

Melting pots and hotspots: genetic variation within *Acanthodactylus erythrurus* (Reptilia: Lacertidae) from the Iberian Peninsula

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Abstract. Identification of high levels of intraspecific genetic variability is common among assessments of reptiles from the Iberian Peninsula, conforming to the “refugia within refugia” hypothesis. Divergent genetic lineages are often found to be allopatric, or with limited contact or hybrid zones; such zones are more widely reported in Northern Europe, leading to the “melting pots not hotspots” hypothesis. However, population level sampling is needed to determine if divergent lineages overlap, something that is often missing in phylogenetic studies. Mitochondrial genetic diversity was assessed within the lacertid lizard *Acanthodactylus erythrurus* across the Iberian Peninsula. Not only were highly divergent lineages recovered in the southern region, but also considerable admixture of these was identified, particularly in northern populations. This has implications for biogeographic scenarios, with patterns typically identified at a larger scale emerging within a single European southern refugium. It also indicates that divergent mtDNA lineages appear to freely mix, at least within this species, which has taxonomic implications.

Keywords: glacial refugia, mitochondrial DNA, ND4, phylogeography.

Within the Iberian Peninsula, high levels of mitochondrial DNA variability within reptile forms that have historically been considered single species are the norm. The *Podarcis hispanica* species complex is a well-studied example of this (e.g. Kaliontzopoulou et al., 2011), but high diversity is found in most widespread groups, including *Psammodromus hispanicus* (now considered three species; Fitze et al., 2011), *Psammodromus algirus* (Carranza et al., 2006), *Tarentola mauritanica* (Rato, Carranza and Harris, 2012) and *Timon lepidus* (Miraldo et al., 2011). These and the many examples from other groups of organisms led to the “refugia

within refugia” biogeographical hypothesis being proposed, with the geological complexity of the Iberian Peninsula allowing distinct separate refugia for temperate forms during the Pleistocene glacial periods, which in turn allowed high levels of genetic diversity to be maintained (Gómez and Lunt, 2006). The diversity originated much earlier than this – most of the examples from reptiles have been dated back to the Miocene – often related to the Messinian Salinity Crisis and the previous fragmentation of Southeast Iberia as an archipelago of distinct islands (Miraldo et al., 2011). Such high levels of genetic diversity, and the recognition of multiple new species as a result, highlight the need for detailed assessments of other species, both to determine if other “cryptic” species may occur, but also to allow comparative biogeographic patterns to be made by comparing the distribution and levels of intraspecific diversity across taxa.

The focus of this study was the fringe-toed lizard *Acanthodactylus erythrurus* (Schinz, 1838). This species is widely distributed in western North Africa and is the only species of *Acanthodactylus* that occurs in the Iberian

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Peninsula. The situation in North Africa is complex, and the relationships with other species unclear (Harris, Batista and Carretero, 2004; Fonseca et al., 2009; Psonis et al., 2016), with the species apparently paraphyletic with respect to both *Acanthodactylus blanci* from Tunisia and *Acanthodactylus lineomaculatus* from Morocco (Tamar et al., 2016). Iberian populations of *A. erythrurus* appear to form a monophyletic lineage within the complex (Fonseca et al., 2009; Tamar et al., 2016), but limited sampling in the Iberian Peninsula meant that, although diverse lineages occur there, the distribution of these is essentially unknown. At the same time, only 1–3 individuals per population were examined, so intra-population variation could not be assessed. The aim of this study was therefore to re-examine genetic diversity within *A. erythrurus* from the Iberian Peninsula, using specimens from across the range and with increased numbers of individuals per population, to compare this with other reptiles from the region.

A total of 138 samples of *A. erythrurus* from 19 populations across the Iberian Peninsula were collected in 2010 and 2011 (fig. 1 and table 1). Tail tips were removed and stored in 100% ethanol for genetic analysis. Two samples

of *Acanthodactylus boskianus* were included as outgroups. DNA was extracted from these using standard high-salt methods (Sambrook, Fritsch and Maniatis, 1989). A partial fragment of the mitochondrial ND4 gene and adjacent tRNAs was amplified using the primers ND4 and Leu (Arévalo, Davis and Sites, 1994). PCR cycling consisted of 92°C for 3 minutes, followed by 35 cycles of 92°C for 30 seconds, 48°C for 40 seconds, 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. All positive PCR products were purified and sequenced by a commercial facility (Macrogen Europe, the Netherlands).

The alignment of the 140 sequences was performed using MUSCLE software implemented in the program Geneious (Biomatters Ltd). Aligned length was 818 base pairs. Phylogenetic relationships were estimated using both maximum likelihood (ML) and Bayesian inference (BI). ML was performed in RaxML 8.1 (Stamatakis, 2014), with node support estimated by nonparametric bootstrapping with 1000 replicates. The model of evolution (TrN + G) was chosen using jModeltest 2.1.7 (Posada, 2008) under the AIC approach, with GTR+G applied in RaxML which does not permit the

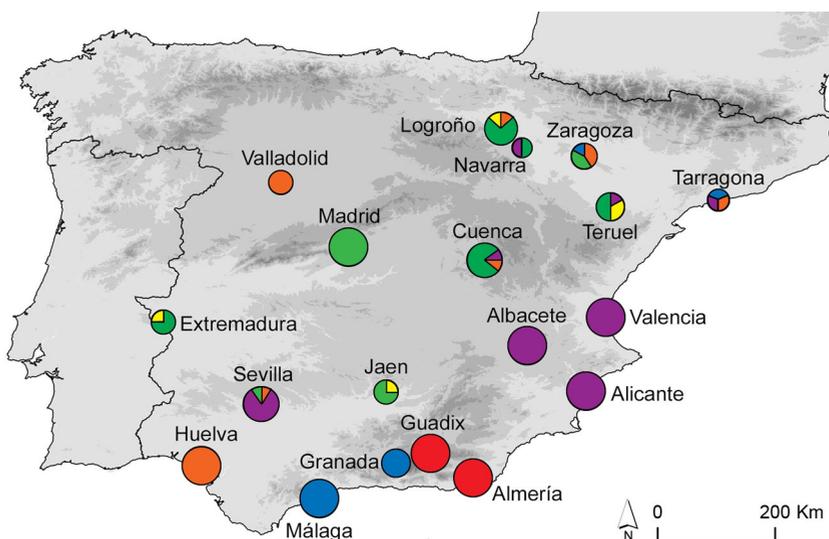


Figure 1. Map showing the sampling locations. At each locality, pie charts indicate the proportion of the population belonging to each of the six identified lineages from fig. 2. Size of the pie charts corresponds to the number of individuals sampled.

Table 1. Indicating the localities sampled, the code numbers employed in the figures, and the number of individuals included per locality.

Code (#)	Municipality	Province	Longitude	Latitude
ALM (10)	Almería	Almería	02° 18.4'W	36° 50.0'N
GUA (10)	La Calahorra	Granada	03° 01.6'W	37° 15.7'N
DON (10)	Almonte	Huelva	06° 31.5'W	36° 59.4'N
MAL (10)	Marbella	Málaga	04° 44.9'W	36° 29.2'N
ALB (10)	C. de Montearagón	Albacete	01° 28.9'W	38° 53.7'N
ALC (10)	Santa Pola	Alicante	00° 35.6'W	38° 11.4'N
MAD (10)	Chapinería	Madrid	04° 13.6'W	40° 22.7'N
VLC (7)	Valencia	Valencia	00° 18.7'W	39° 20.6'N
JAÉ (4)	Linares	Jaén	03° 40.1'W	38° 09.5'N
SEV (9)	S. Nicolás del Puerto	Sevilla	05° 37.0'W	37° 57.8'N
GRA (6)	Monachil	Granada	03° 31.4'W	37° 05.1'N
ZAR (5)	Leciñena	Zaragoza	00° 39.2'W	41° 49.4'N
TER (6)	Alcañiz	Teruel	00° 14.4'W	41° 03.8'N
NAV (2)	Ablitas	Navarra	01° 36.2'W	41° 57.3'N
LOG (8)	Alfaro	La Rioja	01° 51.5'W	42° 12.4'N
VLD (5)	Castroño	Valladolid	05° 15.8'W	41° 24.4'N
EXT (4)	Alburquerque	Badajoz	07° 02.4'W	39° 15.9'N
CUE (9)	Sotorribas	Cuenca	02° 11.1'W	40° 11.6'N
TAR (3)	Torredembarra	Tarragona	01° 26.1'E	41° 09.3'N

TrN model. BI was implemented in MrBayes v3.2.6 (Ronquist and Huelsenbeck, 2001), and run for one hundred million generations, with convergence confirmed using TRACER 1.6 (Rambaut, Suchard and Drummond, 2014). After 25% were discarded as burn-in, remaining trees were combined in a majority rule consensus tree (fig. 2). Uncorrected p -distances within and between lineages were calculated using MEGA6 (Tamura et al., 2013). New sequences were submitted to GenBank (accession numbers MH924624 to MH924761). To estimate the age of divergence between lineages we performed two separate analyses in BEAST (Drummond et al., 2012), with divergence rates from another lacertid lizard, *Podarcis* (Pinho, Harris and Ferrand, 2007), using separately the faster and slower rates proposed (1.74% per My and 2.78%, resulting in mean rates of 0.0087 and 0.0139 respectively, standard deviation of 0.005 in both cases). A strict molecular clock was applied with a lognormal distribution prior, with a HKY model of evolution, and 200,000 MCMC generations.

Considerable genetic diversity within *A. erythrurus* was identified, with six distinct and well-supported lineages (fig. 2, A-F), separated

by between 5.1% (B-C) and 9.8% (A-B and A-C) of genetic distance. Lineage A was the most differentiated (between 8.5% and 9.8%), while other than (B-C) all other lineages were separated to a similar degree (6.7% to 8.1%). Support levels for relationships between the lineages are low (less than 95% BPP), except for a sister taxa relationship between lineages B and C. Low support levels may be due to the use of only a single, relatively short, mtDNA fragment or may indicate that the lineages diverged at similar times. All six lineages occur in populations in the south of the Iberian Peninsula, with five of them also occurring in the central and northern regions. Our dating approach, while only providing a very rough estimate, is not incongruent with previous studies. Tamar et al. (2016) estimated that the *A. erythrurus* group started to differentiate around 7.9 Mya (5.4–11), so that divergence within the Iberian Peninsula should be younger than this. The most closely related lineages (B–C) are estimated to have diverged 1.7–2.7 Mya (depending on the rate used), while the oldest separation (estimated in BEAST to be A versus all other lineages) is estimated at 4.11 to 6.59 Mya. The lineages gen-

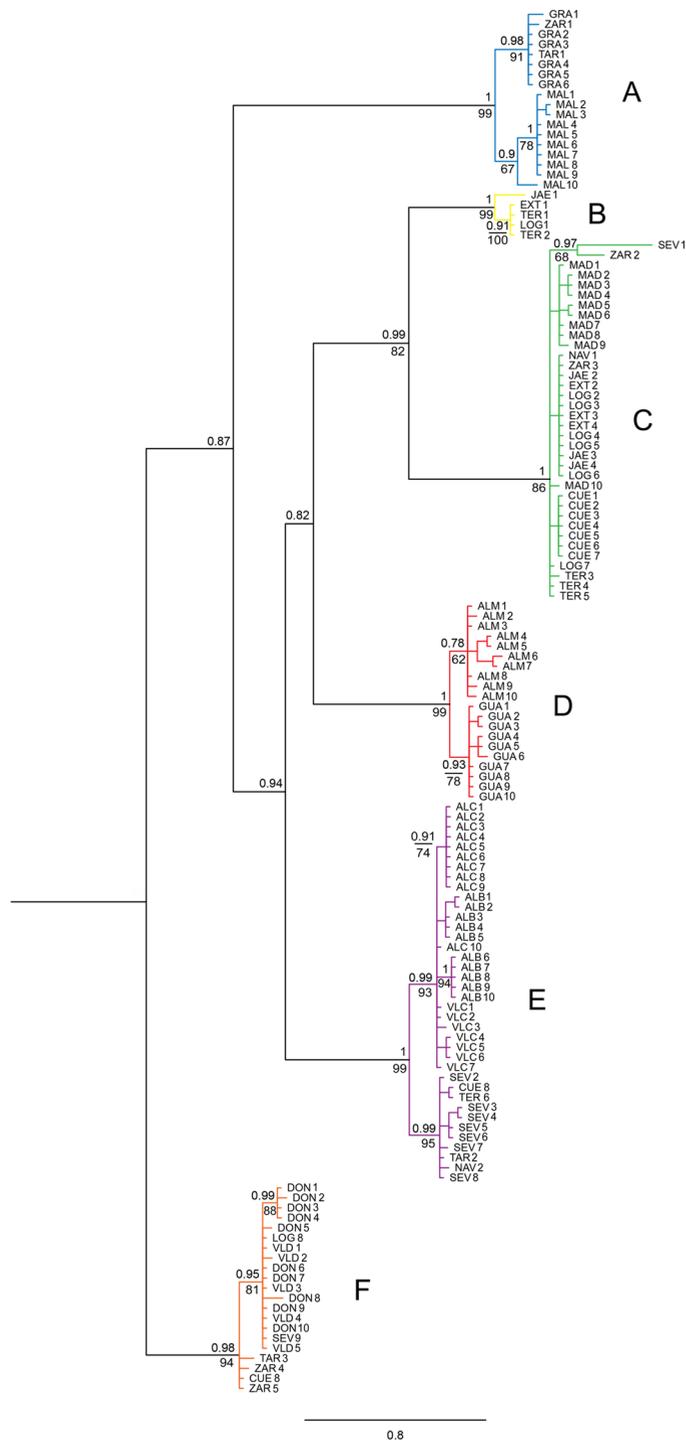


Figure 2. Tree derived from Bayesian analyses of partial ND4 mitochondrial DNA sequences. The outgroup sequences have been removed purely for visual purposes. Bayesian posterior probabilities are shown above corresponding nodes, and ML bootstrap support below them. Colours are representative of each of the six lineages, and codes correspond to localities in fig. 1 and table 1.

erally can therefore be considered as having a Pliocenic origin.

While the existence of high levels of diversity between populations is expected in the south, what is less usual is that several northern populations comprise individuals harbouring distinct mtDNA lineages. For example, within the populations from Almería and Guadix, all 20 individuals have haplotypes from lineage D. On the other hand, the northern population of Zaragoza, with just five individuals sampled, has haplotypes from lineages A, E and F. Because of this, although the southern region includes all the diversity, single populations from the north have greater diversity than populations in the south.

Across Europe as a whole, two paradigms of genetic diversity are widely cited, that of “hotspots”, with southern Peninsulas harbouring much greater diversity than northern regions, and “melting pots” in the North, where divergent southern lineages have expanded and met in Northern regions, leading to some northern populations to be highly diverse (Petit et al., 2003). While many studies have indicated that multiple different refugia existed within each southern Peninsula for many different taxa, the identification of a similar pattern of “melting pots” within these regions is much less common. The highly divergent lineages identified within other reptiles such as Iberian *Podarcis* spp. for example very rarely are found in the same populations, and then typically only in limited contact zone areas (Kaliontzopoulou et al., 2011). The situation within *A. erythrurus*, in which mitochondrial haplotypes from many different distinct southern Iberian populations have become mixed in northern Iberian populations, presumably during the post-glacial colonization of these northern regions, seems to be very different from most other Iberian lizards.

This new biogeographic pattern identified is important for several reasons. Firstly, the mixing of mitochondrial haplotypes in northern

populations is a strong indication that the divergent lineages are exchanging genes freely, although this would need to be confirmed through analysis of nuclear markers. This is important, as the degree of mtDNA divergence – from 5.0% between lineages B and C, up to 9.9% between A and B – is as high or even higher than between some closely related lacertids that are currently considered as distinct species. Recent interspecific assessments using the same marker for *Acanthodactylus* species from Iran report values of 10% and higher between species (Heidari et al., 2014). Divergences of 5.5–16% between lineages within *Atlantolacerta andreanskyi* were considered indicative of a species complex (Barata, Carranza and Harris, 2012). Diversity is lower than this (5%) within *Zootoca vivipara*, which has recently been proposed to consist of two distinct species (Cornetti et al., 2015), and is also much higher than that reported between Iberian mountain lizards of the genus *Iberolacerta* (Carranza, Arnold and Amat, 2004). The finding of multiple divergent mtDNA lineages within a single population highlights the need for caution when using similar levels of mtDNA divergence as evidence in the description of other lizard species. This kind of pattern of admixture in northern populations will only be identified if larger numbers of individuals per population are examined. It may be that the biogeographic pattern is more common than currently realized, simply due to limited sampling in many phylogenetic studies. Finally, *A. erythrurus* can be an excellent model for studying the effects on populations that originate from mixed divergent lineages. By further analysing multiple nuclear markers, any evidence for hybrid advantage, or disadvantage, can be detected. At the same time, with additional markers it would be possible to estimate ancestral areas for the lineages, and how these have spread.

To conclude, *A. erythrurus* demonstrates six distinct mtDNA lineages within the Iberian Peninsula. These show the expected pattern of diversity between populations in the south of

this region, but unexpectedly demonstrate considerable admixture in northern populations, thus mimicking a recognized European pattern of southern hotspots, northern melting pots but within a single southern peninsula. This has therefore the potential to be a useful model for investigating various phylogeographic hypotheses.

Acknowledgements. We thank J. Calatayud, C. de la Cruz, F. Escanero, T. González, F. Molina, L.M. Platero, M. Sempere, J.J. Soler and C. Zaldívar for their help with fieldwork, and R. Antón, A. Bergerandi, M.C. Díaz, A. Gosá, J. Guerrero, F. Martín, C. Navarro, J. Pleguezuelos, M.A. Romeo and F. Ugía for providing information on lizard populations. Thanks to Joana Santos and Daniela Rosada for their help with the figures and analyses, and J. Seabra-Babo and J. Tavares for help in the laboratory. Permission for capturing lizards and collecting samples was provided by the Dirección General de Gestión del Medio Natural, Junta de Andalucía; Instituto Aragonés de Gestión Ambiental, Gobierno de Aragón; Departamento de Desarrollo Rural y Medio Ambiente, Gobierno de Navarra; Dirección General de Medio Natural, Gobierno de La Rioja; Dirección General de Gestión del Medio Natural, Generalitat Valenciana; Servicio Territorial de Medio Ambiente, Junta de Castilla y León; Direcció General de Medi Natural i Biodiversitat, Generalitat de Catalunya; Dirección General del Medio Natural, Junta de Extremadura; Consejería de Medio Ambiente y Ordenación del Territorio, Comunidad de Madrid; and Organismo Autónomo Espacios Naturales de Castilla-La Mancha. DJH was funded through an IF-FCT contract (IF/01627/2014) under the Programa Operacional Potencial Humano – Quadro de Referência Estratégico Nacional funds from the European Social Fund and the Portuguese Ministério da Educação e Ciência. JB and JJC were funded by the European Regional Development Fund and the Spanish Ministerio de Educación y Ciencia (grant CGL2008-00137/BOS).

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Submitted: March 12, 2018. Final revision received: August 20, 2018. Accepted: October 2, 2018.
Associate Editor: Sylvain Dubey.