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Complex biogeographical distribution of genetic variation within *Podarcis* wall lizards across the Strait of Gibraltar

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Abstract

Aim To examine the effect of a known geological barrier on genetic variation within a wall lizard species complex.

Location The Iberian Peninsula and North Africa.

Methods Sequencing of partial 12S rRNA and cytochrome *b* mtDNA.

Results The current distribution of genetic variability is not related to the opening of the Strait of Gibraltar.

Conclusions *Podarcis hispanica* in North Africa is probably a species complex. The Strait of Gibraltar should not be used as a known barrier to gene flow in other land based organisms without careful sampling to test for multiple crossings since its formation.

Keywords
12S rRNA, cytochrome *b*, *Podarcis hispanica*, *Podarcis vaucheri*, phylogeny, Strait of Gibraltar.

INTRODUCTION

One of the focal points of biogeographical studies is the study of clades that occur on both sides of a geographical barrier of known age. Fragmentation of a formerly continuous range by such a barrier may have led to genetic differentiation and eventually vicariant speciation and such barriers enable these processes to be studied within a known time frame. The Strait of Gibraltar is a case in point. Around 5.3–5.5 million years ago (Mya), the Atlantic ocean breached the land present in this region, filling the Mediterranean basin which had dried up during the Messinian salinity crisis caused by the separation of this sea from the Atlantic some 5.6 Mya. The re-establishment of the marine connection separated terrestrial faunal elements in the region into allopatric units (Hsü *et al.*, 1977). However, as with the analogous barrier of the Isthmus of Panama, which has separated the tropical faunas of the west Atlantic and east Pacific oceans, sister taxa on each side of the barrier need to be accurately identified (Lessios, 1998). Ability to do this has been greatly enhanced by recent improvements in phylogenetic analysis and in the use of molecular data, which has led to a renaissance in this field (Avise, 2000). It is also necessary to distinguish taxa that are likely to have been divided by the establishment of the barrier from those that split before or after this event. We consider these points in the context of the wall lizard, *Podarcis hispanica sensu lato* (Steindachner, 1870), a complex of forms that occur on both sides of the Strait of Gibraltar.

The degree of morphological differentiation of *P. hispanica* across the Strait has been historically controversial, with most authors accepting one, admittedly a highly variable form in the Iberian Peninsula and a separate subspecies, *P. h. vaucheri* in all of North-west Africa (Barbadillo *et al.*, 1999). Other authors recommend the recognition of multiple subspecific groups (e.g. Guillaume, 1987). On the basis of high genetic distances of the 12S rRNA gene between one individual from Morocco and from Spain, Oliverio *et al.* (2000) raised the African populations to species rank as *Podarcis vaucheri* (Boulenger, 1905). Using electrophoretic data Capula (1990, 1997) also suggested that the Moroccan populations might merit specific rank, but contrary to this Busack (1986) found very low genetic distances between

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populations from southern Spain and Morocco. Recent assessment of mainly Iberian populations derived from mtDNA sequence data suggest that *P. hispanica* may be a species complex, containing genetically distinct and geographically separate units that warrant species status (Castilla *et al*., 1998; Harris & Sá-Sousa, 2002). In Western Iberia these genetic units correspond with slight differences in morphological features (Harris & Sá-Sousa, 2001) that correlate with different climatic areas (Sá-Sousa, 2000).

Our aim was to try to resolve these discrepancies concerning genetic variation in *Podarcis* across the Strait of Gibraltar. First, it is necessary to establish the populations which are sister taxa, and then by using molecular clocks determine whether these were separated approximately at the time of the opening of the Strait. To carry out this we sequenced partial 12S rRNA and cytochrome *b* gene regions from *Podarcis* in both North Africa and the Iberian Peninsula, and combined this with previously published sequences to estimate the phylogenetic history of these populations.

MATERIALS AND METHODS

The number and geographical locations of the specimens used in this study are given in Table 1 and Fig. 1. Total genomic DNA was extracted from small pieces of tail using standard methods (Sambrook *et al*., 1989). Polymerase chain reaction primers used in both amplification and sequencing were cytochrome *b*1 and 2 and 12Sa and 12Sb from Kocher *et al*. (1989). Amplification conditions were the same as described by Harris *et al*. (1998) and Carranza *et al*. (1999). Amplified fragments were sequenced on a 310 Applied Biosystem DNA Sequencing Apparatus. Two individuals of *Podarcis muralis* (Laurenti, 1768) (Harris *et al*., 1998; Fu, 2000), were included in the analysis and designated as an outgroup. All sequences from *P. hispanica* for which both genes had been previously published were included in the analysis (Castilla *et al*., 1998; Harris & Arnold, 1999; Harris & Sá-Sousa, 2002). The closely related Iberian endemics *Podarcis atrata* (Boscá, 1916), *Podarcis bocagei* (Seoane, 1884) and *Podarcis carbonelli* (Pérez-Mellado, 1981) were also included as these form monophyletic units within a paraphyletic *P. hispanica* complex (Harris & Sá-Sousa, 2001). In all cases sequences from the cytochrome *b* and 12S rRNA belonging to the same individual were merged in the subsequent analysis, and aligned using Clustal W (Thompson *et al*., 1994). Cytochrome *b* and 12S rRNA sequences were, respectively, 306 and 383 base pairs (bp) long. The cytochrome *b* sequences contained no indels. Alignment of the 12S rRNA required insertions in five places. Assessment of saturation in each gene by plotting numbers of transitions and transversions against uncorrected distances indicated that they were not saturated (data not shown). Therefore all positions were included in the analysis. The data were imported into PAUP* 4.0b5 (Swofford, 2001) for phylogenetic analysis. When estimating phylogenetic relationships among sequences a model of evolution is assumed. We used the approach outlined by Huelsenbeck & Crandall (1997) to test fifty-six alternative models of evolution, employing PAUP* 4.0b5 and Modeltest (Posada & Crandall, 1998) described in detail by Posada & Crandall (2001). We concluded that the GTR model with an estimated proportion of invariable sites and a discreet approximation of the gamma distribution was the most appropriate model of evolution for this data. The chosen model was employed to estimate trees using minimum evolution (ME) (Rzhetsky & Nei, 1992). Support for nodes was assessed using the bootstrap technique (Felsenstein, 1985), with 1000 replicates. A maximum parsimony (MP) analysis was also carried out (100 replicate heuristic search) with random sequence addition, and support for nodes estimated by bootstrapping with 1000 replicates.

Table 1 Sample code and locality of specimens sequenced for this study

E31052	Azrou (Morocco)
E16081	N. of Oukaïmeden (Morocco)
E29056	Bab-Berred (Morocco)
E29055	Bab-Berred (Morocco)
E29051	8 km. SW of Zinat (Morocco)
E29052	8 km. SW of Zinat (Morocco)
E290510	El-Had (Morocco)
E31051	Jebel Musa (Morocco)
E29053	15 km. SW of Zinat (Morocco)
E29054	15 km. SW of Zinat (Morocco)
E29058	Taza (Morocco)
E29059	Taza (Morocco)
E16084	Mairena del Aljarace (Spain)
E16085	Sevilla city (Spain)
E30051	N. of Aïn Draham (Tunisia)
E30057	Aïn Draham (Tunisia)
E30052	S. of Aïn Draham (Tunisia)
E30054	S. of Aïn Draham (Tunisia)
E30053	Aïn Draham (Tunisia)
E30056	10 km S. of Tabarca (Tunisia)
E210622	Sierra de Gredos (Spain)
E210621	Andorra
MisD	Mischleiffen (Morocco)
Ouk7	Oukaïmeden (Morocco)
Ate1	Barbate (Spain)
Elv1	Huelva (Spain)
Cin1	Guadalcacin (Spain)
Prg1	Puerto de la Ragua (Spain)
Prg2	Puerto de la Ragua (Spain)
Mot1	Motilla (Spain)
Js1	Jebel Sirwah (Morocco)
Js6	Jebel Sirwah (Morocco)
Tie1	41 km SW of Madrid (Spain)
Maq1	Maqueda, SW of Madrid (Spain)
Bt6	Bab Taza (Morocco)

RESULTS

Including the two outgroups, DNA from sixty-eight specimens from fifty-six populations was analysed. Aligned sequences of the combined gene regions were 689 bp long. Of these, 184 were variable and 166 parsimony informative.

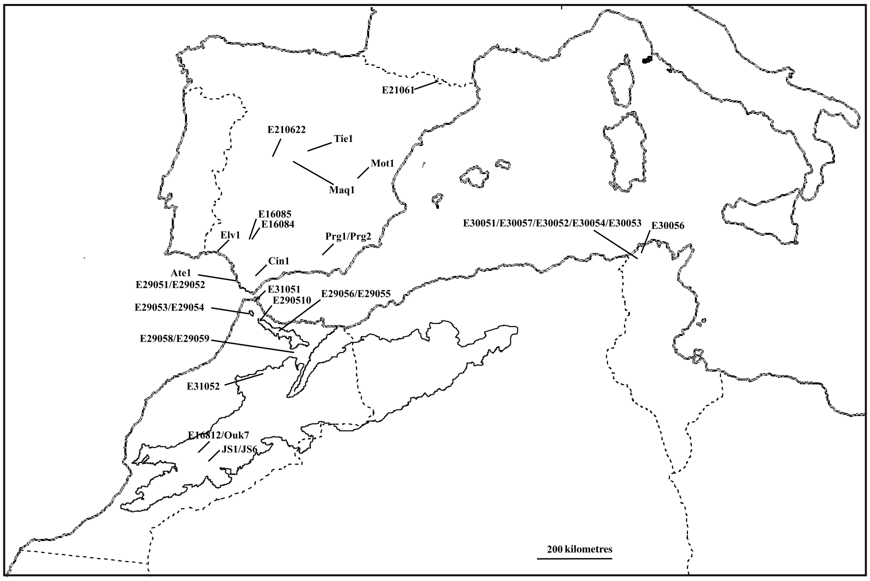

Figure 1 Map showing the localities of sampling points of *Podarcis* sequenced for this study.

New sequences were deposited in GenBank, accession numbers AY134672–AY134741. The sequences used appear to be mitochondrial DNA and not nuclear integrated copies (see Nielsen & Arctander, 2001; Williams & Knowlton, 2001) for several reasons, for instance the protein coding gene contains no premature stop codons, and the free energy of the secondary structure of the 12S rRNA is similar to other lacertid species (data not shown, see Harris, 2001).

Seventy-two ME trees were estimated and one of these is shown in Fig. 2. Only five shallow nodes differed between these. The MP analysis recovered eight trees of 494 steps. As there were no nodes in conflict between the 50% bootstrap consensus trees derived from MP and ME analyses, the MP tree is not shown and the bootstrap values of the MP analysis have been overlaid onto the ME tree (see Fig. 2).

Within the Iberian Peninsula the major groups identified previously using mtDNA sequences (Harris & Sá-Sousa, 2002) are maintained, namely, *P. carbonelli, P. bocagei, P. atrata* and three morphotypes of *P. hispanica*. (Fig. 2). An additional well-supported clade contains all the North African taxa and also some individuals from the southern part of the Iberian Peninsula. Within this clade four very distinct lineages can be recognized (all with 100% bootstrap support). All the samples from Tunisia form a clade. The closest relative of these, although without high bootstrap support, is a population in the isolated mountain of Jebel Sirwah in the Anti-Atlas of southern Morocco. These two lineages are then most closely related to *P. hispanica hispanica* from south-eastern Spain. The fourth lineage contains samples, often referred to *P.* (*hispanica) vaucheri*, from across Morocco other than Jebel Sirwah and from Cadiz and Huelva provinces in southern Spain.

Using our estimate of relationships, parsimony suggests that the *P. hispanica* complex originated in Iberia and later invaded North Africa. We can infer the detailed history of this colonization by incorporating a molecular clock estimate. As the North African samples do not form an exclusive clade and consist of two assemblages each with their closest relatives in Iberia, we infer that there were at least two separate colonization events. Uncorrected sequence divergence, between Iberian *Podarcis h. hispanica* and the Tunisian and Jebel Sirwah form of *Podarcis*, averages approximately 7%. A molecular clock has been calibrated for the same gene regions in other lizards using the age of islands and indicates approximately 2% divergence per million years (Carranza *et al*., 2000). Therefore we estimate that the Tunisian–Jebel Sirwah lineages separated from their Iberian sister group around 3.5 Mya. The Jebel Sirwah population shows approximately 5.5% divergence from the Tunisian populations, implying that these units split perhaps a million years after the initial colonization event. In the second colonization, *P. (hispanica) vaucheri* populations in southern Spain show a divergence from *P. (hispanica) vaucheri* populations in Morocco of approximately 3%, equivalent to 1.5 million years. Our results therefore indicate that the opening of the Strait of Gibraltar was not the cause of genetic differentiation of North African populations from those of the Iberian Peninsula. Instead, they suggest that differentiation followed two independent crossings of the strait some time after it was formed, presumably by natural rafting.

Taxonomic implications

Podarcis hispanica is already known to be a complex of species in the Iberian Peninsula, which can be referred to as *P. hispanica** until its contents can be formally defined (Harris & Sá-Sousa, 2002). In North Africa only *P. (hispanica) vaucheri* has been accepted, although Guillaume (1987) notes morphological differences separating a 'Tunisian form'. As well as supporting this distinction, we report here a genetically distinct form of *Podarcis* so far only

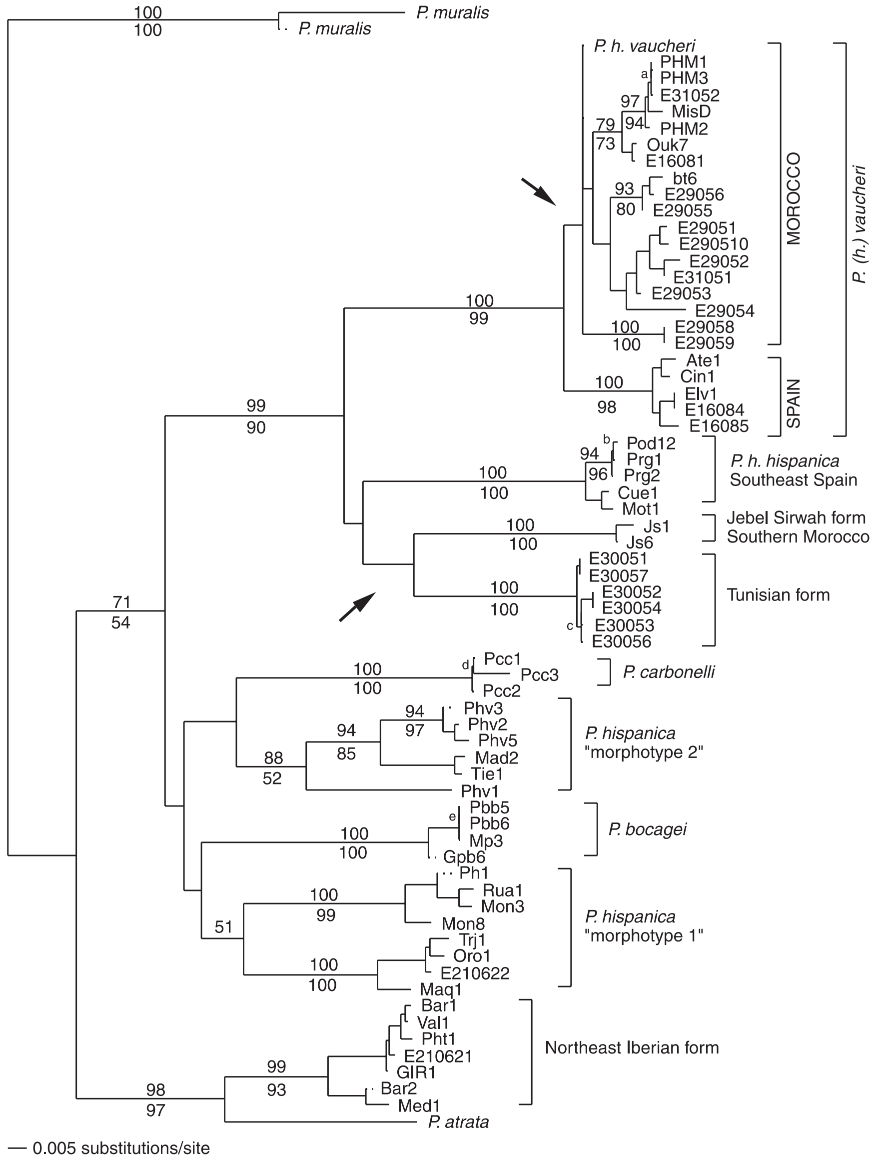

Figure 2 One of seventy-two trees derived from an ME search using the model described in the text. Only five nodes labelled a-e collapse in strict consensus. Bootstrap values (>50%) for ME and MP analyses are given above and below nodes, respectively. The trees were rooted using *Podarcis muralis*. Samples codes refer to Table 1, or are already published (Harris and Sá-Sousa 2002). *P. hispanica* morphotypes are described in Harris and Sá-Sousa (2001). The two arrows indicate independent colonizations from the Iberian Peninsula to North Africa.

known from a single population in Jebel Sirwah. Preliminary analysis of nuclear markers (CP, DJH and NF unpublished) also suggests that this taxon may be a full species. Therefore three distinct taxonomic units can be recognized in North Africa one of which, *P. (hispanica) vaucheri*, also occurs in the Iberian Peninsula.

DISCUSSION

Many reptiles and amphibians from North Africa also show considerable genetic diversity between Moroccan and Algerian—Tunisian forms (Lanza *et al.*, 1986; Mateo, 1990; Mateo *et al.*, 1996; Álvarez *et al.*, 2000), suggesting the presence of an important geographical barrier between eastern and western Maghreb, around the Moulouya river basin in extreme eastern Morocco. Our results also show a separation between northern Morocco and Tunisia, which conforms to this pattern. Our results suggest a possible link between the Anti-Atlas and Tunisia. Investigation of other taxa from these and other regions will be necessary to confirm if this is a general biogeographical phenomenon. One possibility is that the lineage found in Jebel Sirwah is a refugial population of a once more widespread taxa that has been replaced in the rest of Morocco by the more recent invasion of *P.* (*hispanica*) *vaucheri*. Spread of *Podarcis* species is believed sometimes to displace other taxa. For instance there is circumstantial evidence that such spread was associated with restriction of *Algyroides* and small *Lacerta* in extensive areas of southern Europe (Arnold, 1981). The mountain population from Jebel Sirwah is now isolated from the distribution of *P.* (*hispanica*) *vaucheri* by unfavourable desert habitat (D.J. Harris and

C. Pinho, pers. obs.), which could explain its continuing existence.

CONCLUSIONS

The opening of the Strait of Gibraltar does not appear to be a cause of divergence in *Podarcis* lizards. Instead, two transmarine dispersals across the Strait after it was formed appear to be factors in this process. Such crossings are not totally unexpected, as the closely related *Lacerta dugesii* Milne-Edwards, 1829 made a much longer transmarine journey to colonize the Madeiran archipelago (a minimum distance of 1000 km compared with one of 20 km across the Strait of Gibraltar). Within North Africa *Podarcis* fits a previously described pattern where genetically distinct forms occur in much of Morocco and in Tunisia, but the existence of an additional form in the most southerly Anti-Atlas makes the biogeography of *Podarcis* even more complex. What has been recently referred to only as *Podarcis hispanica* contains at least seven genetically distinct lineages, all of which may well deserve species rank. Further assessment of variation in morphological (Harris & Sá-Sousa, 2001) and nuclear markers (Pinho & Ferrand, 2001) will be needed to confirm this. More detailed analysis of other *Podarcis* taxa that have been suggested to be complexes, such as *P. sicula* (Rafinesque-Schmaltz, 1810) (Oliverio *et al.*, 1998, 2000, 2001) is also desirable. This study underlines the importance of adequate sampling when investigating the possible role of geographical barriers in generating differentiation within clades. Without such sampling it may not be possible to correctly identify sister taxa occurring on each side of the barrier. The complexity of events may also be underestimated and dates for the start of differentiation caused by the barrier (either through vicariance or by restricting gene flow after colonization across it) may be erroneously assessed.

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