which equals half the heritability, because only one parent's value is used). A detailed explanation of all the analyses performed can be found in the online appendix S1.

RESULTS

Phylogeographic patterns based on mtDNA variation

We obtained 60 haplotypes from the analysis of 319 mtDNA sequences from 36 populations (Table 1). Of these, only six (haplotypes 1, 11, 18, 24, 42, and 50) were found in two or more populations; the remaining 54 haplotypes were unique to one population each, indicating strong genetic population structure. The resulting haplotype network had a strong phylogeographic signal (Fig. 1), with two main clades matching the eastern and western reciprocally monophyletic clades obtained by Carranza *et al.,* (2006) and Verdú-Ricoy *et al.,* (2010). The western clade had southwestern (W1) and northwestern (W2) haplogroups, whereas the eastern clade had a southeastern haplogroup (E1), a widespread oriental haplogroup (E2) whose distribution range

250 contained and surrounded that of E1, and a central haplogroup (E3) partly overlapping
251 the range of the western clade (Fig. 1b). Interestingly, most populations had all
252 haplotypes from a single haplogroup, the only exceptions being Abrucena and Morata
253 (with E1 and E2 haplotypes), Pallarés (W1 and W2), and Villuercas (E3 and W1). Mean
254 divergence between eastern and western clades was 5.73%; divergence between
255 haplogroups was larger for the eastern clade (2.97, 3.28, and 2.47% for E1-E2, E1-E3,
256 and E2-E3, respectively) than for the western one (1.71% for W1-W2).
257 Visual inspection of the haplotype network revealed two different types of
258 population histories. The topology showed by southern haplogroups (W1 and E1)
259 suggested stable demographic histories, whereas W2, E2 and especially E3 showed star-
260 shaped topologies typical of populations that have experienced a recent demographic
261 expansion. This was supported by R_2 estimates (based on the difference between the
262 number of singleton mutations and the average number of nucleotide differences;
263 Ramos-Onsins and Rozas 2002), which were low and significant for W2 (R_2 = 0.048, P
264 = 0.017), E2 (R_2 = 0.028, P = 0.006) and E3 (R_2 = 0.024, P < 0.001), indicating ratios of
265 singletons that are higher than expected under the assumption of constant population
266 size and consistent with the hypothesis that rapid demographic growth has led to the
267 accumulation of recent mutations in the outermost branches of the network. On the
268 other hand, values for E1 (R_2 = 0.246, P = 0.943) and W1 (R_2 = 0.105, P = 0.220) did
269 not depart significantly from the expectations of constant population size.
270 Latitude was negatively correlated with haplotype diversity (r = -0.322, N = 36,
271 P = 0.055), total number of haplotypes (r = -0.374, P = 0.025), number of unique
272 haplotypes (r = -0.426, P = 0.010), and nucleotide diversity (r = -0.469, P = 0.004),
273 indicating a greater genetic diversity in southern populations.
274

Genetic structure according to microsatellites

All microsatellite loci were polymorphic for all populations under study except for locus Psal4 (it was monomorphic at Abrucena, Aranjuez, Cabañas de Tera, and Pallarés). None of these loci deviated significantly from Hardy-Weinberg equilibrium after applying the sequential Bonferroni correction; ten of 52 cases were significant at the unadjusted level. Twelve of 144 tests of linkage disequilibrium were nominally significant, but none of them remained significant after sequential Bonferroni adjustment, supporting the independent assortment of alleles at different loci.

Microsatellite-based Bayesian population assignment tests performed with Structure, after establishing the most likely number of ancestral clusters at K = 4, identified several groups of populations (Fig. 2): one formed by northwestern lizards (Cabañas de Tera, from haplogroup W2), another one formed by the two southernmost populations sampled, including both western (Pallarés, with W1 and W2 haplotypes) and eastern (Abrucena, with E1 and E2 haplotypes) lizards, and two additional groups located south of the Sistema Central mountain ranges: a central-eastern group including a single population of striped lizards (Aranjuez, from haplogroup E2), and a central-western group comprising four populations of unstriped lizards with E3, W1, and W2 haplotypes (Villuercas, Pelahustán, El Pardo, and Navacerrada). Thus, population structure depicted by microsatellites was inconsistent with the phylogeographic scenario deduced from mtDNA sequences. This was supported by the AMOVA results (Table 2), in which grouping of nuclear markers according to eastern vs. western clades revealed no significant structure between groups (F_{ST} and $R_{ST} = 0$, both $P > 0.75$).

Estimates of gene flow

Migration rates provided by IMa2 (Table 3) confirmed the pattern of genetic structure depicted by microsatellites. Most values of *m* significantly greater than zero (and all *m* values > 1) referred to pairs of populations within the central-western group defined by Structure (El Pardo, Navacerrada, Pelahustán and Villuercas). In contrast, no gene flow was detected between these populations and the nearby location of Aranjuez, despite its relatively short linear distance from El Pardo and Pelahustán.

Phenotypic variation and its association with habitat features

A one-way ANOVA comparing the frequency of striped lizards among the three haplogroups with enough data (E2, E3, and W2, with 8, 7, and 4 populations, respectively) showed that E2 lizards were dominantly striped, whereas E3 and W2 ones were mostly unstriped (ANOVA with the data in Fig. 3, proportions arcsine-transformed: $F_{2,16} = 40.64$, $P < 0.001$; post-hoc comparisons: $P < 0.001$ for E2 vs. E3 and E2 vs.W2, and $P = 0.664$ for E3 vs. W2). Data from four additional populations (Helechosa, Pallarés, Abrucena, and Villuercas; see Table 1) allowed us to tentatively extend this pattern to the remaining haplogroups, leading us to conclude that western and E3 lizards were mostly unstriped, whereas E1 and E2 lizards were striped (Fig. 3); overall, the average percentage of striped lizards was 90.5% for eastern populations (E1 and E2) and 9.5% for central and western ones (E3, W1 and W2).

Concerning habitat associations, a PLS analysis with frequency of striped lizards as the dependent variable yielded a single significant factor that explained 39.6 % of the variance in the response variable ($F_{1,12} = 7.86$, $P = 0.016$). The predictors that contributed significantly to that factor (plant cover 1 m above ground level, tree cover,

and cover of deciduous shrubs) explained 84 % of the variance in X-scores (the values that each population attains along the single factor derived from the PLS analysis; Fig. 4). In addition, X-scores were also positively correlated with longitude ($r = 0.592$, $N = 14$, $P = 0.026$), and they differed significantly among localities with western (W1 and W2), central (E3), and eastern (E1 and E2) haplogroups (Fig. 4: $F_{2,10} = 5.82$, $P = 0.021$), defining an east-west gradient of increasing vegetation cover.

Experiments on visual detectability of lizard phenotypes

Results of our first experiment showed that lizards were detected faster outside their natural environments: at eastern sites unstriped lizards were detected more rapidly than striped ones, and the opposite was true at western sites (site x phenotype interaction in mixed model ANOVA with phenotype as fixed factor and site and observer as random factors: $F_{3,33} = 92.43$, $P < 0.001$; Fig. 5). In our second experiment, detectability at Pelahustán differed significantly among lizards captured at Aranjuez (E2), El Pardo (W2), and native (E3) lizards (population effect in mixed model ANOVA with observer as a random factor: $F_{2,28} = 9.73$, $P = 0.0006$). Striped lizards from Aranjuez were detected faster (mean ± standard error = 19.2 ± 2.7 s) than unstriped lizards, either native (29.7 ± 3.1 s) or captured at El Pardo (39.2 ± 3.7 s; post-hoc comparisons: Aranjuez vs. Pelahustán $P = 0.031$, Aranjuez vs. El Pardo $P = 0.001$, and Pelahustán vs. El Pardo $P = 0.554$).

Heritability of dorsal colouration pattern

Overall, we obtained 27 effective clutches (with at least one viable egg) from 22

females: six from Aranjuez (from five females, one of which laid two clutches), ten from El Pardo (one clutch per female), and eleven from Pelahustán (from seven females, five of which laid two clutches, including one whose first clutch was entirely composed of eggs that failed to hatch). Mean numbers of hatchlings per clutch (± sd) were 2.8 ± 1.9, 4.4 ± 1.1, and 5.5 ± 1.4, respectively. Mean colour scores of adults (± sd) at each of the three populations, pooling together males and females because sexual dimorphism was not significant, were 3.5 ± 1.3, 0.6 ± 0.5, and 0.9 ± 1.1, respectively ($F_{2,37} = 31.97$, $P < 0.001$).

The regression of mean offspring score on mean dam score was significant, with a standardized regression coefficient (± SE) of 0.74 ± 0.06 ($F_{1,119} = 148.38$, $P < 0.001$); that is, juvenile lizards had a dorsal pattern strongly similar to that of their mothers. Within-population regressions were significant for Aranjuez ($\beta = 0.82 \pm 0.15$, $F_{1,15} = 31.81$, $P < 0.001$) and Pelahustán ($\beta = 0.58 \pm 0.11$, $F_{1,58} = 29.53$, $P < 0.001$), but not for El Pardo ($\beta = 0.11 \pm 0.15$, $F_{1,42} = 0.54$, $P = 0.465$), probably due to the lack of variation in mean adult scores of this later population (Levene tests; Aranjuez vs. El Pardo P = 0.021, Pelahustán vs. El Pardo P = 0.023, Aranjuez vs. Pelahustán: P = 0.731).

DISCUSSION

Phylogeographic structure and phenotypic variation

Our phylogeographic analyses of mtDNA confirmed the differentiation of eastern and western Iberian clades whose separation took place between 3 and 3.6 Ma, *i.e.* after the last opening of the Strait of Gibraltar and before the beginning of Pleistocene glaciations (Carranza *et al.*, 2006, Verdú-Ricoy *et al.*, 2010). Similar patterns of east-

west differentiation have been described for other taxa, most noticeably for the related species *Psammodromus hispanicus* (Fitze *et al*., 2011) and for perennial and deciduous oaks (*Quercus spp*.; Rodríguez-Sánchez *et al*., 2010) that form the cleared forests where these lizards reach their highest population densities (western clade: Díaz & Carrascal, 1991; Díaz, 1997; eastern clade: Zamora-Camacho *et al*., 2013). The similarity in the phylogeographic structure of the two *Psammodromus* species suggests a shared history of Plio-Pleistocene events, including long periods of isolation in similar glacial refugia and subsequent latitudinal and altitudinal expansions as the climate became warmer (from the coasts towards the central plateaux, or tracking up suitable microclimates along east-west oriented mountains).

As a result of these historical processes, *P. algirus* shows the typical pattern of latitudinal loss of genetic diversity caused by Quaternary glaciations ('southern richness and northern purity'; Hewitt, 1999): the most basal haplotypes in both clades come from southern localities (Verdú-Ricoy *et al*., 2010), and there are negative correlations between latitude and genetic diversity. Lower diversity at higher latitudes could reflect a rapid colonization of suitable habitat, with repeated founding events along the expansion edge leading to homozygosity and loss of alleles (Hewitt, 2000). However, an alternative explanation for the higher diversity of southern populations could be their secondary contact with anciently differentiated northern haplogroups that, after having survived the ice ages in valley bottoms or coastal areas (Gómez & Lunt, 2007), would have expanded their range in the current interglacial period. This second scenario is supported by the star-shaped topology of northern haplogroups in the haplotype network (W2, E2 and especially E3) and by their low and significant R_2 estimates (Ramos-Onsins & Rozas, 2002), which are typical of populations that have expanded recently and rapidly (Slatkin & Hudson, 1991; Avise, 2000). Such demographic

400 expansions, which could have produced similar patterns in mitochondrial
401 polymorphisms as those detected in our study, seem to have continued until significant
402 geographic barriers have been encountered by dispersing individuals (for example, no
403 W1 haplotypes have been found north of the Tagus river, and no E3 haplotypes have
404 been found north of the Sistema Central mountain ranges). This interpretation implies
405 the existence of multiple Iberian refugia for *P. algirus*, and it is consistent with the role
406 of glacial refugia in southern Europe not only as hotspots but also as melting pots of
407 genetic diversity (Gómez & Lunt, 2007; Godinho *et al*., 2008).
408 Within this context, the E3 haplogroup, which occupies an intermediate position
409 between the western clade (W1+W2) and the remaining eastern haplogroups (E1+E2),
410 deserves special attention because of its unstriped dorsal colouration, its genetic
411 admixture with western lizards, and its transitional type of habitat (see below). The
412 adjustment of mismatch distribution of pairwise differences, which in the case of E3 fits
413 well to the Poisson distribution expected under a model of population expansion (online
414 appendix S2), further supports the recent spread of this group. Such expansion would
415 have produced a secondary contact with western lizards, demonstrated by the
416 mitochondrial polymorphism between W2 and E3 found at Villuercas, but not with
417 eastern, unstriped lizards belonging to the E2 clade. In fact, E2-E3 is the only possible
418 mtDNA polymorphism that has not been found in the southern half of Iberia. All this
419 evidence, together with estimates of recent gene flow showed by the microsatellite data,
420 suggests the contemporary existence of two separate gene pools in central Spain, one
421 corresponding to striped lizards (E2) and the other to unstriped ones (E3, W1 and W2).
422 Thus, phylogeographic structure revealed by the mtDNA data is unable to explain the
423 current distribution of a heritable phenotypic trait that is functionally important and
424 shows convergence between, and divergence within, different branches of the

425 phylogeographic tree. In the following sections we try to shed light on these findings,
426 which seem to support our third initial hypothesis, namely that recent adaptation to the
427 environment by natural selection for crypsis has blurred the effects of ancestral
428 phylogeographic structure.
429
430 **Dorsal colouration as adaptation for crypsis**
431
432 Results of our common-garden breeding experiment indicate that a substantial part of
433 the interindividual variation in the pattern of dorsal stripes is heritable. Thus, dorsal
434 colouration should be able to evolve in response to natural selection. Interestingly, the
435 proportion of striped lizards with a weakly striped pattern was negatively correlated
436 with the overall frequency of striped lizards ($r = - 0.874$, $n = 15$, $P < 0.001$). In other
437 words, populations tend to be composed either mainly of striped lizards with a marked
438 pattern of dorsal stripes, or of unstriped lizards with a minority of weakly-striped
439 individuals. This is the sort of bimodal expression of a phenotypic trait which would be
440 expected under two contrasting scenarios of strong directional selection (Pigliucci *et al*.,
441 2006), perhaps acting on a basis of underlying polygenic inheritance or indicating
442 incomplete penetrance.
443 Concerning habitat associations, the variables included as significant predictors
444 in the PLS factor that best explained geographical variation in the frequency of striped
445 lizards were all related to forest development, giving more negative scores to habitats
446 with lower visibility from above. Because PLS scores decreased in an east-west
447 direction, visual opacity tended to be higher at more western locations, suggesting
448 different selective pressures on lizard colouration and crypsis.
449 The most obvious link between these habitat features and dorsal colouration is

camouflage, because disruptive colour patterns can render a target indistinguishable from its background (Cuthill *et al*., 2005). Thus, when some of the contrasting colours in a striped pattern are coincident with the background, as is the case of dark and clear stripes in grassy habitats, they break up the animal's outline (Merilaita, 1998). The role of crypsis in our system was supported by the results of our two detection experiments, because detectability was always higher for animals with mismatched phenotype. Although strictly speaking our conclusions are restricted to human observers, previous studies have shown a remarkable similarity between humans and birds in the ability to discriminate between different colour patterns under laboratory conditions (Penney *et al*., 2012), and simulations reveal that visual complexity of the background renders 'background matching' methods less reliable than those that address information processing by predators (Merilaita, 2003). Moreover, detection times measured by presenting images of prey on computer screens to humans were significantly correlated with colour contrast in RGB between potential preys and their background (Carrascal *et al*., 2001), vigilance rates of potential preys (Carrascal *et al*., 2001), and estimates of capture probabilities and survival of different colour morphs of free-ranging grasshoppers (Karpestam *et al*., 2013).

The importance of visually-oriented predation as a selective pressure for these lizards is consistent not only with the large number of avian species that prey on them (Salvador, 2014), but also with the influence of antipredator behaviour on other aspects of the ecology of these lizards: 1) thermoregulation, because the choice of compass directions around shrub patches allows basking lizards to remain within shorter reach from the security of shrubs than expected at random (Díaz, 1992); 2) escape tactics, since in deciduous forests lizards change their escape behaviour in response to changes in habitat structure (Martín & López, 1995); and 3) sexual selection, given that larger

and more brightly coloured males are more active, overlap more females, and court them more frequently than smaller and less active ones, but at the cost of a higher risk of predation and increased mortality (Díaz, 1993).

Current genetic structure and maintenance of phenotypic differentiation

The existence of two separate gene pools in central Spain, one corresponding to striped lizards (E2 haplogroup) and the other one to unstriped lizards (E3, W1 and W2 haplogroups), was supported by nuclear markers, given that populations in the southern foothills of the central mountain ranges were pooled together in the same cluster. This gave rise to a typical pattern of mito-nuclear discordance (Toews & Brelsford, 2012), because the largest cluster defined by microsatellite alleles (red cluster in Fig. 2, including all central populations other than Aranjuez) grouped together two W2 populations, one E3 population, and the only eastern-western (W1+E3) polymorphic population detected in our study. In all these populations, the unstriped colour pattern was dominant. IMa2 analyses were also indicative of high levels of gene flow within this cluster. In particular, the two populations acting as main allele donors, El Pardo and Pelahustán, had higher migration rates (Table 3: row averages of 0.86 and 0.93, respectively) than the remaining populations (row averages ≤ 0.23). Remarkably, these two locations are part of the almost continuous corridor of oak forests that cover the southern slopes of the central mountain ranges. We predict that more detailed sampling of central Iberia should provide more examples of mitochondrial admixing between eastern/central (E3) and western clades (W1 and W2), as it has been described for other Iberian lacertids (Miraldo *et al*., 2011, Godinho *et al*., 2008).

What factors could explain the maintenance of the current pattern of population

differentiation? We hypothesize that ecological selection is driving both divergence between E2 (striped) and E3 (unstriped) lizards, and convergence between E3 and western lizards in central Spain. Three lines of evidence support this hypothesis. Firstly, the distribution of the frequency of striped lizards in the 23 populations examined was clearly bimodal: the frequency of the commonest phenotype was smaller than 0.75 in only two of these populations (Fig. 3: localities 22, with 66% striped lizards, and 36, with 60% unstriped lizards), and these sites were both located close to the western and eastern edges, respectively, of the distribution ranges of their haplogroups (E2 and E3). Secondly, the estimated migration rate of microsatellite alleles to and from the Aranjuez population (E2) was extremely low, despite its short linear distance from the nearby populations of El Pardo (W2) and Pelahustán (E3). And thirdly, habitat differences between western (more forested) and eastern (less forested) habitats were significant, with central (E3) sites occupying an intermediate position. Such differences are consistent with the existence of an ecotone matching the division between eastern and western haplogroups of many Iberian taxa with similar phylogeographic patterns (Gómez & Lunt, 2007, Rodríguez-Sánchez *et al*., 2010; Fitze *et al*., 2011). In addition to the dataset analysed in this manuscript, supplementary information on differences in egg incubation times among egg-laying females belonging to different genetic lineages also reveal a close phenotypic link of the E3 haplogroup with the western clade (Díaz *et al*., 2012, online Appendix S3).

Finally, we suggest that ecologically-based divergent selection is causing the evolution of reproductive isolation, either complete or not (Nosil *et al*., 2009), between striped and unstriped lizards. The selective advantage of the matching phenotypes in the habitats where they maximize crypsis can directly promote ecologically-dependent reproductive isolation (Rundle & Nosil, 2005). Pre-zygotic isolation can arise if

525 migrants suffer reduced survival because they are more easily detected by predators in
526 non-native habitat, which could lower the rate of heterologous mating. By contrast,
527 post-zygotic isolation can evolve if hybrid fitness is reduced due to an ecological
528 mismatch between the phenotypes of hybrids and their environment (Rundle & Nosil,
529 2005). The bimodal distribution of the frequency of striped lizards at both sides of the
530 ecotone, together with the negative correlation between the frequency of striped lizards
531 and the proportion of them that show little contrast between stripes, suggest that 'pure'
532 phenotypes have higher fitness than 'hybrid' ones. Secondly, it seems that the
533 development of the sexual colouration of the head is more intense in western males (in
534 which larger and older individuals have a brilliant red-orange patch on the sides of head,
535 mental scutes, and throat) than in eastern males (that show a less conspicuous orange
536 colouration, usually restricted to labial scales) (J. A. Díaz, pers. obs.). Because head
537 colouration is important for sexual selection in western males (Díaz, 1993; Salvador *et*
538 *al*., 1996; López *et al*., 2003) and it may interfere with crypsis in eastern habitats if red-
539 orange heads are more visible from above, such difference could promote assortative
540 mating and therefore prezygotic isolation. However, chemosensory cues are also
541 important for these lizards (López *et al*., 2003), and we encourage future efforts to
542 measure the composition of femoral secretions and the responses of both males and
543 females from different populations along the ecotone.

544 To summarize our findings, our data show how an incipient process of allopatric
545 divergence, defined by the ancient split of eastern and western mtDNA clades, may be
546 blurred by ecological factors acting later on. Moreover, the same environmental factors
547 that seem to be fostering the admixture of the western and E3 haplogroups, thereby
548 promoting their phenotypic and genetic convergence, may also be causing the split of
549 the E3 haplogroup from the rest of the eastern clade in an early process of ecological

speciation. This is remarkable, because to our knowledge this is the first time that ecologically-based divergent selection, which is the basic process involved in ecological speciation, is also documented to promote convergence between phylogeographic lineages. Such duality reinforces the view, emphasized in Futuyma's (1986) definition of evolutionary ecology, that we need ecological data to explain evolution as much as we need evolutionary processes to understand the ecology of extant organisms.

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SUPPORTING INFORMATION

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

Appendix S1 Extended Material and Methods.

Appendix S2 Mismatch distributions of E3 haplogroup.

Appendix S3 Differences in incubation times among haplogroups.

BIOSKETCH

The authors are members and external collaborators of the Evolution and Conservation Biology Research Group of the Complutense University of Madrid (http://www.ucm.es/bcveng). Their research focuses on evolutionary biology, ecophysiology and the evolution of life histories along biogeographical gradients. To deal with the interesting questions that often arise in evolutionary ecology, lizards are used as study systems, bringing together biogeography, behavioural ecology, physiology, evolutionary ecology, population genetics and conservation biology in an integrative approach.

Author contributions: JAD, JVR and AS conceived the study, performed data analyses, and wrote the first version of the manuscript. JVR performed genetic analyses. All

725 authors contributed to data acquisition and manuscript writing.

726

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Table 1. Genetic and phenotypic characterization of the 36 populations studied. For each population, the following data are shown: sample size for mtDNA (N), haplotypes and haplogroups encountered (with the same codes as in Fig. 1a), number of unique haplotypes (N_U), haplotype diversity (H_D), nucleotide diversity (π, equal to the mean number of nucleotide changes among sequences), frequency of striped lizards (F_{STR}, with sample size in parenthesis), and frequency of striped lizards with a weak pattern of dorsal stripes ($F_{W\text{-}S}$). Population numbers as in figure 3b.

Nº	Population	Latitude (º N)	Longitude (º E)	N	Haplotypes	Haplogroup(s)	N_U	H_D	π	F_{STR}	$F_{W\text{-}S}$
1	Navacerrada[a,b]	40.726	-4.023	9	1-3	W2	2	0.417	0.44	0.17 (36)	0.83
2	El Pardo[a,b]	40.511	-3.755	9	1, 4-5	W2	2	0.556	1.39	0.20 (35)	1.00
3	Sotos de Sepúlveda	41.267	-3.567	8	1, 6-7	W2	2	0.714	0.86	-	-
4	Aldea del Fresno[a,b]	40.330	-4.244	8	1	W2	0	0.000	0.00	0.00 (8)	-
5	Cabañas de Tera[a,b]	42.011	-6.056	13	1, 8	W2	1	0.154	0.15	0.17 (23)	0.75
6	Pallarés[a,b]	38.057	-6.184	12	9-16	W1+W2	7	0.924	6.18	0.00 (23)	-
7	Helechosa de los Montes[a]	39.314	-4.900	5	11	W1	0	0.000	0.00	0.00 (5)	-
8	Villuercas[a,b]	39.468	-5.341	16	11, 42	W1+E3	0	0.525	24.68	0.20 (20)	1.00
9	Aranjuez[a,b]	40.016	-3.586	8	17-18	E2	1	0.250	0.25	1.00 (42)	0.29
10	Torrejoncillo del Rey[a]	40.035	-2.599	9	18	E2	0	0.000	0.00	0.95 (21)	0.15
11	Lerma	42.134	-3.650	30	18-20	E2	2	0.628	1.23	-	-
12	Hoces del Cabriel[a]	39.542	-1.518	15	18, 21-24	E2	3	0.562	0.65	1.00 (20)	0.15
13	Pioz	40.443	-3.161	7	18, 25	E2	1	0.286	0.29	-	-

14	San Martín de la Vega[a]	40.187	-3.544	7	18	E2	0	0.000	0.00	0.78 (9)	0.29
15	Villatobas[a]	39.839	-3.259	5	18	E2	0	0.000	0.00	1.00 (5)	0.00
16	Saelices	39.873	-2.788	4	18	E2	0	0.000	0.00	-	-
17	Villar de la Encina	39.627	-2.492	2	18, 26	E2	1	1.000	1.00	-	-
18	Vara de Rey	39.422	-2.320	4	18	E2	0	0.000	0.00	-	-
19	Villares del Saz[a]	39.856	-2.541	8	18, 27	E2	1	0.250	0.25	0.75 (8)	0.33
20	Vellisca[a]	40.139	-2.813	6	18, 28	E2	1	0.333	0.33	1.00 (6)	0.00
21	Honrubia	39.586	-2.220	4	18, 24	E2	0	0.500	0.50	-	-
22	Brihuega[a]	40.766	-2.930	10	18	E2	0	0.000	0.00	0.67 (12)	0.88
23	El Frasno	41.421	-1.489	12	18	E2	0	0.000	0.00	-	-
24	Abrucena[a,b]	37.119	-2.812	13	29-31	E1+E2	3	0.641	10.77	1.00 (21)	0.48
25	Morata	37.588	-1.555	9	18, 32-34	E1+E2	3	0.750	14.33	-	-
26	Valdepeñas	38.901	-3.477	10	35-38	E3	4	0.733	1.64	-	-
27	Despeñaperros	38.375	-3.519	7	39-42	E3	3	0.714	2.19	-	-
28	Pelahustán[a,b]	40.180	-4.607	10	42-43	E3	1	0.200	0.40	0.19 (31)	1.00
29	Monte de Batres[a]	40.224	-3.938	11	42, 44-45	E3	2	0.564	0.62	0.00 (11)	-
30	Talavera de la Reina[a]	39.936	-4.820	7	42, 46-49	E3	4	0.857	1.62	0.00 (7)	-
31	Villarejo de Montalbán[a]	39.760	-4.568	11	42, 50-52	E3	2	0.691	0.95	0.00 (11)	-
32	Malpica de Tajo[a]	39.912	-4.545	8	42, 50, 53-54	E3	2	0.750	1.89	0.00 (8)	-
33	San Román de los Montes	40.084	-4.730	3	42, 55-56	E3	2	1.000	2.00	-	-

34	Espinoso del Rey	39.652	-4.788	3	42	E3	0	0.000	0.00	-	-
35	Noez[a]	39.747	-4.197	10	42, 57-58	E3	2	0.644	0.73	0.00 (10)	-
36	Puerto Lápice[a]	39.328	-3.490	5	59-60	E3	2	0.600	1.20	0.40 (5)	0.50

[a] Populations with frequency data of striped lizards; [b] Populations with microsatellite data

Table 2. AMOVAs with microsatellite data.

	F-statistic	% var	P	R-statistic	% var	P
mtDNA clade						
Among groups	-0.004	-0.40	0.758	-0.030	-3.01	0.828
Among populations within groups	0.058	5.83	< 0.001	0.190	19.61	< 0.001
Within populations	0.054	94.57	< 0.001	0.166	83.40	< 0.001
Groups generated by Structure						
Among groups	0.042	4.25	< 0.001	0.132	13.17	< 0.001
Among populations within groups	0.017	1.65	< 0.001	0.060	5.21	< 0.001
Within populations	0.059	94.10	< 0.001	0.184	81.62	< 0.001

Note: data were classified according to 1) population of origin nested within mtDNA clade (populations with western haplotypes: Cabañas de Tera, El Pardo, Navacerrada, and Pallarés; populations with eastern haplotypes: Abrucena, Aranjuez, and Pelahustán); and 2) population of origin nested within groups generated by Structure (group 1: Cabañas de Tera; group 2: Abrucena and Pallarés; group 3: Aranjuez; group 4: El Pardo, Navacerrada, Pelahustán and Villuercas).

Table 3. Gene flow between pairs of populations, based on IMa2 estimates.

	Aranjuez	**Abrucena**	**Pelahustán**	**Villuercas**	**Pallarés**	**Navacerrada**	**El Pardo**	**Cabañas**
Aranjuez	-	0.19	0.05	0.00	0.09*	0.00	0.01	0.00
Abrucena	0.02	-	0.06	0.00	0.00	0.00	0.00	0.00
Pelahustán	0.01	0.04	-	2.34*	0.05	0.98**	3.09*	0.00
Villuercas	0.00	0.01	0.17	-	0.02	0.14	0.24*	0.01
Pallarés	0.03	0.07	0.08	0.12	-	0.00	0.00	0.01
Navacerrada	0.10*	0.00	0.64*	0.81***	0.04	-	0.00	0.04
El Pardo	0.00	0.03	2.45*	1.23*	0.33*	2.00***	-	0.00
Cabañas	0.00	0.00	0.00	0.02	0.04	0.04	0.06*	-

Note: IMa2 estimates of mutation-scaled migration rate (*m*) between the donor (row) and recipient (column) population. Significance according to log-likelihood ratio tests: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Figure legends

Figure 1: Phylogeography of the lacertid lizard *Psammodromus algirus* in Spain, western Palaearctic. a) Haplotype network obtained from 319 sequences of an 849 bp mtDNA fragment (ND4 and associated tRNAs genes). Circle sizes are proportional to haplotype frequencies. Lines connecting circles represent single mutational steps, black dots represent missing intermediate haplotypes, and dashed lines between circles represent larger number of mutational steps (as indicated with large, black numbers in the Figure). Haplotypes numbers, shown inside the circles, correspond to those in Table 1. Different colours are used for each of the five haplogroups grouped into western (haplogroups W1 and W2) and eastern (haplogroups E1, E2, and E3) clades. b) Physical map of the Iberian Peninsula showing the location of sampled populations, with population numbers as in Table 1; as well as the main mountain ranges. Populations with microsatellite data are shown with larger dots, and identified by acronyms as in Figure 3. Lines show the putative distribution of the five haplogroups in the network, according to sampled populations (continuous lines) and to previous data by Carranza *et al*. (2006) and Verdú-Ricoy *et al*. (2010) (discontinuous lines). Populations with more than a single haplogroup (Pallarés, Villuercas, and the two populations with E1 haplotypes) are indicated with double-coloured dots.

Figure 2: Genetic structure according to seven microsatellite loci of eight Iberian populations of the lizard *Psammodromus algirus*. Results of Bayesian assignment tests with the software Structure uncovered four distinct clusters represented with different colours. Each vertical bar represents a single individual (N = 33, 27, 31, 36, 29, 25, 34, and 29 for Aranjuez, Abrucena, Pelahustán, Villuercas, Pallarés, Navacerrada, El Pardo,

and Cabañas de Tera, respectively; mtDNA haplogroups represented in each population are also indicated). Bar colours represent posterior probabilities of cluster membership. Note the similarity between the four central-western populations located south of the Sistema Central mountain range and assigned mainly to the red cluster.

Figure 3: Left: frequencies of striped lizards (in black) in 23 Iberian populations of the lacertid lizard *Psammodromus algirus*, with population numbers as in Table 1. Right: haplogroup averages (±1 SE) for W2 (N = 4 populations), E3 (N = 7), and E2 (n = 8). Whereas E2 lizards are mostly striped, E3 and W2 ones are mainly unstriped ($F_{2,16}$ = 40.64, $P < 0.001$).

Figure 4: Habitat features associated with the frequency of striped individuals in Iberian populations of the lizard *Psammodromus algirus*: results of a Partial Least Squares analysis (PLS) used to test for the relationship of the habitat variables considered (LL = leaf litter cover, C0-C100 = plant cover 0, 10, 30, 50, and 100 cm in height, HB = grass cover, PSh = cover of perennial shrubs, DSh = cover of deciduous shrubs, and TREE = tree cover) with the frequency of striped lizards. Left: relative contribution of the habitat variables to the multivariate factor (X) predicting the frequency of striped lizards; variables with squared weights > 0.10 (dashed line) are significant. Right: mean X-scores (± 1SE) of western (W1 and W2), central (E3), and eastern (E1 and E2) populations. X-scores differ significantly among these groups ($F_{2,10}$ = 5.82, $P = 0.021$), defining an east-west gradient of increasing vegetation cover development.

Figure 5: Detectability of two phenotypes (striped and unstriped; see Figure S1) of the lacertid lizard *Psammodromus algirus* in four populations located central Spain:

detection times of striped and unstriped lizards (mean ± 1SE) at two eastern localities of the E2 haplogroup where most lizards are striped (Aranjuez and Torrejoncillo) and two western localities of the W2 haplogroup where most lizards are unstriped (El Pardo and Navacerrada). The significant interaction between site and phenotype ($F_{3,33} = 92.43$, $P < 0.001$) shows that lizards are detected faster outside their natural environments, and suggests that dorsal colour pattern is an adaptation for crypsis.

Fig. 1

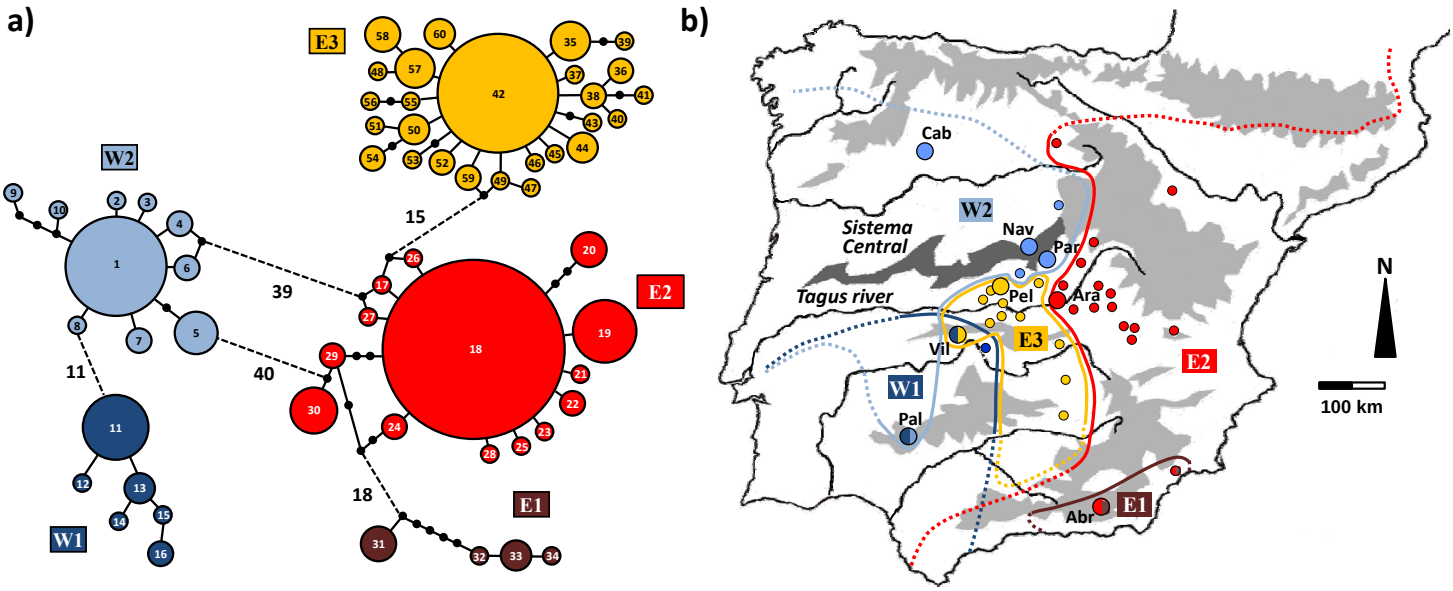

a)
W2
E3
E2
E1
W1
15
39
11
40
18
b)
Cab
W2
Sistema Central
Nav
Par
Tagus river
Pel
Ara
Vil
E3
E2
W1
Pal
Abr
E1
N
100 km

Fig. 2

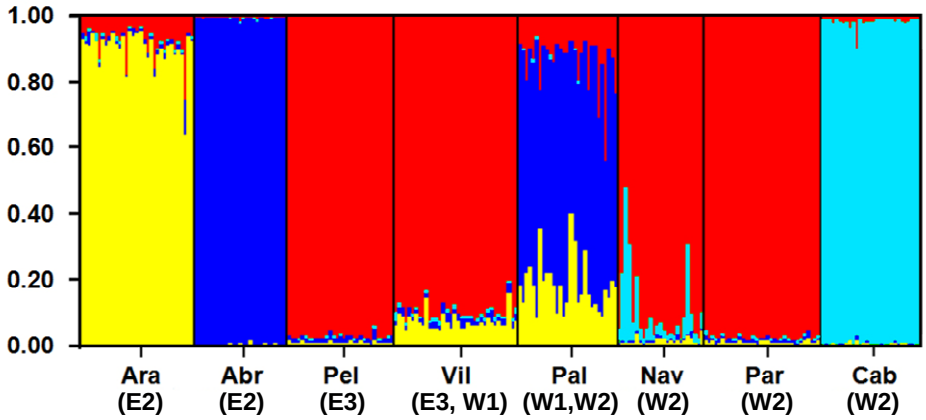

1.00
0.80
0.60
0.40
0.20
0.00
Ara (E2)
Abr (E2)
Pel (E3)
Vil (E3, W1)
Pal (W1,W2)
Nav (W2)
Par (W2)
Cab (W2)

Fig. 3

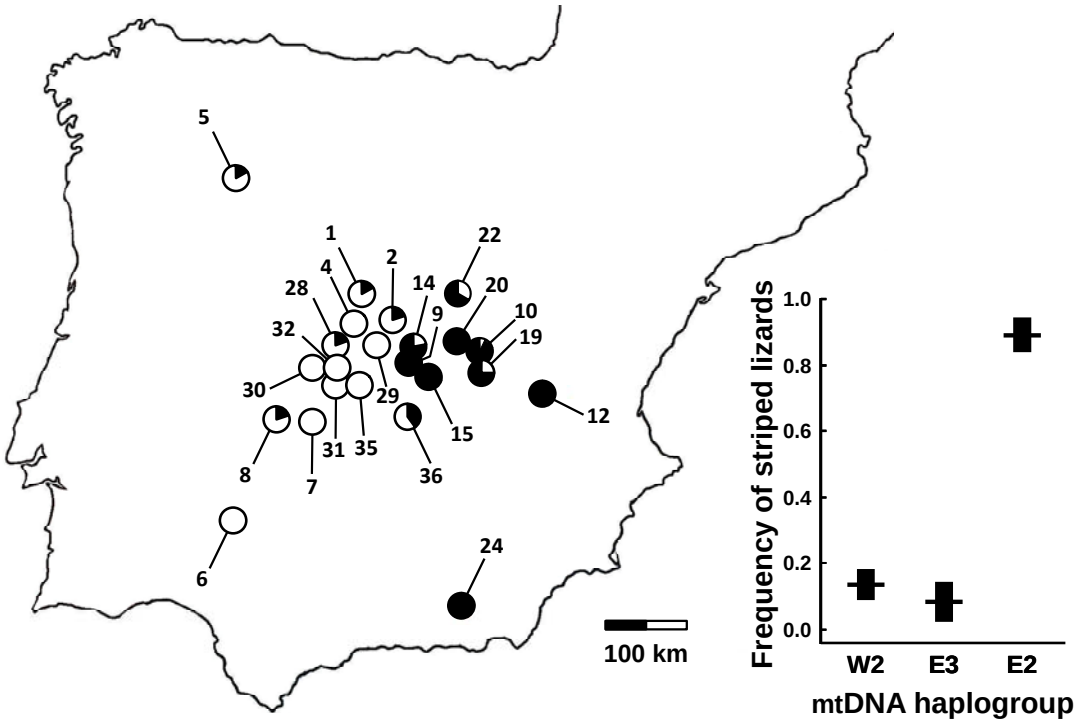

5
1
2
22
4
14
20
28
9
10
32
19
30
29
12
15
8
31
35
36
7
6
24
100 km
Frequency of striped lizards
1.0
0.8
0.6
0.4
0.2
0.0
W2
E3
E2
mtDNA haplogroup

Fig. 4

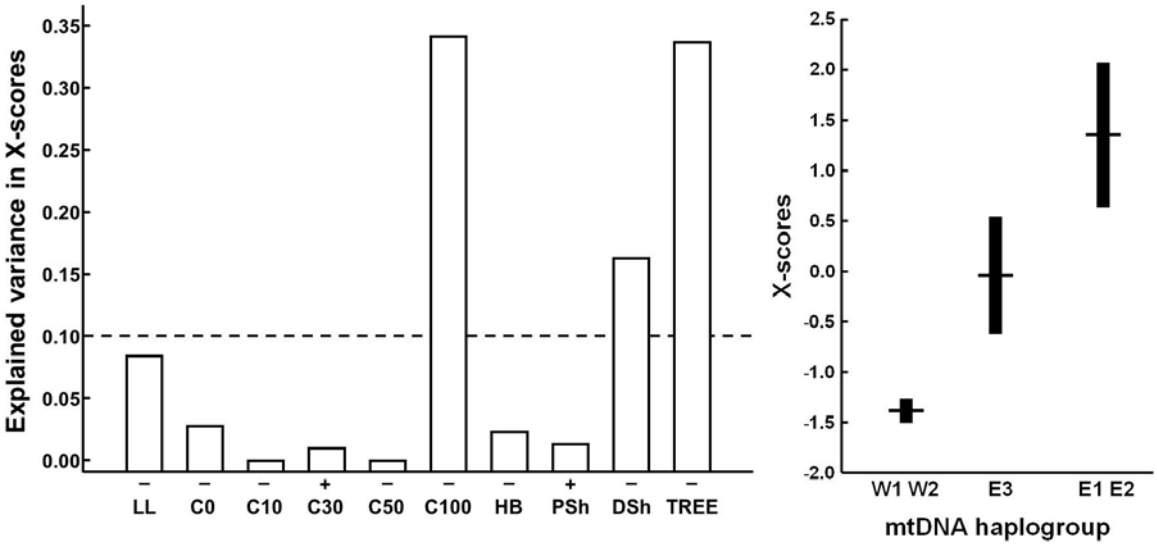
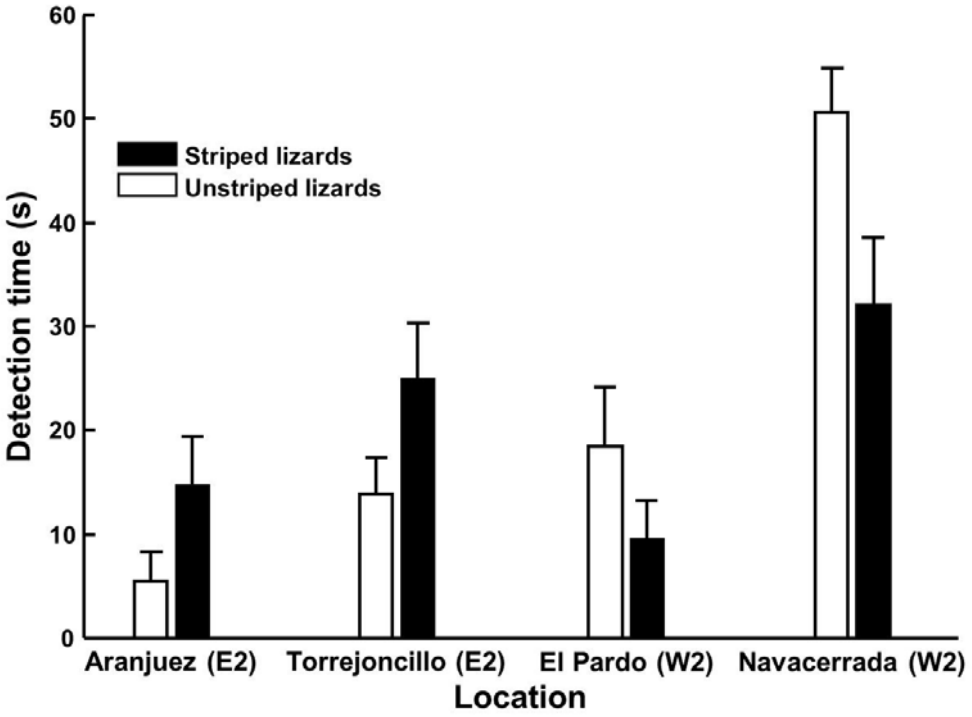

Explained variance in X-scores
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
– – – + – – – + – –
LL C0 C10 C30 C50 C100 HB PSh DSh TREE
X-scores
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
W1 W2 E3 E1 E2
mtDNA haplogroup

Fig. 5


60
50
40
30
20
10
0
Detection time (s)
Striped lizards
Unstriped lizards
Aranjuez (E2)
Torrejoncillo (E2)
El Pardo (W2)
Navacerrada (W2)
Location