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Genetic analysis of a contact zone between two lineages of the ocellated lizard (*Lacerta lepida* Daudin 1802) in south-eastern Iberia reveal a steep and narrow hybrid zone

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Abstract

Measuring the diffusion of genes between diverging taxa through zones of secondary contact is an essential step to understand the extent and nature of the reproductive isolation that has been achieved. Previous studies have shown that the ocellated lizard (*Lacerta lepida* Daudin, 1802) has endured repeated range fragmentation associated with the climatic oscillations of the Plio-Pleistocene that promoted diversification of many different evolutionary units within the species. However, the oldest divergence within the group is estimated to have occurred much earlier, during the Miocene, around 9 Ma and corresponds to the split between the subspecies *Lacerta lepida nevadensis* Buchholz (1963) and *Lacerta lepida lepida* Daudin (1802). Although these two evolutionary units have documented genetic and morphological differentiation, most probably accumulated during periods of allopatry, little is known about patterns of gene flow between them. In this study, we performed a population genetic analysis of a putative area of secondary contact between these two taxa, using mtDNA and microsatellite data. We assessed levels of gene flow across the contact zone to clarify to what extent gene flow may be occurring. Hybridization between the subspecies was observed by the presence of genetically introgressed individuals. However, the overall coincidence of mitochondrial and multilocus nuclear clines and generally steep clines support the idea that this contact zone is acting as a barrier to gene flow. Taken together, these results suggest that *L. l. lepida* and *L. l. nevadensis* are in independent evolutionary trajectories and should be considered as two different species.

Key words: *Lacerta lepida* – hybrid zone – speciation – gene flow

Introduction

Lacerta lepida Daudin (1802) is the largest European lacertid lizard and is the only species from the ocellated lizards to occur in Europe, where it occupies almost all of the Iberian Peninsula, southern France and north-western Italy (Mateo and Castroviejo 1990; Mateo et al. 1996; Castroviejo and Mateo 1998). Currently, four subspecies within *L. lepida* are recognized: *Lacerta lepida iberica* Lopez Seoane (1884), which occurs in the north-western corner of the Iberia peninsula; *Lacerta l. nevadensis* Buchholz (1963), in south-western Spain, mainly associated with the Betic mountain ranges; *Lacerta l. oteroi* Castroviejo and Mateo (1998), which is restricted to the island of Salvora in northern Spain; and finally *Lacerta l. lepida* Daudin (1802), the nominal species and the one with the widest distribution in the group occurring in all remaining parts of the Iberian Peninsula, southern France and north-western Italy. The subspecies designations are based mainly on morphological patterns, allozymes and in one case (*L. l. oteroi*) chromosomes (Mateo 1988; Mateo and Castroviejo 1990; Mateo and López-Jurado 1994; Mateo et al. 1996; Castroviejo and Mateo 1998). Previous studies have shown that *L. lepida* has endured repeated range fragmentation that has promoted diversification within the species (Paulo et al. 2008; Miraldo et al. 2011). Several mitochondrial lineages, which appear to have mainly non-overlapping geographic ranges, were described, suggesting a history of allopatric differentiation in multiple refugia during the Mio-Plio-Pleistocene (Paulo et al. 2008; Miraldo et al. 2011, 2012). The oldest divergence within the group is estimated to have occurred 9 Ma and corresponds to the split between the subspecies *L. l. nevadensis* and *L. l. lepida*. Divergence between these two subspecies is also supported by high levels of allozyme differentiation (Mateo et al. 1996), morphological differences in respect to body size (*L. l. nevadensis* having an increased body size) (Mateo and Castroviejo 1990; Mateo and López-Jurado 1994) and dorsal colour patterns (Mateo and López-Jurado 1994; Mateo et al. 1996; Nunes et al. 2011) and differences in reproductive strategy (*L. l. nevadensis* having an extended reproductive period) (Castilla and Bauwens 1989; Mateo and Castanet 1994). Despite the apparently deep genetic divergence, morphological differentiation and differences in the reproductive period between both lineages, the description of individuals with seemingly hybrid morphology along the transition zone (Mateo and López-Jurado 1994) suggests that hybridization features in the interaction between these two taxa.

It seems most probable that divergence between *L. l. nevadensis* and *L. l. lepida* was initiated by overseas dispersal from the Iberian mainland to the Betic Massif in the late Miocene that, at that time, existed as an island between Iberia and North Africa (Paulo et al. 2008; Miraldo et al. 2011). Under this scenario, subsequent contact between the taxa would have been initiated after the merging of the Betic Massif with Iberian mainland, due to the closing of the Betic corridor 7.6–7.8 Ma (see Rosenbaum et al. 2002a,b for a detailed explanation of the kinematics of the western Mediterranean basin). As there are no obvious geographic barriers between the lineages, gene flow is expected to have occurred after the lineages came into contact. However, despite the apparently deep historical divergence within the species, as revealed by the mitochondrial genealogy, it still remains unclear to what extent these divergent lineages are independently evolving. Recent studies focusing on reptiles have revealed that deep mtDNA divergence does not always correspond to nuclear genomic divergence (Rassmann et al. 1997; Stenson et al. 2002; Lindell et al. 2005, 2008; Thorpe et al. 2008; Ujvari et al. 2008), with male-biased dispersal being invoked as the main explanation for such discrepancies. In such cases, males act as the main agents of gene flow leading to reduced genetic

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structuring in biparentally inherited markers compared with maternally inherited mtDNA.

Evidence for the existence of a secondary contact zone between *L. l. lepida* and *L. l. nevadensis* emerged with the discovery of one population where both mitochondrial lineages occur in sympatry (sampling site 33 in Miraldo et al. 2011) (Fig. 1) located just to the west end of Sierra Nevada mountains in south-eastern Spain. Here, we undertake a population genetic analysis of that putative area of secondary contact between the two taxa, using both mitochondrial and nuclear markers. The specific objectives of this study are the following: (1) to identify the exact geographic location of the putative contact zone between the two subspecies; (2) to assess levels of gene flow across the contact zone, describing the geographical extent of introgression; (3) to investigate whether a barrier to gene exchange exists at the contact zone, by analysing clinal patterns of genetic variation; and finally (4) to clarify whether both lineages may in fact be considered as good species.

Materials and Methods

Sampling strategy collection

Sampling was conducted in 2007 along a south-east–north-west transect perpendicular to the location of a putative contact zone between *L. l. lepida* and *L. l. nevadensis* (hereafter referred to as Lepida and Nevadensis, respectively) based on the distribution limits of each mitochondrial lineage (Fig. 1). Nine populations were sampled along this transect, with the first sampled population (population 1 in Fig. 1) corresponding to the south-eastern limit of Nevadensis' distribution. Additional sampled populations were located to the north-west of population 1, keeping 15–50 km distance between them. In each population, 19–31 samples were collected, making a total of 200 samples. Lizards were captured using tomahawk traps or by hand, and tissue samples were taken by clipping 1 cm of the tail tip that was subsequently preserved in 100% ethanol. To avoid sampling the same individual twice, only lizards with intact tails were sampled. After tissue sampling, animals were immediately released back into the wild in the same place of capture. Geographic coordinates of sampling sites were recorded with a GPS. All lizards were captured with appropriate licences.

Laboratory procedures

Total genomic DNA was extracted from ethanol-preserved muscle tissue using a salt extraction protocol (Sunnucks and Hales 1996; Aljanabi and Martinez 1997). The entire (1143 base pairs) cytochrome *b* gene (cytb) was amplified using primers TRNAGLU(5′- AAC CAC CGT TGT ATTTCA ACT -3′) and TRNATHR(5′- CTT TGG TTT ACA AGA ACAATG CTT TA -3′) (Miraldo et al. 2011). These primers were shown to be specific for the mitochondrial cytb gene and do not amplify NUMTs (Miraldo et al. 2011). Protocols for the amplification of the entire cytb were as described by Miraldo et al. (2011). PCR products were visualized on a 2% agarose gel and purified by filtration through QIAquick® columns (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Purified PCR products were sequenced in both directions using primers L14841 and H15551 (Paulo et al. 2008) and BigDye TERMINATOR v3.1TM (Applied Biosystems, Carlsbad, CA, USA) chemistry. All PCRs and sequencing reactions were performed in a MJ Research thermocycler (PTC-240 DNA Engine Tetrad 2), and sequences were obtained using an ABI 3700 capillary sequencer.

Microsatellites

To obtain genotypic profiles for the lineages under study, eight polymorphic microsatellite loci were amplified for all samples. As there are no microsatellites specifically characterized for *L. lepida*, microsatellite loci characterized for other lacertid lizard species that were previously shown to be polymorphic within *L. lepida* (V.L. Nunes, unpublished data) were amplified: loci C9, B4 and D1, which were characterized in *Podarcis muralis* (Nembrini and Oppliger 2003); loci PB66 and PB73, characterized in *Podarcis bocagei* (Pinho et al. 2004); locus LV-4-72, from *Lacerta vivipara* (Boudjemadi et al. 1999); locus LVIR17 characterized in *Lacerta*

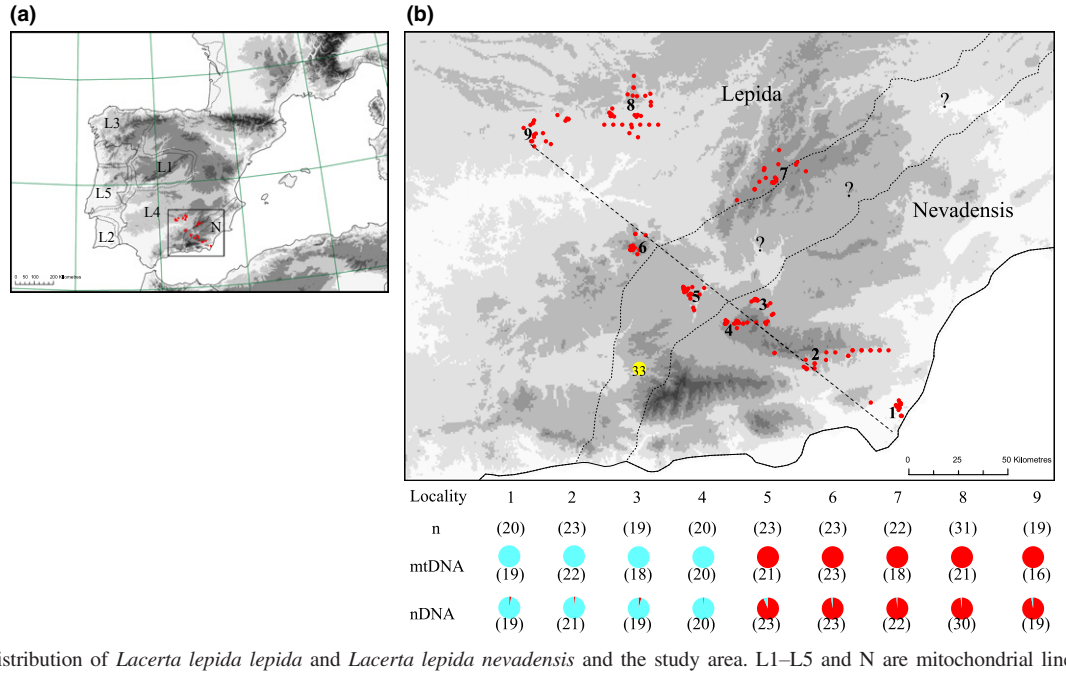

Fig. 1. (a) Distribution of *Lacerta lepida lepida* and *Lacerta lepida nevadensis* and the study area. L1–L5 and N are mitochondrial lineages distributions as described by Miraldo et al. (2011). (b) Sampled transect (dashed line). Shaded areas denote altitude gradients, with darker areas representing higher altitudes. Numbers represent sampling localities along the transect and samples are represented by black dots. The putative zone of secondary contact between the subspecies is indicated by question marks (?). Site 33 represents a population where mitochondrial haplotypes from both lineages were previously detected in sympatry (Miraldo et al. 2011). Pie charts represent: mtDNA – the proportion of mtDNA haplotypes at each site from *L. l. lepida* (red) and *L. l. nevadensis* (blue) mtDNA lineages; nDNA – the proportion of each site assigned to *L. l. lepida* (red) and *L. l. nevadensis* (blue) estimated with the software STRUCTURE using five microsatellite loci. The total number of samples (*n*) at each sampling site is indicated. Numbers below each pie chart represent the number of samples used in the analyses in each population for the specified marker (mtDNA versus microsatellites)

viridis (Böhme et al. 2005) and locus LIZ24 in *Lacerta schreiberi* (O.S. Paulo, unpublished data).

PCRs were carried out in a final volume of 10 µl containing 5× PCR buffer, 2.0 µl of 10× Go Taq® Buffer, 2.0 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of each primer, 2 µg of BSA, 0.5 units of Go Taq® DNA polymerase and approximately 50 ng of DNA. Locus C9 was amplified together with locus PB66, and locus D1 with locus PB73 in two different PCR duplexes. All other loci were amplified individually in independent PCRs. All PCRs were performed in a MJ Research thermocycler (PTC-240 DNA Engine Tetrad 2) using the following profile: initial incubation at 94°C for 3 min followed by 30 cycles of denaturing at 94°C for 30 s, annealing for 30 s (with temperature according to each locus, Table 1) and extension at 72°C for 30 s; plus a final extension incubation at 72°C for 30 min. PCR products of the eight loci were combined in two different mixes that allow distinguishing loci according to fluorescent dye and allele size. Mix A included locus D1, LV-4-72, LVIR17 and PB73; and Mix B included locus B4, C9, LIZ24 and PB66. To determine fragment length, 1 µl of either Mix A or Mix B was added to 8.9 µl of Hi-Di FormamideTM and 0.1 µl of GeneScanTM -500 RoxTM size standard. Each mix was run in an automated ABI Prism 377, and peaks were visualized with GENEMAPPER Software version 4.0 (Applied Biosystems).

Data analyses

Mitochondrial DNA data

DNA sequences were aligned by eye using BioEdit Sequence Alignment Editor 7.01 (Hall 1999). All sequences were trimmed to 627 bp before further analysis, to allow comparisons with published sequences. Median joining (MJ) (Bandelt et al. 1999) and statistical parsimony (SP) (Templeton et al. 1992) networks were constructed to infer the genealogical relationships of the mtDNA sequences. The MJ network was computed with the program NETWORK 4.5.0 (www.fluxus-engineering.com) keeping the parameter $\varepsilon = 0$, which does not allow less parsimonious pathways to be included in the analysis. The SP network was inferred using the program TCS 1.21 (Clement et al. 2000) with a connection limit of 70 mutational steps. For each population, the frequency of each haplotype and nucleotide diversity (π) were calculated. Population structure within each mtDNA lineage was assessed by estimating values of Φ_{ST} (a mtDNA analogue of F_{ST}; Excoffier et al. (1992)) for each locality and performing hierarchical analysis of molecular variance (AMOVAS), using localities as groups. Pairwise Φ_{ST} values between localities were also calculated as a measure of population genetic differentiation. All tests were performed in ARLEQUIN version 3.11 (Excoffier et al. 2005).

Microsatellite data

As this is the first time that this set of eight microsatellite loci has been applied to a population level study in *L. lepida*, and as almost each locus has been characterized in a different species through different independent studies, tests for non-random associations between diploid genotypes at each pair of loci were carried out. Tests for linkage disequilibrium were performed through a log-likelihood ratio statistic (*G*-test) using the Markov chain algorithm as implemented in GENEPOP 4.0 (Rousset 2008). For each locus levels of polymorphism, across and within populations were determined by quantifying allele number and frequency. Deviations from expectations of Mendelian inheritance were tested for each population at each locus using exact tests (Raymond and Rousset 1995) to check for the presence of heterozygote deficits. Departures from Hardy–Weinberg Equilibrium (HWE) can be due to biological factors such as population structure, non-random mating and selection against hybrids. Nevertheless, a departure from HWE can also be due to technical issues that occur during the process of microsatellite amplification, such as the presence of null alleles. To address this issue, the presence of null alleles was assessed using the program MICRO-CHECKER version 2.2.1 (Van Oosterhout et al. 2004). When null alleles were detected, their frequency was estimated using the methodology of Dempster et al. (1977), assuming that any detected heterozygote deficit is due to the presence of null alleles and not to population structure.

Population-based analysis

Allele richness, gene diversity (expected heterozygosity) and the indicator of inbreeding within populations (F_{IS}) were calculated for the microsatellites data set. Levels of genetic differentiation among pairs of populations were assessed by multilocus estimates of F_{ST}. All tests were carried out in GENEPOP 4.0 (Rousset 2008). The Bonferroni procedure (Rice 1989) was used to establish the threshold for statistical significance generated by multiple comparisons. An analysis of molecular variance (AMOVA) was carried out with ARLEQUIN 3.11 to test whether nuclear gene pools correspond to the mtDNA clades. For this analysis, individuals were grouped according to their mtDNA lineage.

Admixture estimation

STRUCTURE version 2.2 (Pritchard et al. 2000) was used to evaluate the extent of admixture between the two mitochondrial lineages using microsatellites data. Two data sets were analysed: one data set comprising all microsatellites loci and a reduced data set with loci for which the presence of null alleles was detected and that showed deviations from HWE for the majority of localities removed (loci Lvir17 and D1). In an exploratory analysis to infer the number (*K*) of genetically homogenous groups of individuals (clusters) along the transect, several analyses were run exploring values of *K* from 1 to 9. The most probable value of *K* was chosen using information from the probability (Ln P (D)) given by the software and by ΔK, a quantity based on the rate of change of the posterior probability with respect to the number of clusters, as defined by Evanno et al. (2005). Analyses were run three times for each *K* with a burn in period of 50 000 followed by 500 000 iterations per run.

Table 1. Primer sequences, annealing temperature (T_A), number of alleles (N_A) and allele size range for each locus in *Lacerta lepida*

Locus	Source	Primers	T_A (°C)	N_A	Allele size range
LIZ24	O.S. Paulo (unpublished)	F: FAM – TCAGTCCAAATATCTCTACAGG R: AGATGAGCAGCATATAGTGATG	50	8	115–139
B4	Nembrini and Oppliger (2003)	F: HEX – AATCTGCAATTCTGGGATGC R: AGAAGCAGGGGATGCTACAG	61	23	122–166
C9	Nembrini and Oppliger (2003)	F: FAM – CATTGCTGGTTCTGGAGAAAG R: CCTGATGAAGGGAAGTGGTG	58	14	130–169
Pb73	Pinho et al. (2004)	F: FAM – GCCCATGTCACTTCAGGTAGAAGC R: GAAAACTAGGAGTTAGGGAGAAGG	58	17	120–152
Pb66	Pinho et al. (2004)	F: NED – GGACAGCTAGTCCCATGGCTTAC R: GGATTGCTGTCACCAGTCTCCCC	58	21	148–192
D1	Nembrini and Oppliger (2003)	F: NED – GAGTGCCCAAGACAGTTGTAT R: GAGGTCTTGAATCTCCAGGTG	58	22	134–209
LVIR17	Böhme et al. (2005)	F: NED – AGCTCTGGATCGAGACAACCTGG R: TCTCTGAAGGAGACCGGCTCC	61	23	221–265
LV-4-72	Boudjemadi et al. (1999)	F: HEX – CCCTACTTGAGTTGCCGTC R: CTTTGCAGGTAACAGAGTAG	63	n.a.	n.a.

Further information regarding each locus can be found in the papers where they were first characterized (Source). Locus LV-4-72 failed to amplify consistently across populations, therefore no information regarding number of alleles or allele size range is provided (n.a., not applicable).

Consistency and convergence of parameter estimates were checked by visualizing the plots of the parameters. For all analyses, the admixture model was applied. According to this model, individuals can have a mixed ancestry, inheriting a fraction of their genome from different ancestors. The posterior mean estimate of those fractions was used to estimate the proportion of membership of each sampling locality in each of the inferred clusters. Levels of admixture for each sampled individual were obtained from the runs corresponding to the most probable value of *K* and using the reduced data set (five loci) with loci where the presence of null alleles was detected removed.

Cline analysis

Mitochondrial cline analysis was performed using information about the frequency of Lepida haplotypes in each sampled locality. For the purpose of nuclear genome cline analysis, we first calculated the mean proportion of membership of each sampled locality to each cluster identified by the STRUCTURE analysis, using five microsatellite loci, as described previously. This data were used to estimate a multilocus cline from which levels of nuclear genome introgression across the contact zone could be inferred. Maximum likelihood clines were fitted independently and plotted with ANALYSE (Barton and Baird 1995). Cline fitting was performed by adding geographic information of sampling localities to the allele frequency data. Localities were collapsed into a one-dimensional transect, with geographic distances measured from the southernmost sampling site (Locality 1) (Fig. 1). As sampling sites 7 and 8 do not fit well with the one-dimensional transect, they were not used for cline fitting purposes. Clines were fitted to a sigmoid tanh curve (Barton and Gale 1993), and the two parameters describing each curve (centre, *c*, and width, *w*) were estimated by the analysis. Estimation of both parameters started from approximate values of *c* calculated from the data and incorporated in the analysis. The centre of the cline is the point where the frequency of alleles switches above 0.5. Cline width was calculated as the inverse of the maximum of the slope of the cline curve (1/maximum slope) as described by Szymura and Barton (1986). The proportion of membership to each cluster, *p*, was allowed to vary between the p_{min} and p_{max} (minimum and maximum gene frequency at the tail ends of the cline) estimated from the data and incorporated in the program.

Results

A total of 200 samples were collected along the transect (see Table S1 for detailed information regarding each sample). Sampling sites and number of samples per site are shown in Fig. 1.

Mitochondrial DNA data

From the 200 samples collected, 178 were successfully sequenced for cytb (Table 2). All cytb sequences represented uninterrupted open reading frames, with no gaps/indels or premature stop codons, suggesting they are functional mitochondrial DNA copies. From the 627 bp analysed, a total of 104 were variable from which 85 were parsimony informative. Fifty-nine unique haplotypes were obtained (GenBank accession numbers: JX626247–JX626305). The genealogical relationships between haplotypes inferred by the two approaches for network construction (MJ and SP) were identical (Fig. 2). The haplotypes form two clusters which correspond to the two very divergent *L. lepida* lineages described by Paulo et al. (2008) and inferred to represent the two subspecies, *L. l. lepida* and *L. l. nevadensis*. No populations were found to be admixed for both mtDNA lineages, with populations 1–4 being fixed for Nevadensis haplotypes, while populations 5–9 were fixed for Lepida haplotypes (Fig. 1, Table 2). Haplotype frequencies within each mitochondrial lineage are represented in Figure S1. Within Lepida some level of

Table 2. Number of samples sequenced (*n*), cytb haplotypes (Haplotypes) and nucleotide diversity (π) in each *Lacerta lepida* sampled locality. Φ_{ST} values for each mitochondrial lineage with respective p-values (p) are also shown. Haplotype numbers are the same as in Fig. 2.

	n	Haplotypes	π	Φ_{ST}	p
Loc 1	19	H1, H2, H3, H4, H5, H6, H7	0.0038		
Loc 2	22	H1, H2, H8, H9, H10, H11, H112, H13	0.0024		
Loc 3	18	H1, H14, H15, H16, H17, H18, H19, H20, H21	0.0057		
Loc 4	20	H1, H2, H10, H22, H23, H24, H25	0.0052		
				0.0141	0.1603
Loc 5	21	H26, H27, H28, H29, H30, H31, H32	0.0024		
Loc 6	23	H26, H28, H33, H34, H35, H36, H37, H38	0.0028		
Loc 7	18	H33, H35, H39, H40, H41, H42, H43, H44	0.0039		
Loc 8	21	H28, H33, H34, H45, H46, H47, H48, H49, H50, H51, H52	0.0029		
Loc 9	16	H28, H33, H45, H53, H54, H55, H56, H57, H58, H59	0.0040		
Total	178			0.1145	<0.001

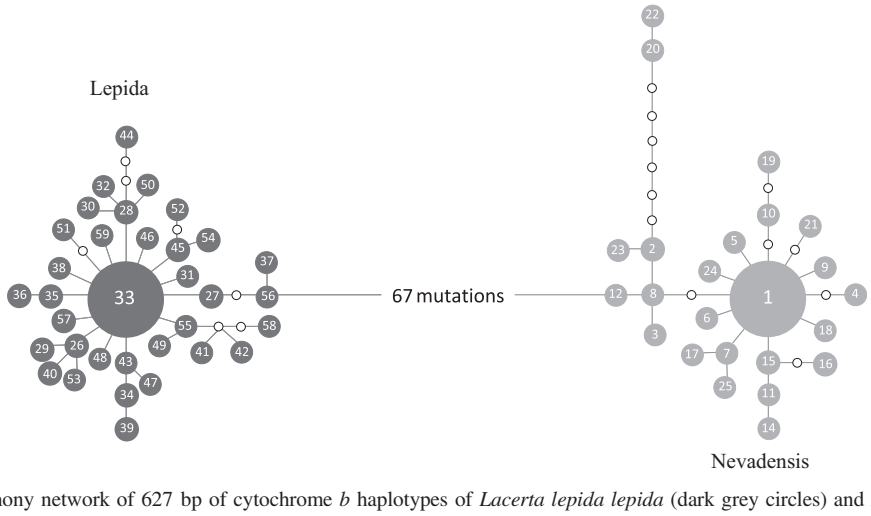

Fig. 2. Statistical parsimony network of 627 bp of cytochrome *b* haplotypes of *Lacerta lepida lepida* (dark grey circles) and *Lacerta lepida nevadensis* (light grey circles). White circles represent unsampled or extinct haplotypes. Size of circles does not correspond to frequency

genetic structure was detected, with 11.45% (Φ_{ST} = 0.1145, p < 0.001) of the genetic variation occurring between sampled localities. This structure was not found in Nevadensis (Φ_{ST} = 0.014, p = 0.16). Pairwise Φ_{ST} values between localities are shown in Table 3 (below diagonal).

Nuclear DNA data

From the 200 samples collected, we successfully genotyped 196 samples. From all microsatellite loci used in this study, only one locus, LV-4-72, failed to amplify consistently across all populations and was therefore eliminated from further analyses. All loci had moderate to high levels of polymorphism with the number of alleles over all populations ranging from 8 (LIZ24) to 23 (B4 and LVIR17) (Table 1). Allele frequencies per locus within each mitochondrial lineage are shown in Figure S2. The most frequent allele at each microsatellite locus is different in each mitochondrial lineage, with the exception of the least polymorphic locus (LIZ24), where allele 1 is the most frequent in both lineages, showing a gene frequency of 85–99% in Lepida and Nevadensis, respectively. Three loci (Locus C9, LVIR17 and PB73) exhibit a clear difference in allele frequencies between the mitochondrial lineages. Among these three loci, the clearest picture of variation between clusters occurs at locus C9 where lineages show almost non-overlapping allele size ranges, with few shared alleles of intermediate size and low frequency. The remaining loci show a broad overlap in allele sizes between Nevadensis and Lepida mitochondrial lineages with the majority of high-frequency alleles being shared among them (Figure S2).

Significant linkage disequilibrium was detected for 11 pairs of loci in six localities, with most of the non-random associations being detected at locality 4 (Table 4). Generally, pairs of loci in linkage disequilibrium were not detected at more than one locality. High deviations from HWE were detected in the majority of loci when considering all populations, with the exception of locus LIZ24 (χ^2 = 13.6, df = 10, p = 0.19). Tests of HWE for each locus/locality combination revealed 16 cases of significant heterozygote deficit (F_{IS} > 0; p < 0.05) relative to what is expected under HWE (Table 6). With the exception of localities 6 and 9, which show heterozygote deficits for the majority of loci, heterozygote deficits were usually detected at only a single locus within each locality. For loci Lvir 17 and D1, the presence of null alleles was detected in several localities (Table 6), which could explain the observed heterozygote deficits. As the incorporation of null alleles can influence downstream analyses, these loci were removed from the data set.

Table 4. Linkage disequilibrium for each pair of microsatellite loci in each *Lacerta lepida* sampled locality. Only significant non-random associations between pairs of loci are shown.

Locality	Locus 1	Locus 2	p-value
1	PB66	D1	0.029
2	LVIR17	PB73	0.030
4	B4	LVIR17	0.036
4	B4	C9	0.000*
4	LVIR17	C9	0.000*
4	LVIR17	PB73	0.044
4	PB73	C9	0.034
4	B4	PB66	0.000*
4	LVIR17	PB66	0.006
4	C9	PB66	0.004
6	LVIR17	PB73	0.014
6	C9	PB66	0.029
8	C9	PB73	0.042
8	B4	CYTB	0.002
9	LIZ24	C9	0.045

*Comparisons that remain statistically significant after sequential Bonferroni correction (Rice 1989) for 189 multiple tests.

Table 5. Results from AMOVA using nuclear markers (five microsatellite loci) with test groups corresponding to mtDNA lineages of *Lacerta lepida* sampled along a transect in south-eastern Spain

Source of variation	Sum of squares	Variance of component	Variation (%)	p-value
Among groups (Loc1–4, Loc5–9)	55.643	0.531	14.1	0.007
Among localities within groups	38.949	0.111	2.9	0.001
Among localities	587.668	3.143	83.0	

Population-based analysis

Pairwise F_{ST} values between pairs of localities and p-values for the genetic differentiation tests are shown in Table 3 (above diagonal). Levels of genetic differentiation among localities 1–4 are low, but these localities are significantly different from localities 5–9. Genetic differentiation among localities 5–9 varies between low to moderate. This suggests a homogeneous group in the south-eastern part of the transect that is genetically distinct from another slightly less homogenous group in the northwestern part of the transect. Differentiation between localities based on mtDNA haplotypes was higher than estimates based on

Table 3. F_{ST} values between pairs of localities of *Lacerta lepida* and p-values from the genetic differentiation test (in brackets) estimated with five microsatellites data (above diagonal); and Φst values estimated for 627 bp of mtDNA cytb gene sequences (below diagonal). See Fig. 1 for sampling localities. Statistically significant pairwise F_{ST} values (p < 0.05) after sequential Bonferroni correction for 36 multiple tests are denoted with bold font. The result HS is reported when at least one of the individual tests being combined in the Fisher's method yielded a zero p-value estimate.

	1	2	3	4	5	6	7	8	9
1	–	0.026 (0.005)	0.020 (0.007)	0.026 (0.001)	0.090 **(HS)**	0.106 **(HS)**	0.140 **(HS)**	0.145 **(HS)**	0.092 **(HS)**
2	0.021	–	0.024 (0.227)	0.022 (0.006)	0.087 **(HS)**	0.119 **(HS)**	0.153 **(HS)**	0.153 **(HS)**	0.113 **(HS)**
3	0.037	0.011	–	0.012 (0.011)	0.085 **(HS)**	0.104 **(HS)**	0.131 **(HS)**	0.133 **(HS)**	0.093 **(HS)**
4	−0.013	0.025	0.007	–	0.090 **(HS)**	0.124 **(HS)**	0.158 **(HS)**	0.156 **(HS)**	0.111 **(HS)**
5	0.974	0.979	0.966	0.967	–	0.024 **(4E-6)**	0.048 **(HS)**	0.040 **(3E-5)**	0.026 **(2E-6)**
6	0.972	0.977	0.965	0.966	0.087	–	0.025 **(5E-4)**	0.027 (0.001)	0.020 **(6E-4)**
7	0.967	0.974	0.959	0.961	0.227	0.093	–	0.009 (0.133)	0.025 **(HS)**
8	0.971	0.977	0.964	0.965	0.125	0.012	0.135	–	0.024 **(6E-4)**
9	0.967	0.974	0.958	0.960	0.166	0.107	0.132	0.059	–

HS, highly significant.

Table 6. Measures of genetic diversity at seven microsatellite loci in *Lacerta lepida*: expected (H_E) and observed (H_O) heterozygotes, F_{IS} values and null allele frequency for each locality/locus combination (null). Shaded values are statistically significant ($p < 0.05$) and denote significant heterozygote deficits. The presence of null alleles detected by MICROCHECKER is denoted with bold italic font.

Locus	B4			LVIR17			LIZ24			C9		
Loc.	H_E (H_O)	F_{IS}	Null	H_E (H_O)	F_{IS}	Null	H_E (H_O)	F_{IS}	Null	H_E (H_O)	F_{IS}	Null
1	14.3 (13)	0.10	0.00	15.0 (12)	0.20	0.07	0 (0)	–	–	17.1 (16)	0.06	0.07
2	15.4 (14)	0.09	0.00	15.8 (17)	−0.08	0.00	0 (0)	–	–	19.0 (19)	0.00	0.08
3	16.14 (13)	0.20	0.07	14.5 (11)	0.25	0.10	1 (1)	–	0.00	17.1 (15)	0.12	0.04
4	16.1 (13)	0.20	0.07	17.1 (15)	0.12	0.06	0 (0)	–	–	16.4 (17)	−0.04	0.00
5	21.1 (22)	−0.04	0.00	20.8 (18)	0.14	0.05	3.8 (4)	−0.05	0.00	17.4 (15)	0.14	0.06
6	20.6 (17)	0.18	0.07	29.8 (16)	***0.20***	0.09	4.6 (4)	0.14	0.14	16.2 (12)	***0.26***	0.11
7	15.2 (12)	0.21	0.06	18.7 (19)	−0.01	0.00	9.4 (8)	0.15	0.11	14.1 (12)	0.15	0.05
8	25.2 (26)	−0.03	0.01	27.2 (21)	***0.25***	0.12	7.4 (8)	−0.08	0.00	12.9 (13)	−0.01	0.00
9	16.3 (14)	0.15	0.07	17.0 (11)	***0.36***	0.16	5.5 (4)	0.28	0.00	12.8 (9)	0.30	0.11

Locus	PB73			PB66			D1		
Loc.	H_E (H_O)	F_{IS}	Null	H_E (H_O)	F_{IS}	Null	H_E (H_O)	F_{IS}	Null
1	16.9 (16)	0.06	0.04	17.2 (17)	0.01	0.00	16.1 (11)	***0.32***	0.12
2	18.3 (19)	−0.04	0.00	18.2 (17)	0.07	0.00	18.2 (15)	***0.18***	0.06
3	15.8 (15)	0.05	0.00	16.2 (18)	−0.12	0.00	16.4 (11)	***0.34***	0.14
4	16.5 (16)	0.03	0.01	17.8 (17)	0.05	0.03	16.1 (10)	***0.39***	0.16
5	19.8 (21)	−0.06	0.00	20.3 (22)	−0.09	0.00	20.4 (20)	0.02	0.00
6	17.3 (12)	***0.31***	0.13	20.9 (20)	0.04	0.00	19.7 (20)	−0.02	0.00
7	18.9 (18)	0.05	0.00	18.8 (19)	−0.01	0.00	19.5 (19)	0.03	0.00
8	24.5 (23)	0.06	0.01	26.8 (24)	0.11	0.04	27.2 (24)	0.12	0.05
9	16.1 (13)	0.20	0.10	16.7 (16)	0.04	0.00	17.9 (17)	0.05	0.04

microsatellite allele frequencies (Table 3). Results from AMOVA (Table 5) support the existence of nuclear genetic differentiation between mtDNA lineages. Although most of the genetic variation (83%) is distributed within sampled localities, some genetic variation (14.1%) is distributed among groups (mtDNA lineages) despite little or no differentiation (2.9%) among localities within each mtDNA lineage.

Admixture estimation

Similar results were obtained when analysing the microsatellite data with STRUCTURE, which revealed $K = 2$ as representing the most probable number of clusters (Figure S3 and Table S2). Both analysed data sets (data set with all loci and reduced data set with LVIR17 and D1 removed due to the presence of null alleles and deviations from HW expectations) gave similar results. The proportion of each locality assigned to each cluster using the reduced data set is shown in Fig. 1. Localities 1–4 were assigned to cluster 1, while localities 5–9 were assigned to cluster 2. These results are concordant with the mitochondrial data.

For each individual analysed, the proportion of assignment to each cluster can be seen in Fig. 3. Generally, the majority of individuals have a high proportion of assignment to one of the clusters (higher than 95%), but nine individuals show some level of admixture (Fig. 3, Table 8). Among these nine individuals with admixed ancestry, five show a high proportion of their genome (80–95%) assigned mainly to one of the clusters (Table 8). However, in localities 5 and 3, three individuals were identified as having more extensive admixture levels. In locality 5, individual '5–19' represents a putative F1 hybrid between the two taxa, having half of its nuclear genome assigned to each cluster. The other two individuals with mixed ancestry (individuals '5–10' and '3–18') likely represent a backcross of an F1 hybrid with a pure cluster 2 form, having 24% and 22% of their genome assigned to cluster 1 and the remaining assigned to cluster 2.

Cline analysis

Maximum likelihood fitted clines are presented in Fig. 4. Cline centres and widths are generally coincident and concordant, with

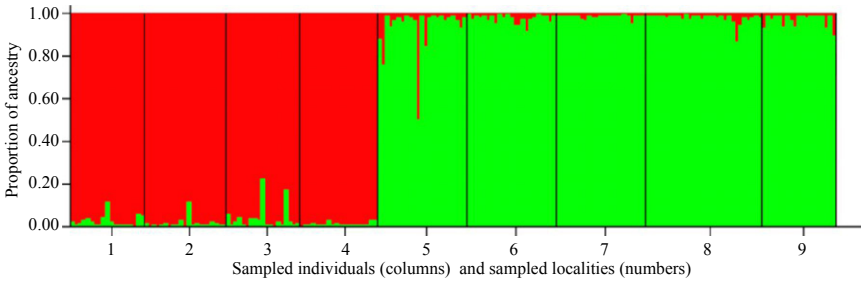

Fig. 3. Proportion of ancestry of each sampled individual (columns) as inferred with STRUCTURE for five microsatellite loci, assuming the admixture model

the nuclear cline being slightly wider than the mitochondrial one (Fig. 4 and Table 7). The centre of the contact zone is located between locality 4 and 5.

Discussion

Paulo et al. (2008) postulated that populations of *L. lepida* from Sierra Nevada Mountains, in south-eastern Spain, should be elevated to a different species from the remaining populations in the Iberian Peninsula, on the basis of high mitochondrial genetic differences between these. Nevertheless, due to the broad sampling and the low number of genetic markers employed in that study, the extent of gene exchange between these apparently genetically divergent populations could not be evaluated. Our sampling here reveals that the two divergent lineages meet at a narrow contact zone that probably runs all the way from the coastal region of Granada, where both mitochondrial lineages were previously found to occur in sympatry (locality 33 in Fig. 1), contouring the northern side of the Sierra Nevada mountains in a north-east direction and reaching the western part of Sierra de Baza, somewhere between localities 4 and 5. Further inferences about the eastern extent of the contact zone cannot be made due to lack of sampling. However, previous studies suggest that the contact zone might extend to the east as individuals with hybrid morphology occur in the limits between the provinces of Murcia and Albacete and also in the province of Valencia (Mateo and López-Jurado 1994).

The fine detailed geographic sampling allowed us to assess the patterns of mitochondrial and nuclear genetic variation across the contact zone, supporting Paulo et al. (Paulo et al. 2008) suggestion that the two subspecies in contact reflect in fact two different species. Despite the presence of one putative F1 hybrid in locality 5, our results show that admixture between *L. l. lepida* and *L. l. nevadensis* is limited and there is little if any gene exchange between them.

Table 7. Maximum likelihood estimates of cline centres (*c*) and widths (*w*) for cytochrome *b* (mtDNA) and for nuclear data. Overall nuclear multilocus cline parameters were estimated using five microsatellites loci (nDNA)

Locus	*C* (km)	*W* (km)
mtDNA	106.87	2.70
nDNA	109.72	10.73

Table 8. Estimated membership coefficients (*Q*) in each cluster for each admixed individual. Membership coefficients were calculated in STRUCTURE using the admixed model.

		Ancestry estimates (*Q*)	
Population	Sample ID	Cluster 1	Cluster 2
1	1–19	0.880	0.120
2	2–2	0.880	0.120
3	3–18	0.777	0.223
3	3–6	0.828	0.172
5	5–1	0.118	0.882
5	5–10	0.236	0.764
5	5–19	0.497	0.503
5	5–20	0.152	0.848
8	8–32	0.133	0.867

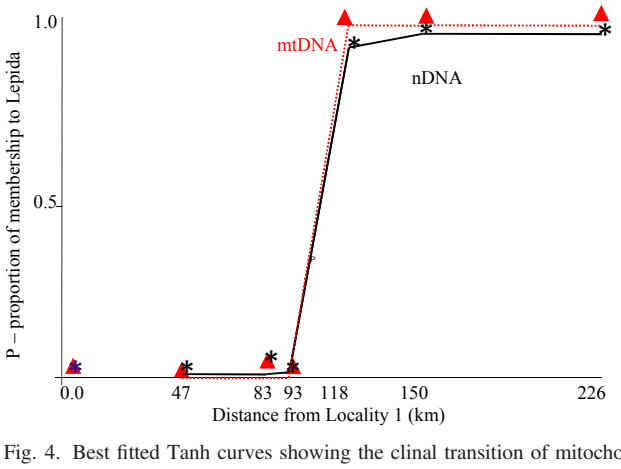

Fig. 4. Best fitted Tanh curves showing the clinal transition of mitochondrial and nuclear loci through the contact zone between *Lacerta lepida nevadensis* and *Lacerta lepida nevadensis*. Changes in proportion of membership (P) to *L. l. lepida* along the transect based on five microsatellite loci (black stars) and changes in frequency of *L. l. lepida* mitochondrial haplotypes (red stars)

Genetic structure of the contact zone: tension zone versus neutral diffusion

Several taxa show phylogenetic breaks associated with the Betic mountains caused by an history of allopatry in this region (see Gomez and Lunt 2007 and references therein), which also seems to be the case for *L. lepida*. Divergence between *L. l. lepida* and *L. l. nevadensis* started during the Miocene (9 Ma), most probably associated with a period of allopatry with the dispersal of founder individuals from Iberian mainland to the Betic Massif that, 9 Ma, existed as a large island between Iberia and North Africa (Rosenbaum et al. 2002b). Contact between the divergent taxa would have been initiated after the merging of the Betic Massif with Iberian mainland 7.6 –7.8 Ma (Rosenbaum et al. 2002b). Nevertheless, it seems likely that the profound climatic oscillations during the Pleistocene have induced further differentiation between the taxa. It is probable that the present contact between the lineages occurred after the Last Glacial Maxima (LGM) when an increase in temperature allowed populations to expand their ranges from refugial areas. In fact, a recent detailed phylogeographic study of *L. lepida* in the Iberian Peninsula revealed signatures of demographic and spatial expansions between 0.10 and 0.15 Ma in all lineages (Miraldo et al. 2011). Present contact is thus estimated to have occurred approximately 15 000 BP, or perhaps even more recently due to the effect of the Younger Dryas (YD). The YD was a period of rapid climatic change during the interglacial characterized by a dramatic fall in temperature re-establishing conditions similar to the last glacial period. The YD ended approximately 10 000 BP, with the start of the preboreal when the climate warmed markedly. Although the climatic changes during the YD are thought to have been less dramatic in southern Spain, an increase in steppe type vegetation in the region is registered during this period, especially at higher altitudes (Carrión and Dupre 1996; Carrión et al. 1998). It is therefore likely that the YD had an impact on the distribution of *L. lepida* in the region, likely delaying or interrupting contact between the diverging lineages until the end of the climatic reversal, around 10 000 BP. Given that contact between the two evolutionary units must have been possible 10 000 BP, it is reasonable to assume that the hybrid zone formed at least 3300 generations ago [the generation time of *L. lepida* is of 3 years

(Mateo 1988; Mateo and Castanet 1994)] and is now at or near dispersal/selection equilibrium.

The width (w) of a neutral diffusion cline is a function of the time (in generations) since secondary contact (T) and the dispersal rate (d) as described by the equation:

$$T = 0.35(w/d)^2 \text{(Endler, 1977)}$$

To generate a cline width of 10 km (the width detected using microsatellite loci) assuming neutral diffusion, a dispersal rate of 100 m per generation would have to be invoked. One hundred metres dispersal per generation (3 years) is likely to be a very small distance for *L. lepida*. Dispersal rates for *Chioglossa lusitanica*, a relatively much smaller salamander, have been estimated to be 120 m per generation (Sequeira et al. 2005). This species dispersal might be restricted due to habitat requirements such as high dependence of juveniles on water streams, which is not the case for *L. lepida*. *Lacerta lepida* territories are on average 3500 m^2 for females and 11 000 m^2 for males (Salvador et al. 2004), suggesting dispersal rates (variance in parent/offspring distance) to be much higher than 100 m. This is supported by the overall multilocus F_{ST} values within each lineage which are relatively small (0.02), suggesting high levels of gene flow. Therefore, higher dispersal between the lineages would be expected implying much wider nuclear clines, which is not observed. It seems likely that further sampling between localities 4 and 5 (25 km apart) will probably reveal much steeper cline widths, requiring even smaller dispersal rates for the nuclear genome to conform to neutral diffusion.

Although our evidence is indirect, it seems likely that selection against hybrids is acting at the centre of the zone, keeping the clines steep. The fact that only three putative hybrids were found in populations near the centre of the contact zone, the coincidence of mtDNA and nuclear clines and the existence of linkage disequilibrium in populations near the contact zone also support this. As is the case for many hybrid zones, this contact zone seems to conform to the 'tension zone' model where clines are maintained by a balance between selection and dispersal (Barton and Hewitt 1985).

At the time of secondary contact, genetic introgression is initiated, but any co-adaptation of lineage-specific alleles may enhance the effect of divergence between the lineages through epistatic interactions among loci. New recombinants generated from hybridization between the lineages may be less fit, under these conditions epistasis and linkage can promote cline coincidence (Barton and Hewitt 1989).

Selection forces that are influencing cline shape in this contact zone are probably endogenous as there is no clear evidence for clinal environmental variation through the zone or any geographic barrier to dispersal. Hybrid fitness is therefore most probably determined by genome interactions, such as heterozygote disadvantage and epistasis, independent from the environment.

Prezygotic mechanisms can also be involved in the observed pattern of cline widths. In fact, differences in the reproductive activity between both subspecies have been identified. *L. l. nevadensis* shows an extended reproductive period in concordance with the longer period of male sexual activity and has the ability of producing two clutches per year, whereas *L. l. lepida* only produces one (Mateo 1988; Castilla and Bauwens 1989; Mateo and Castanet 1994). It would be interesting to examine mating and reproduction through the zone. In total, this evidence indicates that overall reproductive isolation between the lineages is very strong.

The historical dynamics of contact and introgression

It should be noted that there may have been episodes of introgression during earlier Pleistocene interglacial periods of contact between the lineages, and even before in the Pliocene, especially if during those previous contacts selection against hybrids was weaker or if the contact was maintained for longer. The deeply divergent mtDNA lineages, corresponding to a divergence time of approximately 9 Ma, indicate that a substantial period of geological time has been available for climatically mediated allopatry and parapatry. Earlier contact and hybridization may have allowed for the exchange of alleles between lineages, and therefore, some degree of similarity in allele frequencies between the lineages is expected, concomitant with the 'evolutionary filter' role played by the contact zone. It is more likely that the similarities in allele frequencies registered are the result of ancestral gene flow and do not indicate contemporary gene flow among lineages. However, we cannot exclude incomplete lineage sorting as the justification for the presence of shared alleles.

Taxonomic and conservation implications

The existence of clear significant morphological differences between the pure forms (Mateo and Castroviejo 1990; Mateo and López-Jurado 1994; Mateo et al. 1996), clinal variation in genotype frequencies and the very low frequency of hybrids detected suggest that *L. l. lepida* and *L. nevadensis* are genetically independent. Divergence between the subspecies seems to have passed the threshold whereby introgression is greatly reduced, and therefore, coalescence between the subspecies is unlikely. The two subspecies should therefore be considered as different evolutionary units and conservation efforts should bear this in mind.

Lacerta lepida is widely distributed across Spain and Portugal and there are no specific conservation measures for its protection. The IUCN considers the existence of only one species within the group, which is classified as in significant decline mainly due to habitat loss. In the last Mediterranean Red list assessment, *L. lepida* was classified as Near Threatened (NT), a status that is more alarming if the existence of two species within it is to be considered. More worrying is the case of *L. nevadensis* which presents a very restricted distribution, associated with zones of high touristic pressure and where current changes in land use (e.g. the increasing density of greenhouses in the province of Almeria) are most likely to be detrimental for the species survival, and the threats posed by habitat loss might be more alarming.

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Supporting Information

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

Figure S1. Frequency of mitochondrial DNA haplotypes (cytb gene) in each *Lacerta lepida* population.

Figure S2. Allele frequencies per locus for each *Lacerta lepida* mtDNA lineage. Dark grey bars represent Lepida (populations 5–9) and light grey bars represent Nevadensis (populations 1–4).

Figure S3. Magnitude of ΔK, as defined by Evanno et al. (2005), as a function of K.

Table S1. List of *Lacerta lepida* samples collected from nine populations in south-eastern Spain. For each sample, the cytochromeb haplotype based on 627 base pairs (Cytb Hap.) and the genotypes (allele size) scored in five loci (B4, Liz24, C9, PB73 and PB66) are shown. Samples that were not successfully amplified for cytb or genotyped are denoted by n.a. (not applicable).

Table S2. Posterior probabilities of K for each run.

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