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When cryptic diversity blurs the picture: a cautionary tale from Iberian and North African *Podarcis* wall lizards

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Evolutionary inference based on molecular phylogenetic methods has profoundly modified the way that we understand biological diversity, unravelling a higher evolutionary diversity than previously considered. An exemplary case of this is the group of Iberian and North African *Podarcis* wall lizards. More investigated than any other reptile group in Europe, the *Podarcis hispanica* species complex comprises unexpectedly high levels of phylogenetic diversity and illustrates how the discovery of further cryptic diversity may entangle evolutionary inference. In the present study, we report on the discovery of two new mitochondrial lineages in this species complex, reassess the phylogeny of the group, infer the age of major phylogenetic splits, and provide a detailed description of the geographical distributions of all known mitochondrial DNA lineages. Our data show that the differentiation of major lineages is older than previously considered, in most cases predating the Messinian salinity crisis. The new lineages discovered and their position in the phylogeny of the group profoundly modify previous biogeographical scenarios, clearly showing that the area today corresponding to the south-eastern corner of the Iberian Peninsula is a very important centre of diversification. The dating obtained for the differentiation of the lineages currently inhabiting this area coincides with the complex geological events that took place during the Miocene/Pleistocene transition, supporting the idea that both land movements and dramatic climatic oscillations during that period could be involved. Finally, the discovery of these new lineages, together with the observed distribution patterns, not only further augments the uncertainty associated to our understanding of the evolutionary history of this group of lizards, but also points to new areas of interest for future investigation. *Biological Journal of the Linnean Society*, 2011, **103**, 779–800.

ADDITIONAL KEYWORDS: biogeography – distribution – Lacertidae – mtDNA – phylogeny – species complex.

INTRODUCTION

The extensive application of molecular phylogenetics for the investigation of biological patterns and processes has profoundly modified the way that we study and understand organismal diversity. Traditionally, organisms were classified into groups based on their phenotypic (usually morphological) properties and the relationships between such groups were inferred on the basis of phenotypic similarity. Evolutionary scenarios were then built on these inferences, trying to explain how biological diversity emerges and is distributed across different temporal and geographical scales. The arrival of molecular phylogenetics supplied a new way of inferring evolutionary relationships, with the advantages of a larger number of unambiguous characters available, the ease and

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higher speed of data acquisition, and the suitability of molecular data for analysis using transformational models (Scotland, Olmstead & Bennett, 2003). Among a wide range of applications, the molecular phylogenetics approach is commonly used to infer the degree of evolutionary relatedness between populations, species or higher-order taxa, aiming to describe how biological diversity is distributed geographically (Kidd & Ritchie, 2006), to relate these distributions to major geological events, and to attempt to understand the evolutionary processes that led to the spatial patterns of biodiversity that we observe today (Wiens & Donoghue, 2004). Additionally, molecular phylogenetics are also widely used for species delimitation in systematics, preferably in combination with other biological evidence (Wiens & Servedio, 2000; Wiens, 2007).

In parallel to the above-mentioned direct applications, the use of molecular phylogenetics has also changed our quantitative notion of organismal diversity. The change of framework from phenotypic to molecular characters was naturally followed by the discovery of cases of discordance between both approaches, most commonly towards identifying higher levels of molecular diversity than previously described on a morphological basis. This has led to an explosion of description of cryptic diversity and species complexes, when organisms that are morphologically very similar and thus classified as a single taxonomic unit are shown to be evolutionarily divergent on the basis of molecular evidence (Beheregaray & Caccone, 2007; Bickford *et al*., 2007). On the 'cryptic' side, such cases have served as paradigms in the study of morphological evolution, by bringing to light processes previously considered to be scarce or secondary, such as phenotypic convergence, stasis or plasticity (Sáez & Lozano, 2005; Bickford *et al*., 2007). On the 'diversity' side, however, we may have failed to fully appreciate the consequences of documenting a mismatch between the human sensory machine and the actual biological units operating. The discovery of unexpected levels of diversity should be treated with caution because it inflates the level of uncertainty for all biological questions considered. However, the description of new cryptic diversity should be taken not as an obstacle but rather as an opportunity to augment our understanding of how species complexes evolve and formulate new hypotheses and detect fascinating areas of interest for future investigation.

The *Podarcis hispanica* (Steindachner, 1870) species complex (Squamata; Lacertidae) is archetypal of this flux in the perception of biological diversity. This group of wall lizards has been more studied from a phylogenetic and phylogeographical perspective than almost any other reptile group in Europe (Camargo, Sinervo & Sites, 2010). Traditionally, two species have been recognized in this complex (Arnold & Ovenden, 2002): *Podarcis bocagei* (Seoane, 1884), in westernmost Iberia, and *P. hispanica*, inhabiting all of the Iberian Peninsula and North Africa. Early studies on mitochondrial (mt)DNA indicated that Iberian and North African *Podarcis*, with the exception of *Podarcis muralis* Laurenti, 1768 from Northwest Iberia, form a monophyletic clade (Harris & Arnold, 1999; Oliverio, Bologna & Mariottini, 2000). Later assessments of mtDNA variation uncovered high levels of differentiation, which, when combined with morphological differences, led to the elevation of two forms to species level: *Podarcis carbonelli* Pérez-Mellado, 1981 (Harris & Sá-Sousa, 2001, 2002; Harris, 2002) and *Podarcis atrata* (Boscá, 1916) from the Columbretes islands (Castilla *et al*., 1998a; Sá-Sousa & Harris, 2002), and further defined the ranges of these species (Harris *et al*., 2002a). This left *P. hispanica* as a paraphyletic assemblage of distinct genetic lineages (Harris & Sá-Sousa, 2002). The inclusion of North African specimens further highlighted genetic diversity in this region (Harris *et al*., 2002b), leading to the recognition of *Podarcis vaucheri* (Boulenger 1905) in North Africa and parts of Southern Spain (Busack, Lawson & Arjo, 2005). Subsequent phylogenetic assessments recovered even more hidden variation: first, a previously undescribed, highly divergent lineage was detected in south-eastern Spain (Pinho, Ferrand & Harris, 2006) and, subsequently, the assessment of Algerian populations revealed the existence of two new lineages (Lima *et al*., 2009), thereby increasing the number of 'forms' in North Africa to five.

At the same time as these phylogeographical scenarios were developed using mtDNA sequences, various nuclear makers were used to test for concordance in defining forms. Polymorphic allozyme loci were studied in over 500 individuals and corroborated to a great extent the major splits that are observed in mtDNA analyses (Pinho, Harris & Ferrand, 2003, 2007a). Similarly, analyses of nuclear DNA sequences indicated that, although considerable ancestral polymorphism persisted, the identified lineages were cohesive and could be considered as incipient species (Pinho, Harris & Ferrand, 2008). Using a combined morphological and genetic approach, Geniez *et al*. (2007) redefined and delimited *Podarcis hispanica hispanica* as a first step towards a taxonomic reassessment of the whole group and, subsequently, Renoult *et al*. (2010a) recognized *Podarcis liolepis* (Boulenger 1905) as the form in the Northeast Iberia, synonymizing *P. atrata* from the Columbretes islands. Despite the general concordance between mtDNA lineages and units delimited using nuclear markers or morphological characters, Pinho *et al*. (2007a), Pinho *et al*. (2008), and Renoult *et al*. (2009) identified cases of discordance and gene flow between forms in

south-east Iberia. In particular, Renoult *et al*. (2009) analyzed nuclear markers and morphological characters, which were used to identify three evolutionary units within this region, whereas analysis of mtDNA sequences recovered four. It was suggested that this was likely a result of ancient introgression originating from a fourth evolutionary unit, either unsampled or now extinct.

In the present study, we report on the discovery of two additional mtDNA lineages from south-eastern Spain within the *P. hispanica* species complex. We conduct a reassessment of the phylogenetic relationships of the group following the robust molecular scheme applied by Pinho *et al*. (2006), including sequences from five mtDNA gene regions. Additionally, we use relaxed molecular clocks to reassess the concordance of divergence between lineages with the known ages of geological events. We combine the results obtained with a detailed description of the geographical distributions of different lineages to re-evaluate previous scenarios of historical biogeography proposed for the group. Finally, we examine the results obtained by recent systematic studies in the light of this new evidence, aiming to evaluate how the discovery of further cryptic diversity may modify the biogeographical, systematic, and evolutionary hypotheses proposed and open the way to a renewed vision of the diversity observed in this group of lizards.

MATERIAL AND METHODS

The present study focuses on the distribution and phylogenetic relationships of mtDNA lineages of Iberian and North African *Podarcis*. Although the present analyses are based on a single genetic marker, potentially suffering from the limitations that are associated with such an approach (Zhang & Hewitt, 2003; Galtier *et al*., 2009), the extensive background information previously obtained for this particular system (i.e. general concordance with nuclear markers: Pinho *et al*., 2007a, 2008; morphology: Kaliontzopoulou, Carretero & Llorente, in press; behaviour and ecology: Carretero, 2008) enables us to draw important inferences from this study, regardless of any limitations.

Sampling and compilation of bibliographical sources

One of the goals of the present study was to more accurately describe the distribution of the various mtDNA lineages described in previous studies. Accordingly, we collected 205 new samples throughout the Iberian Peninsula and North Africa, focusing especially on regions where previous sampling was limited. Individual lizards were caught by hand, and the tip of the tail removed and stored in 100% ethanol. Specimens were then released at the site of capture. All the new samples used in the present study are described in Table 1. Additionally to the new sampling, we compiled all published data including mtDNA sequences from the Iberian and North African group of *Podarcis* wall lizards, for which detailed geographical information was available (Castilla *et al*., 1998a, b; Harris & Arnold, 1999; Oliverio *et al*., 2000; Harris & Sá-Sousa, 2001, 2002; Harris *et al*., 2002a, b; Carranza, Arnold & Amat, 2004; Busack *et al*., 2005; Pinho *et al*., 2006, 2007a, b, 2008; Renoult, 2006; Sanz-Azkue *et al*., 2006; Arntzen & Sá-Sousa, 2007; Lima *et al*., 2009; Renoult *et al*., 2009, 2010b), aiming to obtain a complete image of the distribution of existing lineages.

DNA extraction, mitochondrial DNA sequencing, and lineage assignment

DNA was extracted using the Qiagen DNeasy tissue kit. Because mtDNA lineages are highly divergent, obtaining the sequence from part of a single mtDNA gene or region is sufficient for the unambiguous assignment of individuals to a known mtDNA lineage. Therefore, as a standard procedure, we amplified and sequenced a portion of the 12S ribosomal RNA (rRNA) region in at least one of the samples collected from each locality. In a small minority of cases, other genes were sequenced instead of 12S rRNA to perform this assignment (Table 1).

Although lineage assignment does not require a large amount of sequence data, establishing a well-resolved phylogenetic tree does (Pinho *et al*., 2006). The preliminary analysis of 12S rRNA sequences suggested that some samples might belong to previously undescribed mtDNA lineages (see below). To confirm this hypothesis and to evaluate the placement of such new samples in the phylogenetic tree, we additionally obtained partial sequences of four other mitochondrial DNA regions [partial 16S rRNA, control region, NADH dehydrogenase subunit 4 (ND4) and adjacent tRNAs, and cytochrome *b*] in some of the samples. In addition, we completed the same five-region dataset in three individuals that had been included in a previous study reporting novel lineages in North Africa (Lima *et al*., 2009), which had been previously analyzed only for 12S rRNA and ND4. This five-region data set was compiled for a total of nine new individuals to combine with the dataset reported by Pinho *et al*. (2006) and thus obtain a robust and updated view on the phylogeny of mtDNA lineages in this system. In all of the above analyses, primers and amplification conditions strictly followed those given in Pinho *et al*. (2006), with the exception of the ND4

Table 1. Accession numbers of the sequences produced for this study. Accession numbers of the remaining sequences incorporated in the phylogenetic analyses can be found in Pinho *et al.* (2006)

Sample code	Mitochondrial DNA lineage	Locality	Region	Country	Genbank accession numbers				
					12S rRNA	16S rRNA	Control region	Cytochrome *b*	ND4
3.296	PB	Castro Laboreiro	Viana do Castelo	Portugal	HQ898061				
3.302	PB	Castro Laboreiro	Viana do Castelo	Portugal	HQ898062				
Gi30	PB	Gião	Porto	Portugal					HQ898004
DB8665	PB	Maia	Porto	Portugal	HQ898063				
3.1383	PB	Mindelo	Porto	Portugal	HQ898064				
3.1457	PB	Mindelo	Porto	Portugal	HQ898065				
3.292	PB	Palacios del Compludo	León	Spain	HQ898066				
3.295	PB	Palacios del Compludo	León	Spain	HQ898067				
DB8760	PB	Permedelos, Vila Verde	Braga	Portugal	HQ898068				
3.175	PB	São Mamede do Coronado	Porto	Portugal	HQ898069				
3.211	PB	São Mamede do Coronado	Porto	Portugal	HQ898070				
3.223	PB	Subportela	Viana do Castelo	Portugal	HQ898071				
3.253	PB	Subportela	Viana do Castelo	Portugal	HQ898072				
DB4292	PB	Torneros de la Valdería	León	Spain	HQ898073				
4.159	PC	El Acebuche	Huelva	Spain	HQ898074				
4.176	PC	El Acebuche	Huelva	Spain	HQ898075				
DB9670	PC	S. Jacinto	Aveiro	Portugal	HQ898076				
5.143	PH1A	Alvão NP, next to dumm	Vila Real	Portugal	HQ898077				
DB8653	PH1A	Barrocal do Douro	Bragança	Portugal	HQ898078				
DB8409	PH1A	Celanova	Ourense	Spain	HQ898079				
DB8671	PH1A	Chavães	Porto	Portugal	HQ898080				
DB8398	PH1A	Chelos, Gaia	Porto	Portugal	HQ898081				
DB8609	PH1A	Cidadelhe	Guarda	Portugal	HQ898082				
DB1734	PH1A	Crestuma Castle	Porto	Portugal	HQ898083				
DB1730	PH1A	Fornillos (de Aliste)	Zamora	Spain	HQ898084				
DB8322	PH1A	Gerês	Braga	Portugal	HQ898085				
5.247	PH1A	Ledesma	Salamanca	Spain	HQ898086				
5.259	PH1A	Ledesma	Salamanca	Spain	HQ898087				
DB8669	PH1A	Lourosa	Porto	Portugal	HQ898088				
DB8411	PH1A	Murça	Vila Real	Portugal	HQ898089				
DB1751	PH1A	Near Sta. Eulalia	Zamora	Spain	HQ898090				
DB1763	PH1A	Near Sta. Eulalia	Zamora	Spain	HQ898091				
DB8399	PH1A	Oliveira do Hospital	Coimbra	Portugal					HQ898005

DB1753	PH1A	Rio Casares	León	Spain	HQ898092		
DB1760	PH1A	Rio Casares	León	Spain	HQ898093		
DB1758	PH1A	Rio Negro, Peque	Zamora	Spain	HQ898094		
DB8612	PH1A	Serra d'Arga	Viana do Castelo	Portugal	HQ898095		
DB8400	PH1A	Sobreira (Chaves)	Vila Real	Portugal	HQ898096		
5.262	PH1A	Sta. Eulalia	Zamora	Spain	HQ898097		
DB8672	PH1A	Sto. Estevão	Vila Real	Portugal	HQ898098		
5.225	PH1A	Tudera	Zamora	Spain	HQ898099		
5.232	PH1A	Tudera	Zamora	Spain	HQ898100		
And3	PH1A	Vale do Rossim	Serra da Estrela	Portugal		HQ898054	
DB8401	PH1A	Vila Chã (Vale de Cambra)	Viana do Castelo	Portugal	HQ898101		
DB8403	PH1A	Vinhais	Bragança	Portugal	HQ898102		
DB8416	PH1A	Zamora	Zamora	Spain	HQ898103		
5.203	PH1B	Alba de Tormes	Salamanca	Spain	HQ898104		
DB8614	PH1B	Arévalo	Ávila	Spain	HQ898105		
DB8461	PH1B	Bejar	Salamanca	Spain	HQ898106		
5.194	PH1B	Ciudad Rodrigo	Salamanca	Spain	HQ898107		
5.198	PH1B	Ciudad Rodrigo	Salamanca	Spain	HQ898108		
DB8621	PH1B	El Piornal	Cáceres	Spain	HQ898109		
DB8615	PH1B	Las Ventas c/Peña Aguilera	Toledo	Spain	HQ898110		
DB8903	PH1B	Torrejon de la Calzada	Madrid	Spain	HQ898111		
6.161	PH2	Albacete city	Albacete	Spain	HQ898112		
DB9647	PH2	Almoster	Santarém	Portugal	HQ898113		
DB2862	PH2	Area Recreativa de Gil Cobo	Jaén	Spain	HQ898114		
DB2871	PH2	Area Recreativa de Gil Cobo	Jaén	Spain	HQ898115		
DB2911	PH2	Area Recreativa de Gil Cobo 2	Jaén	Spain	HQ898116		
DB1779	PH2	Area Recreativa de los Estrechos	Toledo	Spain	HQ898117		
PH76	PH2	Arroyo Brezoso	Castilla la Mancha	Spain	HQ898118		
DB1728	PH2	Arroyo de la Luz	Cáceres	Spain	HQ898119		
PH50	PH2	Arroyo del Chorro – Los Navalucillos	Toledo	Spain	HQ898120		
PH80	PH2	Balazote	Albacete	Spain	HQ898121		
DB9667	PH2	Casar de Cáceres	Cáceres	Spain	HQ898122		
DB9669	PH2	Castanheira de Pera	Leiria	Portugal	HQ898123		
DB2642	PH2	Cobeta	Guadalajara	Spain	HQ898124		
6.128	PH2	Cornalvo NP	Badajoz	Spain	HQ898125		

Table 1. *Continued*

Sample code	Mitochondrial DNA lineage	Locality	Region	Country	Genbank accession numbers				
					12S rRNA	16S rRNA	Control region	Cytochrome *b*	ND4
DB1776	PH2	Cortijo de Angelita	Jaén	Spain	HQ898126				
DB1769	PH2	Cortijo de los Petrolos	Jaén	Spain	HQ898127				
DB1778	PH2	Cortijo de los Petrolos	Jaén	Spain	HQ898128				
DB1783	PH2	Cortijo El Maguillo	Jaén	Spain	HQ898129				
DB1736	PH2	Cueva del Santillo	Jaén	Spain	HQ898130				
PH87	PH2	El Chorro (Cabañeros NP)	Toledo	Spain	HQ898131				
DB9676	PH2	El Laminador, Sierra de Aljubar	Albacete	Spain	HQ898132				
DB1837	PH2	Fuente de Cueva Ahumada	Albacete	Spain	HQ898133				
DB1787	PH2	Fuente del Macho	Jaén	Spain	HQ898134				
PH55	PH2	Fuente Nueva – Villarubia de Santiago	Toledo	Spain	HQ898135				
PH53	PH2	Fuente Vieja – Villarubia de Santiago	Toledo	Spain	HQ898136				
PH89	PH2	Fuertescusa	Cuenca	Spain	HQ898137				
PH95	PH2	La Roda	Albacete	Spain	HQ898138				
DB1862	PH2	Laguna de Arroyofrío	Albacete	Spain	HQ898139				
PH91	PH2	Lagunas de la Ruidera	Albacete	Spain	HQ898140				
DB8905	PH2	Louriçal	Leiria	Portugal					HQ898006
DB2641	PH2	Mazarete	Guadalajara	Spain	HQ898141				
DB9607	PH2	Monte Real	Leiria	Portugal	HQ898142				
PH66	PH2	Ocaña	Toledo	Spain	HQ898143				
DB9603	PH2	Olmeda de Cobeta	Guadalajara	Spain	HQ898144				
DB8904	PH2	Ourém	Santarém	Portugal					HQ898007
DB1876	PH2	Palacio Gosalvez, Villalgordo del Júca	Albacete	Spain	HQ898145				
DB1877	PH2	Palacio Gosalvez, Villalgordo del Júca	Albacete	Spain	HQ898146				
DB1781	PH2	Peña del Olivar	Jaén	Spain	HQ898147				
DB1828	PH2	Piedra de los Endrinales	Albacete	Spain	HQ898148				
DB2960	PH2	Rio Borosa (La Iruela)	Jaén	Spain	HQ898149				

PH49	PH2	Rio Estena Hontanar	Toledo	Spain	HQ898150					
PH52	PH2	Rio Frio – Sevilleja de la Jara	Toledo	Spain	HQ898151					
DB1890	PH2	Río Linares- Riba de Saelices	Guadalajara	Spain	HQ898152					
6.313	PH2	Riopar el Viejo	Albacete	Spain	HQ898153					
DB9658	PH2	S. Pedro de Moel	Leiria	Portugal	HQ898154					
PH72	PH2	Saelices	Albacete	Spain	HQ898155					
DB2866	PH2	Sierra de Segura, 5 km W of Embalse del Tranco	Jaén	Spain	HQ898156					
DB2785	PH2	SW of Embalse del Tranco	Jaén	Spain	HQ898157					
DB2846	PH2	SW of Embalse del Tranco	Jaén	Spain	HQ898158					
PH98	PH2	Valencia del Ventoso	Badajoz	Spain	HQ898159					
6.317	PH2	Villanueva de Córdoba	Córdoba	Spain	HQ898160					
6.320	PH2	Villanueva de Córdoba	Córdoba	Spain	HQ898161					
6.36	PH2	Virgen de la Cabeza, Andujar	Jaén	Spain	HQ898162					
DB1817	PHAM	Camino del Tobalejo	Albacete	Spain	HQ898184					
9.76	PHAM	Cañada del Provencio	Albacete	Spain	HQ898185					
9.77	PHAM	Cañada del Provencio	Albacete	Spain	HQ898186					
DB1841	PHAM	El Pardal	Albacete	Spain	HQ898187					
DB1878	PHAM	Montealegre del Castillo	Albacete	Spain	HQ898188					
DB3861	PHAM	Sierra de Callosa del Segura	Alicante	Spain	HQ898189					
9.79	PHAM	Sierra de la Oliva	Albacete	Spain	HQ898190	HQ898056	HQ898046	HQ898037	HQ898010	
9.89	PHAM	Sierra de la Oliva	Albacete	Spain	HQ898191					
DB1285	PHAM	Sierra de la Pila	Murcia	Spain	HQ898192	HQ898057	HQ898047	HQ898038	HQ898011	
DB1286	PHAM	Sierra de la Pila	Murcia	Spain	HQ898193					
Aza879	PHAza	Azazga	Tizi Ouzou	Algeria	GQ856131*	HQ898052	HQ898043	HQ898034	GQ856106*	
Aza881	PHAza	Azazga	Tizi Ouzou	Algeria	GQ856132*	HQ898053	HQ898044	HQ898035	GQ856107*	
Ham1	PHBat	Hamla	Batna	Argelia	GQ856127*	HQ898051	HQ898042	HQ898033	GQ856102*	
9.68	PHGal	Caravaca de la Cruz	Murcia	Spain	HQ898173					
9.60	PHGal	Cartagena	Murcia	Spain	HQ898174	HQ898055	HQ898045	HQ898036	HQ898008	
9.64	PHGal	Cartagena	Murcia	Spain	HQ898175					
DB3841	PHGal	Embalse de La Pedrera	Almeria	Spain	HQ898176					
DB3849	PHGal	Embalse de La Pedrera	Almeria	Spain	HQ898177					
DB8647	PHGal	Láujar de Andarax	Almeria	Spain	HQ898178					

Table 1. *Continued*

Sample code	Mitochondrial DNA lineage	Locality	Region	Country	Genbank accession numbers				
					12S rRNA	16S rRNA	Control region	Cytochrome *b*	ND4
DB3851	PHGal	Rambla del Cañar-Cartagena	Murcia	Spain	HQ898179				
DB2961	PHGal	Rio Castril river source	Granada	Spain	HQ898180				
9.10	PHGal	Sierra de Espuña	Murcia	Spain	HQ898181				
9.7	PHGal	Sierra de Espuña	Murcia	Spain	HQ898182				
DB1663	PHJS	Road to Jbel Siroua	Taroudannt	Morocco	HQ898183				
DB11031	PHJS	Tizi-n′-Melloul	Ouarzazate	Morocco					HQ898009
DB1791	PHSS	500 m from Los Negros Camping	Jaén	Spain	HQ898194				
DB1879	PHSS	Barranco de Guadalentín	Jaén	Spain	HQ898195				
DB1899	PHSS	Barranco de Guadalentín	Jaén	Spain	HQ898196				
9.22	PHSS	Boniche	Cuenca	Spain	HQ898197				
DB8646	PHSS	Bunyol	Valencia	Spain	HQ898198				
DB1834	PHSS	Calar de Mundo	Albacete	Spain	HQ898199				
9.8	PHSS	Castillo de la Calahora	Granada	Spain	HQ898200				
10.45	PHSS	Cazorla, Nava de San Pedro	Jaén	Spain	HQ898201				
10.53	PHSS	Cazorla, Nava de San Pedro	Jaén	Spain	HQ898202				
CU	PHSS	Ciudad Encantada	Cuenca	Spain	HQ898203				
DB8630	PHSS	Cortijo Becerra, Guadix	Granada	Spain	HQ898204				
DB1887	PHSS	Fte la Reina	Jaén	Spain	HQ898205				
DB3167	PHSS	Fte la Reina	Jaén	Spain	HQ898206				
DB1898	PHSS	Fuente de la Garganta	Jaén	Spain	HQ898207				
DB1735	PHSS	Guadalquivir river source	Jaén	Spain	HQ898208				
DB1748	PHSS	Guadalquivir river source	Jaén	Spain	HQ898209				
B2	PHSS	La Casella, Alzira	Valencia	Spain	HQ898210				
DB3053	PHSS	Pico Cabañas	Jaén	Spain	HQ898211				
DB8628	PHSS	Puebla del Salvador	Cuenca	Spain	HQ898212				
DB3857	PHSS	Revolcadores	Murcia	Spain	HQ898213				

DB1853	PHSS	Rio Madera	Jaén	Spain	HQ898214						
DB3858	PHSS	Sierra Espuña-Zona Pozos de Nieve	Murcia	Spain	HQ898215						
B3	PHSS	St Esperit, Gilet Sagunt	Valencia	Spain	HQ898216						
B4	PHSS	St Esperit, Gilet Sagunt	Valencia	Spain	HQ898217						
DB1895	PHSS	Venta Benito	Jaén	Spain	HQ898218						
9.28	PHSS	Xátiva	Valencia	Spain	HQ898219						
9.29	PHSS	Xátiva	Valencia	Spain	HQ898220						
8.553	PHTA	Cap Negro	Jendouba	Tunisia							HQ898012
8.480	PHTA	Feidja NP	Jendouba	Tunisia							HQ898013
DB1595	PHTA	Jbel Goraa	Teboursouk	Tunisia	HQ898221						
DB1625	PHTA	Jbel Goraa	Teboursouk	Tunisia	HQ898222						
DB1716	PL	Aiguamolls del Empordà	Girona	Spain	HQ898163						
DB9604	PL	Alcolea del Pinar	Guadalajara	Spain	HQ898164						
DB8664	PL	Bordils	Girona	Spain	HQ898165						
1.15	PL	Calomarde	Aragón	Spain	HQ898166						
DB8613	PL	Castrillo de la Vega	Burgos	Spain	HQ898167						
DB1762	PL	Les Solans	Barcelona	Spain	HQ898168						
DB1731	PL	Monasterio de Moreruela	Zamora	Spain	HQ898169						
DB8605	PL	Near Sopeira	Huesca	Spain	HQ898170						
1.113	PL	Rio Segre	Lleida	Spain	HQ898171						
DB1732	PL	Torredembarra	Tarragona	Spain	HQ898172						
DB5048	PVMA	Mines	Al Haouz	Morocco							HQ898024
DB9130	PVMA	Beni Amint	Taza	Morocco							HQ898025
DB1449	PVMA	Ceuta	Ceuta	Spain							HQ898026
DB8843	PVMA	Ifrane	Ifrane	Morocco							HQ898027
8.252	PVMA	Imlil	Al Haouz	Morocco							HQ898028
DB5042	PVMA	Jebel Owlime	Taroudannt	Morocco							HQ898029
DB5040	PVMA	Road Tadert/Tizin Tichka	Al Haouz	Morocco							HQ898030
DB1611	PVMA	road to Jbel Siroua	Taroudannt	Morocco	HQ898228						
DB5087	PVMA	Talzemt	Boulemane	Morocco							HQ898031
8.377	PVMA	Tislit Lake	Errachidia	Morocco							HQ898032
DB1047	PVMA	Tizi-n-Tleta	Taroudannt	Morocco	HQ898229						
DB1098	PVMA	Tizi-n-Tleta	Taroudannt	Morocco	HQ898230						
8.80	PVSCSp	Alcalá la Real	Jaén	Spain	HQ898231	HQ898059	HQ898049	HQ898040	HQ907939		
8.83	PVSCSp	Alcalá la Real	Jaén	Spain	HQ898232	HQ898060	HQ898050	HQ898041	HQ907940		

Table 1. *Continued*

Sample code	Mitochondrial DNA lineage	Locality	Region	Country	Genbank accession numbers				
					12S rRNA	16S rRNA	Control region	Cytochrome *b*	ND4
DB1208	PVSCSp	Between Benalua de las Villas and Iznalloz	Jaén	Spain					HQ907941
8.104	PVSCSp	Jaén city	Jaén	Spain					HQ907942
DB1873	PVSCSp	Linares	Jaén	Spain	HQ898233				
DB2863	PVSCSp	Linares	Jaén	Spain	HQ898234				
DB2869	PVSCSp	Linares	Jaén	Spain	HQ898235				
DB1754	PVSCSp	Pradollano, Sierra Nevada	Granada	Spain	HQ898236				
8.59	PVSCSp	Santa Ana	Jaén	Spain	HQ898237				
DB1811	PVSSp	Bonanza	Cádiz	Spain	HQ898223				
DB51	PVSSp	Castillo de la Duquesa	Cádiz	Spain	HQ898224				
DB1251	PVSSp	Castro del Rio	Córdoba	Spain					HQ898014
DB1254	PVSSp	Castro del Rio	Córdoba	Spain					HQ898015
8.119	PVSSp	Córdoba city	Córdoba	Spain					HQ898016
DB1380	PVSSp	Granada city	Granada	Spain					HQ898017
DB1874	PVSSp	Linares	Jaén	Spain	HQ898225	HQ898058	HQ898048	HQ898039	HQ898018
8.26	PVSSp	Matalascañas	Huelva	Spain					HQ898019
DB446	PVSSp	Parque Nacional Los Alcornocales	Cádiz	Spain	HQ898226				
8.122	PVSSp	Peñarroya-Pueblonuevo	Córdoba	Spain					HQ898020
8.69	PVSSp	Playa de la Víbora	Málaga	Spain					HQ898021
DB3871	PVSSp	Sanlucar La Mayor	Sevilla	Spain	HQ898227				
DB1390	PVSSp	Sierra de Aljibe	Cádiz	Spain					HQ898022
8.57	PVSSp	Torcal de Antequera	Málaga	Spain					HQ898023

*Denotes previously published sequences (Lima *et al*., 2009).

Lineages: PB, *Podarcis bocagei*; PC, *Podarcis carbonelli*; PH1A, *Podarcis hispanica* type 1A; PH1B, *P. hispanica* type 1B; PH2, *P. hispanica* type 2; PHAM, *P. hispanica* Albacete/Murcia; PHAza, *P. hispanica* Azazga; PHBat, *P. hispanica* Batna; PHGal, *P. hispanica* Galera; PHJS, *P. hispanica* Jbel Siroua; PHSS, *P. hispanica s.s.*; PHTA, *P. hispanica* Tunisia/north-east Algeria; PL, *Podarcis liolepis*; PVMA, *Podarcis vaucheri* Morocco/Algeria; PVSCSp, *P. vaucheri* southern-central Spain; PVSSp, *P. vaucheri* southern Spain. For the phylogenetic position and geographical distribution of each lineage, see Figs 1, 2. ND4, NADH dehydrogenase subunit 4.

region in sample 9.60, belonging to a lineage detected in south-eastern Spain ('Galera' lineage: Pinho *et al*., 2007a, 2008), *P. hispanicus sensu* Renoult *et al*. (2009)]. To avoid amplification of a nuclear pseudogene similar to ND4, which is known to exist in this lineage (Pinho *et al*., 2006), we used the primers GalND4F and GalND4R (Pinho *et al*., 2008), with similar conditions to those used for standard amplification of the ND4 locus, both for amplification and sequencing. Polymerase chain reaction products were purified enzymatically and sequenced in an ABI 3130xl Genetic Analyzer (Applied Biosystems). All new sequences have been deposited in GenBank (accession numbers are provided in Table 1).

Sequences were aligned manually using BIOEDIT, version 7.0.5.3 (Hall, 1999). As a general procedure, sequences were assigned to a particular lineage by observing clustering patterns in a Neighbour-joining tree (Saitou & Nei, 1987) constructed in MEGA, version 4.1 (Tamura *et al*., 2007).

Phylogenetic analysis

Although most samples examined were easily assigned to one of the previously known lineages based on a diagnostic portion of the 12S rRNA, the survey also revealed divergent samples from previously unsampled geographical areas. These were further investigated using partial sequences of a total of five mitochondrial gene regions (Pinho *et al*., 2006). Because the ND4 sequence for sample 9.60 was significantly shorter than the other samples' (as a result of its amplification with an internal set of primers), we excluded from the alignment the tRNAs that are adjacent to the ND4 gene. The final alignment thus included a total of 2291 bp (corresponding to 383 bp from 12S rRNA, 510 bp from 16S rRNA, 418 bp from the control region, 306 bp from cytochrome *b*, and 675 bp from ND4).

For the five-loci dataset, comprising a total of 41 individuals, namely the 32 individuals analyzed by Pinho *et al*. (2006) plus the nine newly sequenced samples, we used three different approaches to investigate evolutionary relationships: maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). For MP analyses, sequences were imported into PAUP* 4.0b10 (Swofford, 2002). Ten heuristic searches were performed using random sequence addition and tree bisection – recognition branch swapping. Gaps were treated as a fifth state. Nodal support was assessed by nonparametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates.

ML analyses (Felsenstein, 1981) were conducted using the method implemented in GARLI, version 0.96 (Zwickl, 2006), which simultaneously searches parameter space for tree topology, branch lengths, and substitution model parameters. On the basis of preliminary analyses using the Akaike information criterion in MODELTEST, version 3.06 (Posada & Crandall, 1998), we allowed the software to estimate parameters within the general time reversible model of sequence evolution, using a discrete approximation to the gamma model of among-site rate variation (with four rate categories) and an estimate of the proportion of invariant sites. GARLI runs were automatically terminated when no new significantly better scoring topology was encountered in 50 000 generations of the Markov chain Monte Carlo. Ten replicate searches were performed. Bootstrap support (Felsenstein, 1985) was evaluated by performing 1000 pseudoreplicates under the same conditions. Bayesian phylogenetic analyses were performed using MrBAYES, version 3.1.2 (Huelsenbeck & Ronquist, 2001). Because the choice of an appropriate partition strategy is expected to influence the outcome of phylogenetic estimates (Brown & Lemmon, 2007), we performed three separate runs: one assumed the same substitution model for the complete data set (unpartitioned analysis), and the remaining two employed different partition strategies, allowing for substitution models to vary among distinct character sets: (1) partition in the five mitochondrial segments and (2) partition in the five mitochondrial segments, plus each of the three codon positions of the two protein-coding genes (cytochrome *b* and ND4). Each run included two independent replicates. Runs started from randomly-generated trees and were sampled every 1000 generations along the Markov chain. Runs were allowed to proceed until convergence to the stationary distribution was accomplished and sufficient samples had been obtained after stationarity (between 20 and 30 million steps, depending on the run). This was assessed using AWTY (Wilgenbusch, Warren & Swofford, 2004; Nylander *et al*., 2008), which provides, among other measures, plots of the cumulative posterior probabilities for the different clades. Trees sampled before these measures stabilized (corresponding to 10–12 million first iterations along the Markov chain, depending on the partitioning strategy) were discarded as burn-in. The two sets of post-burn-in trees sampled from the replicate runs were then combined. *Sensu* Brown & Lemmon (2007), we used Bayes factors to choose the most appropriate partitioning strategy to perform phylogenetic inference.

Divergence time estimates

Inferring the timing of splitting events in Iberian and North African wall lizards has been complicated by the lack of suitable molecular clock calibrations. Typi-

cally, these inferences have relied on evolutionary rates adapted from studies in other reptiles (Harris *et al*., 2002b; Pinho *et al*., 2006); hence, they correspond to approximations. Ideally, one should use instead a specific calibration for the system in hand, based on a paleogeographical event suspected to have caused diversification. In the case of Iberian and North African wall lizards, one could in principle take advantage of the fact that the tectonic history of the western Mediterranean is fairly well-known (Rosembaum, Lister & Duboz, 2002). However, the distribution of genetic variation around the Strait of Gibraltar in wall lizards is not easily explained and appears to have been driven by dispersal as much as by vicariance (Harris *et al*., 2002b; Pinho *et al*., 2006), making the patterns difficult to interpret and the use of such events to calibrate a molecular clock highly problematic. However, this problem does not appear to affect other groups of wall lizards, namely those that differentiated on islands (Poulakakis *et al*., 2005; Brown *et al*., 2008). For example, Poulakakis *et al*. (2005) used the isolation of Crete from the Peloponnesus [reflected in the differentiation of *Podarcis cretensis* (Wettstein, 1952) from *Podarcis peloponnesiaca* (Bibron and Bory, 1833)], which occurred at the end of the Messinian Salinity Crisis (MSC), as a calibration point. Brown *et al*. (2008) considered that the same event caused differentiation between the two Balearic lizards *Podarcis pityusensis* (Boscá, 1883) and *Podarcis lilfordi* (Günter, 1874) and also used this event to calibrate their divergence time estimates.

Because our data set partially overlaps with those used in the above mentioned studies, we were able to use the same calibration points to infer divergence times in Iberian and North African *Podarcis*. Accordingly, we retrieved from GenBank, cytochrome *b* and 16S sequences from clades B3 and B5 from Poulakakis *et al*. (2005) [belonging to *P. peloponnesiaca* and *P. cretensis*, respectively; although note that in that publication *P. cretensis* was still referred to as a clade of *Podarcis erhardii* (Bedriaga, 1882)]. Because the phylogenetic position of *Podarcis levendis* Lymberakis *et al*. 2008, from the island of Pori (clade B4 in Poulakakis *et al*., (2005) appears dubious (Lymberakis *et al*., 2008), we did not include this clade in our analysis. Similarly, we obtained all *P. pityusensis* and *P. lilfordi* cytochrome *b*, control region, and 12S rRNA sequences from Brown *et al*. (2008). A complete list of the used sequences' accession numbers is supplied in the Supporting information (Table S1). These sequences were then used to build three distinct datasets: (1) INAG (including cytochrome *b* and 16S sequences from Iberian, North African, and Greek *Podarcis* – 801 bp); (2) INAB (including the common portions of the cytochrome *b*, 12S and control region from Iberian, North African, and Balearic *Podarcis* – 1062 bp); and (3) ALL (including only the cytochrome *b* from the three groups of lizards – 306 bp).

These datasets were analyzed using BEAST, version 1.5.3 (Drummond & Rambaut, 2007). This software implements a Bayesian Markov chain Monte Carlo method to perform a number of demographical and phylogenetic inferences, using a wide array of evolutionary and mutation models. For these analyses, we first defined sets of taxa corresponding to the clades of interest. The analyses allowed for different substitution models for different character sets (for the INAG and INAB analyses) and started from randomly-generated trees. We used the Yule process speciation tree prior, which is more appropriate for between species sequence divergence, throughout the analyses. Divergence times were calculated assuming relaxed molecular clocks, for which an uncorrelated log-normal model was applied (Drummond *et al*., 2006), imposing a prior on the time to most recent common ancestor (TMRCA) of the 'Greek' (*P. cretensis* + *P. peloponnesiaca*) and 'Balearic' (*P. lilfordi* + *P. pityusensis*) clades coinciding with the end of the Messinian salinity crisis. The refilling of the Mediterranean was a rapid event (García-Castellanos *et al*., 2009), such that it could be considered as a point in geological time. However, because the most precise estimate for the dating of this event is 5.33 Mya (Krijgsman *et al*., 1999), we used a uniform distribution in the range 5.325–5.335 Mya as the prior for these TMRCA. After running several preliminary, shorter analyses to optimize running conditions and evaluate convergence of different runs to similar output values, BEAST was run for 100 million steps for each data set, with genealogies sampled every 1000th generation. TRACER (Rambaut & Drummond, 2007), version 1.4 was used to visualize the results and assess if the effective sample size of estimated parameters was satisfactory. We discarded the initial 10% of sampled trees as burn-in.

RESULTS

Estimates of relationships based on five mtDNA regions

Of the 2291 characters of the alignment, 612 are polymorphic and 557 parsimony informative. Given our efforts to avoid known nuclear pseudogenes in this group of lizards and the absence of double peaks, premature stop codons, nucleotide composition and substitution pattern abnormalities (*sensu* Harris, 2002 and Podnar *et al*., 2007) in the two protein-coding gene sequences, we are confident that our sequences represent true mitochondrial DNA instead of nuclear pseudogenes.

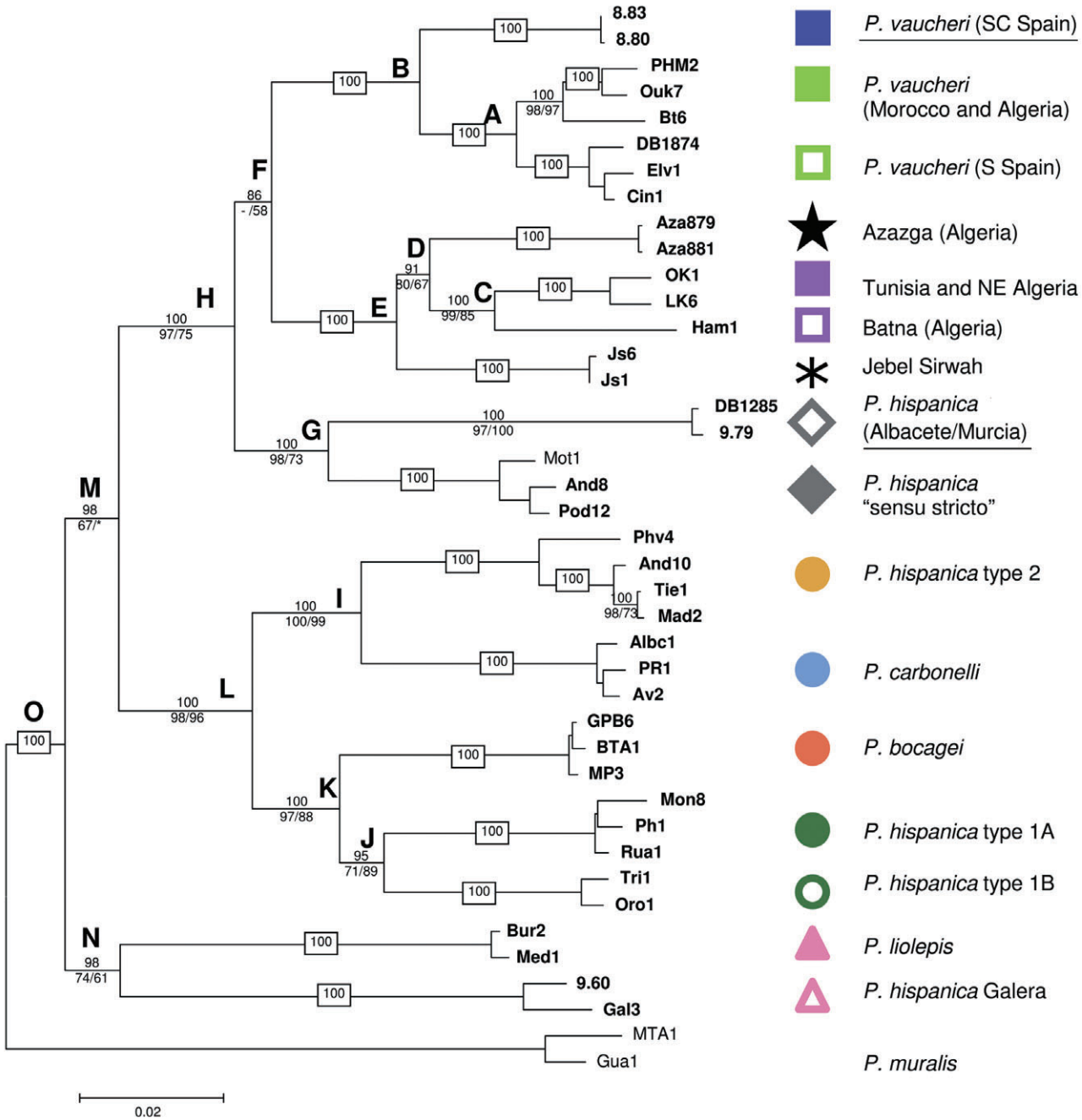

Figure 1. Estimates of relationships between Iberian and North African *Podarcis* based on maximum likelihood analyses of 2291 bp of mitochondrial DNA. This tree is rooted using *Podarcis muralis*. Bayesian posterior probabilities are given above nodes; maximum likelihood and maximum parsimony bootstraps, respectively, are below the nodes. When all three were identical, one value is given in a box. –, bootstrap values lower than 50%. An asterisk (*) indicates branches where different methods yielded different topologies. Underlined names indicate the two recently-discovered lineages. Note that branch lengths represent substitution rate, not geological time; for the estimated age of nodes under different calibration strategies, see Table 2.

The ML tree obtained using GARLI is depicted in Figure 1. Because the results obtained with GARLI were highly consistent across the multiple replicate runs performed, it is unlikely that the recovered estimate of relationships depicts trapping in local optima. Furthermore, analyses based on Bayesian inference recovered exactly the same tree topology as ML analyses, independently of the partition strategy;

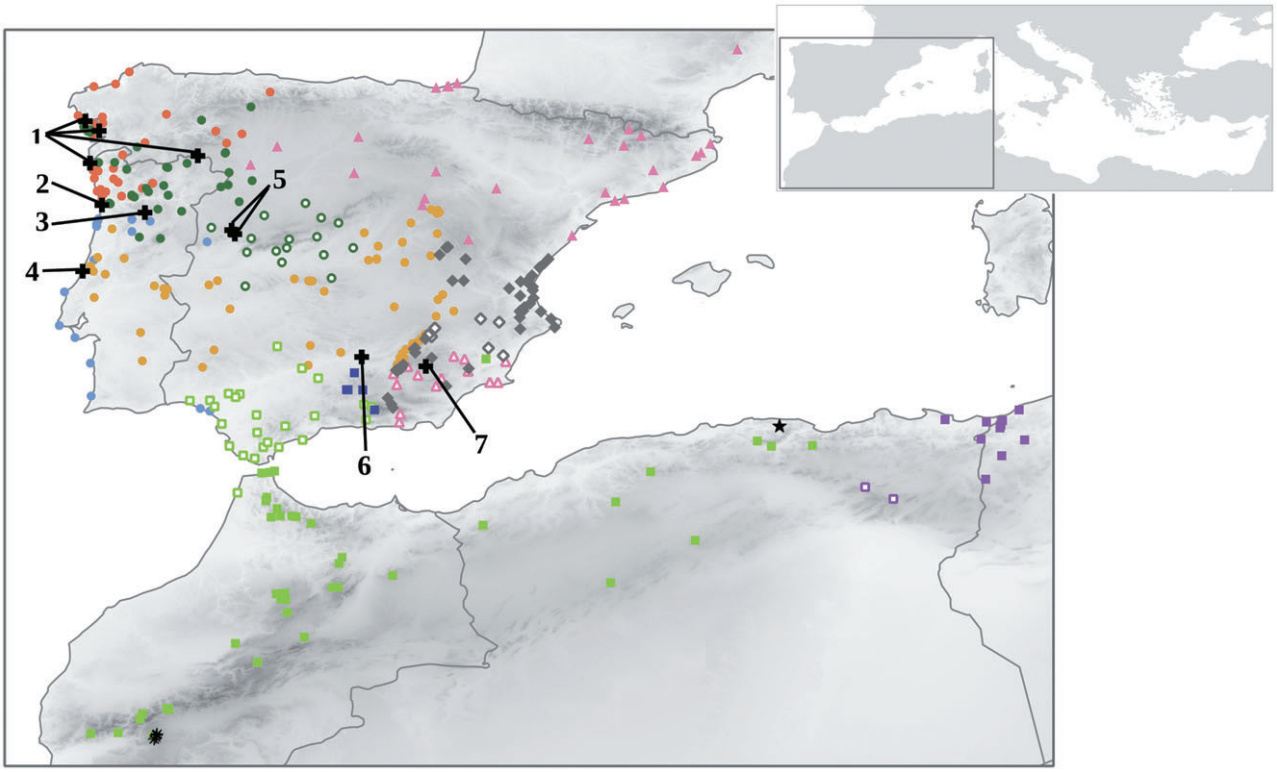

Figure 2. Geographical distributions of the 16 mtDNA lineages of the *Podarcis hispanica* species complex, based on a total of 362 locality data. Colours and symbols correspond to those in Fig. 1. Black crosses indicate localities of syntopy between different lineages, specifically between *Podarcis bocagei* and *P. hispanica* type 1A (1); *P. bocagei* and *Podarcis carbonelli* (2); *P. carbonelli* and *P. hispanica* type 1A (3); *P. carbonelli* and *P. hispanica* type 2 (4); *P. carbonelli* and *P. hispanica* type 1B (5); *Podarcis vaucheri* southern Spain and *P. vaucheri* southern-central Spain (6); and *P. hispanica s.s.* and *P. hispanica* Galera type (7).

nevertheless, not unexpectedly, Bayesian factors analyses showed that the partition into the five mtDNA regions plus distinct codon positions in the protein coding genes was the most appropriate for the data, and the post-burn-in trees resulting from this run were therefore used to obtain an estimate of the phylogeny and compute posterior probabilities. Both ML and BI estimates of the phylogeny are largely concordant with previous assessments of the mtDNA tree in Iberian and North African *Podarcis* (Pinho *et al*., 2006; Lima *et al*., 2009). Apart from the introduction of previously unknown lineages, the relationships between forms remain virtually unchanged. Three main clades can be identified: one clade that appears as sister to all other Iberian and North African forms (clade N; Fig. 1) encompasses *P. liolepis* and the 'Galera' form from Pinho *et al*. (2006) (referred to as the 'hispanicus' clade in Renoult *et al*., 2009); another clade includes all forms from western and central Iberia (clade L); finally, a third clade includes forms inhabiting both North Africa and the south-eastern region of Iberian Peninsula (clade H). It is within the latter that the two newly-discovered lineages fall.

The first of these lineages (depicted as '*P. hispanica* Albacete-Murcia' in Fig. 1) appears as a sister taxon to *P. hispanica s.s.* (*sensu* Pinho *et al*., 2006; 'Valencia' lineage in Renoult *et al*., 2009) and exhibits, on average, approximately 9.5% uncorrected distance in cytochrome *b* from the latter. This lineage was detected in only seven localities and is geographically associated with the area of confluence between the Spanish provinces of Albacete, Alicante, and Murcia (see below and also Fig. 2). A second, previously undetected lineage, sampled from southern Spain, appears as sister to all *P. vaucheri* (depicted as *P. vaucheri* 'South Central Spain' in Fig. 1) and is at present known from six different localities from the Granada and Jaén provinces. This lineage shows, on average, a 9.4% uncorrected distance from its sister taxon (clade A, composed by both North African and south-eastern Spanish *P. vaucheri*).

MP analyses recovered the same overall topology as depicted above, with the exception of the relative relationships between clades H, L, and N (instead of a sister taxon relationship between clades H and L, MP analyses recovered a group encompassing clades H and N).

Dating the major splits

The means and 95% high posterior density (HPD) intervals for the various clades in the tree are shown in Table 2. The 95% HPD for the various estimates overlap between the three dating strategies; however, the inferred mean of the TMRCA for each clade varies according to the calibration employed, especially for those MRCA that are more distant in time. Estimates based on the separation between *P. peloponnesiaca* and *P. cretensis* (INAG) are generally older than those based on the divergence between *P. pityusensis* and *P. lilfordi* (INAB), although both differentiation episodes have been described to happen as a consequence of the same event: the end of the MSC. The calibration strategy based on both events (ALL) suggests in general intermediate values.

Distribution of lineages of Iberian and North African *Podarcis*

The detailed geographical survey conducted resulted in the assignment of 205 samples (Table 1) to previously or newly-identified lineages of the *P. hispanica* species complex (Pinho *et al*., 2006). This, together with a review of previously published data, allowed the compilation of 362 locality data that provide a thorough description of the distribution of different mitochondrial lineages in the Iberian Peninsula and North Africa (Fig. 2) (a detailed table of all compiled locality data is available from the authors upon request). The number of records per lineage ranges from 1 (in the case of the lineage from Azazga, in Algeria, known from a single locality) to 67 (in the widespread *P. hispanica* type 2), with *P. hispanica s.s.* ($N = 45$), *P. vaucheri* from Morocco ($N = 42$), *P. hispanica* type 1A ($N = 40$), and *P. bocagei* ($N = 39$) also sampled in detail. The reduced number of localities comprising records of some forms reflects their geographically restricted nature coupled with their recent detection.

Table 2. Estimates of the times to the most recent common ancestor (in million years) of selected clades of Iberian and North African wall lizards, calculated using a relaxed molecular clock implemented in BEAST, based on different molecular clock calibration strategies

	Calibration strategy		
Clade	INAG	INAB	ALL
A	2.65	2.30	2.69
	1.51–3.89	1.39–3.31	1.31–4.26
B	4.97	4.06	4.01
	3.10–7.06	2.53–5.72	2.06–6.15
C	3.36	2.88	3.33
	1.99–4.90	1.69–4.14	1.38–5.50
D	5.26	3.05	3.64
	3.39–7.26	1.90–4.30	1.71–5.81
E	5.27	4.14	4.09
	3.38–7.24	2.65–5.74	2.02–6.36
F	7.61	6.85	6.56
	5.19–10.34	4.67–9.20	3.66–9.73
G	6.08	4.86	4.27
	3.49–8.79	2.63–8.08	1.46–7.52
H	9.44	6.99	7.00
	6.38–12.63	4.75–9.40	4.05–10.28
I	6.19	3.81	3.94
	3.76–8.87	2.34–5.42	1.87–6.30
J	5.97	4.45	5.33
	3.70–8.48	2.63–6.43	2.44–8.47
K	6.05	5.17	5.64
	3.86–8.62	3.36–7.19	2.84–8.74
L	8.03	6.37	6.38
	5.36–11.00	4.28–8.63	3.48–9.48
M	11.71	8.98	10.11
	8.02–15.86	6.25–12.02	5.83–14.55
N	10.09	9.15	9.69
	6.21–14.36	5.95–12.66	4.33–14.94
O	13.94	9.44	10.41
	9.85–18.43	6.52–12.66	6.07–15.04

Calibration strategies: INAG: based on cytochrome *b* and 16S rRNA sequences and calibrating with the separation of *Podarcis peloponnesiaca* and *Podarcis cretensis* (approximately 5.33 Mya; Poulakakis *et al*., 2005); INAB: based on cytochrome *b*, 12S rRNA, and control region and calibrating with the separation of *Podarcis lilfordi* and *Podarcis pityusensis* (approximately 5.33 Mya; Brown *et al*., 2008); ALL: based on cytochrome *b* sequences only and including the two above-mentioned calibration points. 95% high posterior density limits are shown below the mean values. Clades correspond to those in Fig. 1. Details on the calibration strategies are provided in the text.

DISCUSSION

In the present study, we obtained a new estimate of the Iberian and North African *Podarcis* phylogeny, which fully coincides with previous estimates considering the already known lineages (Pinho *et al*., 2006; Lima *et al*., 2009) and includes two new mtDNA lineages, whose position in the known phylogeny is recovered with a high support (Fig. 1). The discovery of these two additional mtDNA lineages within the Iberian and North African group of *Podarcis* wall

lizards has important implications for the biogeographical scenarios related to the evolutionary history of the group, and clearly illustrates the profound effect that unsampled cryptic diversity may have on paleobiogeographical inference.

Polarity and timing of colonization events around the Strait of Gibraltar

The Strait of Gibraltar is a known centre of diversity for numerous animal taxa, functioning either as a vicariant agent or as a transmarine dispersal corridor. Curiously, for herpetofaunal species, the effectiveness of the Strait as a barrier to gene flow widely varies among taxa (Busack, 1986; Carranza & Arnold, 2004; Carranza, Arnold & Pleguezuelos, 2006a; Carranza *et al*., 2006b; Fonseca *et al*., 2009). In the case of *Podarcis*, the role of the Strait of Gibraltar in shaping diversity has long been an intriguing centre of attention. From early examinations of phylogenetic variation in the area, it became clear that, to explain the patterns of diversity around the Strait, one would need to invoke two independent events (either two transmarine dispersal episodes or one such episode coupled with vicariance promoted by the opening of the Strait of Gibraltar or other geological event). Initially, it was proposed that two transmarine dispersal events across the Strait, at 3.5 Mya (by the ancestral of the Tunisian and Southern Moroccan forms) and 1.5 Mya (by *P. vaucheri*), both from the Iberian Peninsula to North Africa, were responsible for the observed patterns of variation (Harris *et al*., 2002b). With the refinement of the knowledge of mtDNA phylogenetic relationships around the area (Pinho *et al*., 2006), it was suggested that an additional scenario, that of a vicariant separation (e.g. caused by the opening of the Strait) followed by a colonization of *P. vaucheri* from North Africa into the Iberian Peninsula, could also not be discarded. Indeed, based on dating estimates which placed the separation between *P. hispanica s.s.* and the other forms in the clade approximately 5.5 Mya, at approximately the end of the MSC, this second hypothesis appeared to be favoured, although evidence was obviously circumstantial (Pinho *et al*., 2006). A major difficulty in such analyses was that, because *P. vaucheri* groups from both sides of the Strait were consistently recovered as monophyletic units, the polarity of this colonization event could not be directly inferred from the patterns of genetic variation; therefore, this forced researchers to rely on other sources of evidence (such as dating estimates) to make biogeographical inferences. The newly-discovered lineage of *P. vaucheri* from the area of Granada and Jaén, recovered as a sister taxon to all other *P. vaucheri* in phylogenetic analyses, renders Spanish *P. vaucheri* as a paraphyletic group, therefore presently providing a clear indication that *P. vaucheri* invaded North Africa from the Iberian Peninsula and not the other way around. However, given the instability of previous inferences, we must also consider the possibility of further, until now undiscovered, cryptic lineages being described in the future, a fact that may again modify the proposed biogeographical scenario. This is particularly true, for example, if new *Podarcis* lineages are discovered in North Africa, as has been the case recently (Lima *et al*., 2009). Our estimates of the timing of this event (Table 2) would be slightly younger, at between 2.3 and 2.69 Mya, depending on the calibration used, than previously inferred.

On the basis of the knowledge that the ancestor of *P. vaucheri* inhabited the Iberian Peninsula, we can therefore parsimoniously assume that the previous colonization event was carried out by the ancestor of clade E (which comprises all North African forms except *P. vaucheri s.s.*) also with a north–south polarity. According to our estimates, this colonization predates the opening of the Strait of Gibraltar (between 6.56 and 7.61 Mya depending on the calibration used), although 95% HPD limits include the end of the MSC at 5.33 Mya. This therefore suggests that the eventful geological history of the Betic–Rifean regions may have played an important role in shaping the diversity of these lizards, more than the opening of the current Strait itself.

Such a modification of the biogeographical scenario concerning the differentiation of *P. vaucheri* across the Strait of Gibraltar further influences both our knowledge on the evolutionary history of this species and more generalized biogeographical hypotheses proposed for the area. Specifically, an invasion of *P. vaucheri* to North Africa merely 2.3–2.69 Mya, as suggested by the present study, may further enhance our understanding of the high intra- and interpopulation genetic diversity that characterizes this species in the North African portion of its range (Pinho *et al*., 2007b). Previous analyses of population subdivision and historical demography indicated a coalescence time for Moroccan populations of *P. vaucheri* between 1 and 1.6 Mya (Pinho *et al*., 2007b) and suggested that the high diversity observed may be associated with the temporal isolation of distinct subpopulations during the warm–cold cycles during the Pleistocene (Zagwijn, 1992). New evidence supporting the colonization of North Africa by *P. vaucheri* at approximately 2.5 Mya, together with the presently known distribution of the species, which ranges from the Strait of Gibraltar eastwards to the Béjaïa province in Algeria and southwards to the south-eastern edge of the High Atlas mountains in Morocco (Fig. 2), may point to an additional mechanism of diversification, through the expansion of this species across Morocco and northern

Algeria. Additionally, such an invasion and expansion through North Africa by *P. vaucheri* may have also caused geographical fragmentation and isolation within other forms previously occupying this area, as suggested by the isolates of the Jbel Siroua and Azazga lineages (Fig. 2).

From a more global persepective, the modification of the biogeographical scenario concerning the colonization of North Africa by *P. vaucheri* may also alter our view concerning the role of connections between Iberia and North Africa as an agent shaping patterns of diversification. For example, in an analysis of intra- versus intercontinental variation in most reptile species across the Strait, Busack & Lawson (2008) reported low intracontinental variation relative to intercontinental variation in groups including *Blanus*, *Timon*, and *P. vaucheri*. However, recent studies have reported high mtDNA variation within *Timon* in the south-east Iberian Peninsula (Paulo *et al*., 2008) and North Africa (Perera & Harris, 2010), and a new species of *Blanus* from south-west Iberia (Albert & Fernández, 2009), which were not considered by Busack & Lawson (2008). In the present study, we report an additional *P. vaucheri* mtDNA lineage, which, similar to the examples of *Blanus* and *Timon*, was not sampled and which greatly changes such assessments of variation across the Straits. It is clear, therefore, that extensive sampling is needed if such generalized biogeographical comparisons across the Strait of Gibraltar are going to be meaningful, especially when groups that are known to present high levels of cryptic diversity are involved.

Cryptic diversity in south-eastern Iberia

Although the colonization of North Africa by *P. vaucheri* may alter previous scenarios explaining patterns of diversity and historical distribution of this species, it appears that the opening of the Strait of Gibraltar has had a relatively secondary role considering the whole group of Iberian and North African *Podarcis*. By contrast, the area presently corresponding to the south-eastern corner of the Iberian Peninsula appears to be of major importance for the evolutionary history of the group, as already indicated by previous studies (Harris *et al*., 2002b; Pinho *et al*., 2006; Carretero, 2008). The discovery of a new lineage of *P. hispanica* in this area not only reinforces this view, but also augments the uncertainty related to the formulation of evolutionary scenarios. This area, approximately corresponding to the Spanish provinces of Jaén, Granada, Albacete, Murcia, Almería, and Alicante, hosts as many as six different *Podarcis* forms (omitting an introduced population of Moroccan *P. vaucheri*; Renoult *et al*., 2010b): *P. vaucheri* southern Spain, *P. vaucheri* southern-central Spain, *P. hispanica* type 2, *P. hispanica s.s.* (*sensu Pinho et al*., 2006), *P. hispanica* Galera, and the newly-discovered *P. hispanica* from Albacete and Murcia (Figs 1, 2). The lineages of *P. vaucheri* appear to have diverged in southern Iberia at approximately 4.01 and 4.97 Mya (Table 2) and later invaded North Africa (see above). On the other hand, *P. hispanica* type 2 clearly belongs to the western Iberian clade that started to diversify between 6.37 or 8.03 Mya, depending on the calibration used (Table 2), and shows a geographical affinity with the central latitudes of the Iberian Peninsula, being delimited eastwards at the southern part of its range by the Segura mountains (Fig. 2), which emerged as a result of the contact between the Betic–Riffean and Iberian plates (Weijermars, 1991).

However, when examining the remaining lineages present in the area, inference is less straightforward, given that distributions are extremely patchy and lineage divergence quite deep. Two distantly-related clades are involved: one comprising *P. liolepis* and *P. hispanica* Galera, which constitute the sister clade to all other Iberian and North African *Podarcis*, and the other including *P. hispanica s.s.* (*sensu* Pinho *et al*., 2006) and the newly-discovered *P. hispanica* lineage, which together are sister taxa to all North African forms (Fig. 1). The evolutionary history of both clades can probably be traced back to the geological events that took place in the area that is now the Alborán sea and that eventually led to the MSC. The pair consisting of *P. liolepis* and *P. hispanica* Galera (clade N in Fig. 1) diverged, according to our estimates, at approximately 9.15 or 10.09 Mya (Table 2), depending on the calibration used, almost simultaneously to the formation of the other two major clades of the complex, corresponding to the western (clade L) and southern (clade H) groups. This period coincides with the opening of the Betic marine corridor and the fragmentation of the area today forming the Betic region (approximately 8–10 Mya: Weijermars, 1991; Rosembaum *et al*., 2002), which have also been suggested to have promoted the diversification of *Salamandra* (Steinfarz, Veith & Tautz, 2000), *Discoglossus* (García-París & Jockush, 1999; Fromhage, Vences & Veith, 2004) and *Alytes* (Fromhage *et al*., 2004; Martínez-Solano *et al*., 2004) species. Further on, at approximately 6.56 or 7.61 Mya (Table 2), the split occurs between the pair consisting of *Podarcis hispanica s.s.* and the new lineage of *P. hispanica* (clade G) and the clade including *P. vaucheri* and all North African forms (clade F), during a period characterized by land connection and disconnection processes observed in what are today the Betic and Rif mountain ranges and caused by the deposition of sediments in these areas as a result of an increase in water salinity (Krijgsman *et al*., 2000; Krijgsman

& Langereis, 2000). *Podarcis hispanica s.s.* and the new lineage of *P. hispanica* then diverge from each other at approximately 4.27 or 6.08 Mya (Table 2), just around the abrupt climatic modifications related to the closing of the Betic and Rif marine corridors, which triggered the Messinian salinity crisis at 5.96 Mya and the subsequent opening of the Strait of Gibraltar at 5.33 Mya (Hsü *et al*., 1977; Krijgsman *et al*., 1999; Duggen *et al*., 2003; Rouchy & Caruso, 2006).

The geological events that took place in what is now the south-eastern corner of the Iberian Peninsula therefore appear to fairly coincide with the major phylogenetic splits involving the *Podarcis* forms that occupy this area at present. It is important, however, to notice that the patterns of distribution observed today in this area (Fig. 2) are quite inconsistent geographically and do not allow to assess in further detail the potential historical distributions of different lineages. Furthermore, it is likely that the climatic oscillations that occurred during the Pleistocene and post-glacial aridification of the climate have changed distribution patterns in the area, as appears to be the case with other members of this group (Pinho *et al*., 2007b; Pinho *et al*., 2011). These observations, together with the finding of an additional lineage of *P. hispanica* in the area, further question the systematic arrangement and evolutionary scenarios proposed for the forms that currently occur in the region.

Renoult *et al*. (2009) suggested that three evolutionary lineages could be identified in south-east Iberia using morphological characters and nuclear loci, whereas analysis of mtDNA data revealed four lineages. It was thus proposed that extensive past mtDNA introgression could explain this pattern; subsequently, specific status was formally attributed to *P. liolepis* (Renoult *et al*., 2010a), which would then correspond to two mtDNA lineages: the one treated in the present study as *P. liolepis* (filled pink triangles in Fig. 2) and the northern range of the lineage treated in the present study as *P. hispanica s.s.* (*sensu* Pinho *et al*., 2006; filled grey diamonds in Fig. 2). According to Renoult *et al*. 2009, *P. hispanica s.s.*, which they refer to as *P. hispanicus*, and which is still considered as the nominal subspecies of the entire complex, would then include what is treated in the present study as *P. hispanica* Galera, and the southern range of the lineage treated in the present study as *P. hispanica s.s.* (Geniez *et al*., 2007; Renoult *et al*., 2009). The geographical break between these two species would then be located at the south-western part of the provinces of Valencia and Alicante (Renoult *et al*., 2009: fig. 2).

The newly-identified lineage of *P. hispanica* (open grey diamonds in Fig. 2) therefore further challenges an already contrived scenario, by fully coinciding geographically with the presumed limit between the above mentioned taxa. Furthermore, a detailed analysis of morphological variation in all the mtDNA lineages of *Podarcis* shows that this area is characterized by extremely high levels of morphological variability that may be related to local adaptation (Kaliontzopoulou *et al*., in press). Additionally, it indicates a very high morphological similarity between the new *P. hispanica* lineage from south-east Iberia and *P. hispanica* Galera, whereas it also supports the morphological distinction between these two and *P. hispanica s.s.* and *P. liolepis* lineages (Kaliontzopoulou *et al*., in press). This new lineage remains to be analyzed in terms of its nuclear genome. Given the complex nature of genetic variation in the area, the fragmented sampling scheme used in previous studies and the contradictory results obtained by different studies both regarding genetic (Pinho *et al*., 2007a versus Pinho *et al*., 2008 versus Renoult *et al*., 2009) and morphological (Renoult *et al*., 2009 versus Kaliontzopoulou *et al*., in press) variation, further investigation is clearly necessary. Particularly, it appears premature to attempt to define formal taxonomic units in this area and to try to determine whether introgression occurs between them, or even if there are discrepancies between the number of lineages proposed using either morphological, mtDNA or nuclear datasets. Rather, still more geographical sampling is needed to determine how many lineages occur based on the different datasets, and only when this number has stabilized can concordance between them be determined and taxonomic revisions performed.

CONCLUSIONS

Iberian and North African *Podarcis* wall lizards are an example of a group with a complex evolutionary history that clearly illustrates how cryptic diversity may challenge biological inference. As new data become available, our understanding of the biogeography and evolutionary history of the group rapidly increases; in some cases confirming and in others rejecting previous conclusions. Clearly, the major phylogenetic groups of *Podarcis* now inhabiting the Iberomaghrebian region are quite older than initially considered (Harris *et al*., 2002b; Pinho *et al*., 2006) and their formation coincides with or predates the Messinian salinity crisis. An interesting biogeographical consequence deriving from this is that, unexpectedly for a group of Iberomaghrebian affinity, the Strait of Gibraltar does not appear to have played a major role in shaping diversity within the *P. hispanica* species complex. By contrast, the geological events that took place at the end of the Miocene in the

area now corresponding to south-eastern Iberia, the Rif mountain range in Morocco, and the north of Algeria (Rosembaum *et al*., 2002), followed by the abrupt climatic modifications observed during the Miocene/Pliocene transition (Jiménez-Moreno, Fauquette & Suc, 2010), appear to have influenced the evolution of this group of lizards much more profoundly. Combining these paleobiogeographical scenarios with present distribution patterns and morphological evidence, it becomes clear that, without additional advances in the understanding of the phylogenetic history of this group, stable evolutionary hypotheses and systematic arrangements cannot be formulated. Future research concerning the evolution of this group still needs to evaluate genetic diversity based on nuclear markers to enhance our understanding of the biological potential of the mitochondrial lineages investigated in the present study; to explore present and past distribution patterns of those well supported lineages and relate them to environmental variation; to describe their ecological affinities and robustly infer how climatic oscillations may have shaped diversity across space; and, finally, to investigate the phenotypic variation to aid our comprehension of the potential role of local adaptation in shaping morphological patterns observed in this group of lizards.

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SUPPORTING INFORMATION

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

Table S1. GenBank accession numbers of the sequences used for molecular clock calibration.

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