Systematic and phylogeographical assessment of the *Acanthodactylus erythrurus* group (Reptilia: Lacertidae) based on phylogenetic analyses of mitochondrial and nuclear DNA

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**ABSTRACT**

We have used mitochondrial 12S rRNA, 16S rRNA and nuclear β-fibrinogen (intron 7) sequences to investigate the phylogenetic and phylogeographic relationships between *Acanthodactylus erythrurus* group species (except for *A. bouei*). The phylogenetic analyses of the *Acanthodactylus* genus did not cluster *A. guenennis* and *A. savignyi* with the remaining species of the group (*A. blanci, A. lineomaculatus* and *A. erythrurus*). Within the *A. erythrurus* group, the results of the mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) showed a complex phylogeny with geographic structure, but it was not congruent with the present taxonomy. Some taxonomic units, such as *A. blanci, A. lineomaculatus, A. e. atlanticus* and *A. e. bellii* did not form monophyletic genetic units. The application of a molecular clock suggested that the uplift of the Atlas Mountains in the mid-late Miocene and the reopening of the Strait of Gibraltar could be major biogeographic events responsible for the genetic differentiation in the group. Additionally, diverse microevolutionary patterns due to the recent contraction/expansion phases of the habitats in North Africa associated with the high dispersal capabilities of these lizards could be related to the complex phylogenetic patterns observed.

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1. Introduction

The Maghreb is a North-West African region located in the Mediterranean Basin, which includes the coastal northern parts of Morocco, Algeria and Tunisia. It is situated at the interception of two major landmasses, Eurasia and Africa. The Eastern and the Southern frontiers are surrounded by an arid zone, which extends for several thousand kilometers across the Libyan and the Saharan deserts, respectively. It is also limited to the West by the Atlantic Ocean and to the North by the Mediterranean Sea.

Although the study of evolutionary history of the Maghrebian fauna is very recent when compared to the North-American or European ones (Hewitt, 2004), the biogeography of this region is of great interest due to its (i) complex geological and climatic histories, (ii) heterogeneous landscape, (iii) diversity of habitats, (iv) well defined barriers (Atlantic Ocean, Mediterranean Sea and the Libyan and Saharan deserts) and (v) the known age for some geological events (formation of the Strait of Gibraltar around 5.3 million years ago (Ma) (Hsü et al., 1977) and of the uplift of the Atlas Mountains in the mid-late Miocene). In addition, the Maghreb region has high diversity of endemic reptiles (Busack, 1986). Therefore, this North-African region may provide interesting case studies to investigate how these biogeographical features molded the genetic and demographic evolution of its contemporary species.

The Strait of Gibraltar is thought to have played an essential role in shaping demographic and genetic patterns across several taxa, acting as a biogeographical barrier between the Iberian Peninsula of Southwest Europe and the Maghreb (e.g. Busack, 1986). However, it shows dissimilar degrees of porosity between taxa. On one hand, it promoted deep divergence between populations located in both continents. This pattern was found in amphibians (e.g. Martínez-Solano et al., 2004; García-Paris and Jockusch, 1999), in reptiles (e.g. Vaconcelos et al., 2006; Pinho et al., 2006), in birds (e.g. Salzburger et al., 2002) and also in mammals (e.g. Juste et al., 2004; Castella et al., 2000). On the other hand, recent studies have detected natural colonization events from either the Maghreb or Iberia after the reopening of the Strait of Gibraltar (e.g. Carranza and Arnold, 2004; Cosson et al., 2005; Guillaumet et al., 2006).

The influence of the Sahara desert in shaping the history of the North-African taxa is an important biogeographical question that needs to be addressed, through the study of different species...
inhabiting the desert or its surrounding areas (Douady et al., 2003). The Sahara desert can be a major barrier to the distribution of organisms that cannot, biologically, live in its most arid areas. However, the limits of the desert changed rapidly and repeatedly in the past. The arid periods occurred during the mid-Miocene (15 Ma; Le Houérou, 1997). Between Miocene of hyper-arid desert habitats and the Pleistocene (1.6 Ma) of steppe habitats, at least four humid periods alternated with periods of desertification, as a consequence of the climatic oscillations (Jolly et al., 1998; Le Houérou, 1997). After the Last Glacial Maximum (18,000 years ago), where the climate was again arid (Schuster et al., 2006), repeated oscillations occurred from desert to vegetated land and vice versa, and in some periods they spanned no more than a few 100 years (Sarnthein, 1978). These changes have probably shaped species distribution, by inducing isolation or connectivity among populations during arid and less arid periods. Recently, a few phylogeographic studies addressed this question, hypothesizing that the Sahara desert did not act as a permanent barrier to the dispersal either of birds (Galerida cristata; Guillamet et al., 2006) or reptiles (Acanthodactylus pardalis group; Fonseca et al., 2008). Nevertheless, it was suggested that the rapid oscillations of the desert limits might have contributed to the complex micro-evolutionary patterns inferred by the phylogeny of its reptiles (Fonseca et al., 2008).

The Maghreb region itself also suffered biogeographic oscillations during the same periods that the Sahara desert did, but in the opposite direction. These fluctuations may have led to the isolation of populations in refuges, potentiating allopatric differentiation (Schleich et al., 1996). For example, the NW–SE biogeographical pattern found in the agamid lizards might be explained by vicariance events mediated by the formation of the Atlas Mountains (Brown et al., 2002). There are other North-African species showing strong geographical patterns of genetic differentiation between Western (Morocco) and Eastern (Algeria and Tunisia) populations. This pattern of vicariance, which could be caused by isolation in glacial refugia, was found in invertebrates (Helix land snails, Guiller et al., 2001), in amphibians (Pleurodeles spp. salamanders, Veith et al., 2004), in reptiles (Podarcis lizards, Pinho et al., 2006), in mammals (Crocidura shrews, Cosson et al., 2005) and also in birds (Galerida larks, Guillamet et al., 2006). However, other organisms do not reveal this pattern (Apodemus woodmouse, Libois et al., 2001; Fringilla chaffinch, Griswold and Baker, 2002). Therefore, more phylogeographical studies are needed in order to understand the role of biogeography on current distributions of genetic diversity of the Maghrebian organisms.

The common fringe-toed lizards of the Acanthodactylus erythrurus group are a very interesting model to address phylogeographic and taxonomic questions. The members of this group are associated with mesic environments, not entering more xeric environments (like loess, hamadas and ergs; Schleich et al., 1996), and its wide distribution includes the Maghreb (with the Tunisian (A. blanci), the Algerian (A. savignyi) and the Moroccan endemics (A. erythrurus atlanticus, A. lineomaculatus), and A. e. beli in Morocco and Algeria), the Southern two thirds of the Iberian Peninsula (A. e. erythrurus) and also Northwestern nonarid regions of Sub-Saharan Africa (A. boueti and A. guineensis).

The taxonomy of the A. erythrurus group is controversial and no consensus exists on the systematic status of some forms. The systematics of the genus is generally based on morphological traits, osteological characters and morphology of the hemipenis (Salvador, 1982; Arnold, 1983, 1986; Harris and Arnold, 2000), although molecular data are available (Blanc and Cariou, 1987; Squalli-Houssani, 1991; Harris and Arnold, 2000; Harris et al., 2004). This high morphological variability within the group has led to different redescriptions between conflicting forms (Salvador, 1982; Arnold, 1983; Bons and Geniez, 1995). Salvador (1982) and Arnold (1983) do not retain either the subspecies A. e. atlanticus or the specific status of A. lineomaculatus, considering the latter a subspecies of A. erythrurus. On the other hand, Squalli-Houssani (1991) suggested that none of the Moroccan subspecies (A. e. atlanticus, A. e. beli, A. e. lineomaculatus) should be accepted as a taxonomic unit, because their differences represent superficial adaptations to different local habitats. In the same study, it was suggested that the Iberian A. e. erythrurus should be a distinct (monotypic) species. More recently, Bons and Geniez (1995) considered A. lineomaculatus sufficiently differentiated to justify specific status. Arnold (1983) considered A. blanci a subspecies of A. savignyi. However, based on a recent phylogenetic inference using mitochondrial DNA (mtDNA) of A. erythrurus and A. blanci specimens, A. erythrurus was paraphyletic, with A. blanci arising inside the A. erythrurus clade (Harris et al., 2004).

The aim of this study was (i) to determine the influence of the Quaternary climatic fluctuations and the role of geological barriers (i.e. Atlas Mountains, Strait of Gibraltar, Sahara desert) on the evolutionary history of the A. erythrurus group; and (ii) to clarify the systematics of the A. erythrurus group and the relationships among different geographic populations. For that purpose we have used a phylogeographic approach based on the estimation of the phylogenetic relationships among populations. The phylogenetic analyses were done based on partial fragments of two mitochondrial genes (12S and 16S rRNA) and one portion of a nuclear gene (β-fibrinogen gene intron 7, β-fib? from specimens of all species of the A. erythrurus group (samples from A. boueti were not available, and nDNA for A. guineensis and A. savignyi could not be amplified). This nuclear fragment is expected to be phylogenetically informative, as it was in studies of other lacertids (Paulo et al., 2008; Pinho et al., 2008; Godinho et al., 2005). Mitochondrial DNA gene fragments from the Sub-Saharan A. guineensis and the Algerian endemic A. savignyi were sequenced for the first time.

2. Material and methods

2.1. Sampling

The morphological identification of the specimens sampled for this study (Fig. 1 and Table 1) was based on Bons and Geniez (1995, 1996). Tail tips were removed and stored in 100% alcohol from the specimens collected in the field and from the Bonn Museum, Germany. Additional data were included from previously published sequences (Harris and Arnold, 2000; Harris et al., 1998, 2004; Gonzalez et al., 1996).

For the mitochondrial DNA analyses of the A. erythrurus group within the genus, we have used two species of Mesalina (Arnold, 1989) and Lacerta dugesii dugesii (Gonzalez et al., 1996; Harris et al., 1998) as outgroups. For the analyses of phylogenetic variability (mtDNA) within the A. erythrurus group we have used A. tristrama, A. aureus (Harris and Arnold, 2000) and A. maculatus (Fonseca et al., 2008) as outgroups. For the β-fib? analyses, A. maculatus (this study), Lacerta media and Lacerta lepida (Godinho et al., 2005) were used as outgroups.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the alcohol-preserved tails using standard methodologies (Harris et al., 1998). Fragments of 12S and 16S rRNA genes were amplified using published primers 12Sa and 12Sb for 12S rRNA gene (Kocher et al., 1989) and 16SL1 and 16SH2 for 16S rRNA gene (Hedges and Bezy, 1993). Both amplifications were performed in 25 μL of 10-fold reaction buffer (Ecogen), 3.2 mM MgCl2, 1.6 mM each dNTP, 4.0 μM each primer, 1 U of EcoTaq DNA polymerase (Ecogen) and approximately 100 ng genomic DNA (PCR profile: pre-denaturating 94 °C (5 min)
Fig. 1. Geographic locations of specimens from the Acanthodactylus erythrurus group used in the study. Locations of A. guineensis specimens are not shown, but geographic coordinates are given in Table 1. Arrows point to Algerian specimens from different localities but with the same mtDNA haplotype of Aeb3.

Table 1

<table>
<thead>
<tr>
<th>Current species assignation</th>
<th>Code used</th>
<th>References</th>
<th>Country</th>
<th>Region</th>
<th>Latitude</th>
<th>Longitude</th>
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<td>E-06°22.000'</td>
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Specimens sequenced for the nuclear marker. References: 1, New for this study; 2, Harris et al. (2004); 3, Harris and Arnold (2000); 4, Fu (2000). All the Acanthodactylus not described in this table were used from Harris and Arnold (2000).
and 35 cycles with 94 °C (30 s) denaturing, 50 °C (30 s) annealing and 72 °C (40 s) extension temperatures. A final extension was conducted at 72 °C for 5 min.

A fragment of the β-fibint7 gene was amplified using primers BF8 (Pinho et al., 2008) and BFXF (Sequeira et al., 2006) in 19.55 μL of 10× reaction buffer (Ecogen), 2.0 mM MgCl2, 1.6 mM each dNTP, 2.0 μM each primer, 1 U of Ecotaq DNA polymerase (Ecogen) and approximately 75 ng genomic DNA [PCR profile: pre-denaturating 94 °C (3 min) and 35 cycles with 94 °C (30 s) denaturing, 52 °C (30 s) annealing and 72 °C (45 s) extension temperatures]. A final extension was conducted at 72 °C for 5 min.

The PCR products were purified by enzymatic cleaning and sequenced using the ABI Prism Big Dye Terminator Cycle sequencing protocol in an ABI Prism 310 automated sequencer (Applied Biosystems).

2.3. Phylogenetic analysis

The multiple alignments of the DNA sequences were performed using MAFFT v.5 (Katoh et al., 2005) with the “Q-INS-I” strategy. To avoid bias in refining alignments and to remove regions without specific conservation we have used Gblocks (Castresana, 2000) with the following parameter settings: minimum number of sequences for a conserved position—14; minimum number of sequences for a flanking position—14; minimum length of a block—5; allowed gap positions—“with half”. In the nDNA, β-fibint7, some sequences showed heterozygote positions for which it was not possible to determine each allele solely by the analysis of the chromatogram. Therefore, the program PHASE v.2.1. (Stephens et al., 2001) was used to estimate haplotypes for each individual with uncertain phase sites. This analysis was run multiple times (3) with different seeds for the random-number generator and checked if haplotype estimation was consistent across runs. Each run was conducted for 1.0 × 10⁶ iterations with the default values. The sites with phase probabilities above 0.70 were maintained in the final sequence dataset.

The maximum parsimony (MP) analyses were performed with PAUP*-4.0b10 (Swofford, 2003) and the trees were estimated using the heuristic search algorithm with tree-bisection–reconnection (TBR) branch swapping and 100 random addition replicates. All changes had the same weight, and gaps were treated as a fifth state.

The most appropriate models of sequence evolution were selected employing PAUP*- and Modeltest v.3.8 (Posada and Crandall, 1998; Posada, 2006) under the Akaike information criterion, following Posada and Buckley (2004). The Maximum Likelihood (ML) analyses were performed using PHYML (Guidon and Gascuel, 2003). The program estimated the base frequencies, the ts/tv ratio, the γ distribution (four rate categories) parameter and the proportion of invariable sites. The input tree was determined with the BioNJ algorithm and tree topology was optimized. Bootstrapping (1000 pseudo-replicates) was used to evaluate the stability of nodes of the phylogenetic trees (Felsenstein, 1985) for MP and ML analyses.

The Bayesian inference (BI) was implemented using MrBayes v.3.1.2. (Huelsenbeck and Ronquist, 2001), which calculates Bayesian posterior probabilities using a Metropolis–coupled, Markov Chain Monte Carlo (MC-MCMC) sampling approach. All analyses started with randomly generated trees and ran for 11 × 10⁶ generations, saving one tree every 100 generations. Two independent runs were generated for each dataset (mtDNA and nuclear DNA). Although each run was carried out using different random starting seeds, they should have similar properties at convergence (Brooks and Gelman, 1998). Therefore, different diagnostic analyses (stationarity for each run, convergence between runs, symmetric tree-difference score within and between runs) were carried out using the web application of the AWTY program (Nylander et al., 2007). This program uses as input the phylogenetic trees generated as output by Mrbayes for each run. All analyses reached stationarity and showed convergence. In addition, outputs from AWTY program were analyzed to determine the samples that should be discarded before calculating summary statistics for each run. For all the analyses the burn-in period covered the first 20%. The convergence diagnostic (PSRF) approached 1 in all analyses. To determine the most appropriate BI model, we have used the Bayes factors. A 50% majority-rule consensus tree was generated combining the remaining trees of the analyses with the most appropriate model. The frequency of any particular clade of the consensus tree represents the posterior probability of that clade (Huelsenbeck and Ronquist, 2001).

We have tested the homogeneity between gene fragments (Incongruence Length Difference test—ILD; Farris et al., 1995) and the homogeneity of base frequencies across taxa (χ² test). Both tests were implemented in PAUP*. The ILD test was run after removing all invariant characters (Lee, 2001). Topological constraints were also generated, when necessary, to test alternative tree topologies using the Shimodaira–Hasegawa test (SH test, Shimodaira and Hasegawa, 1999) implemented in PAUP* 4.0b10 and employing one-tailed test, RELL bootstraps with 1000 replicates.

A dispersal–vicariant analysis was performed with DiVA 1.0 (Ronquist, 1996) to generate hypotheses about the geographical distribution of ancestors within the A. erythurus group.

The likelihood ratio test (LRT; Huelsenbeck and Crandall, 1997) was performed to assess the statistical significance between the log-likelihood of the trees calculated with or without enforcing a molecular clock. If twice the difference between the likelihoods was not significant, it could indicate that the dataset tested was evolving in a clocklike behavior and genetic distances between taxa could be used to establish approximate dates for some nodes of the inferred phylogenetic trees. Finally, the analyses for estimating divergence times among clades were performed with BEAST v.1.4.7 (Drummond and Rambaut, 2007) and data on 12S-rRNA gene. The calibration rate was estimated using the age of El Hierro Island in the Canary Islands, which is estimated as 1 Myr (Guillou et al., 1996) and assuming that the resident lacertid lizard Gallotia caesars caesars colonized this island immediately after its formation, from neighboring La Gomera Island, where G. c. gomerae occurs (Macameyer et al., 2003). Although several studies have used these taxa to estimate divergence times among clades (p.e. Carranza et al., 2004; Paulo et al., 2008), factors that could distort clock calibrations (i.e. stochastic variation at low levels of sequence divergence, extinct or unsampled lineages; Emerson, 2002) cannot be excluded. Nevertheless, there is no evidence that these facts occurred in Gallotia (Macameyer et al., 2003). The rate of 0.5% sequence divergence per million years per pair of lineages (which correspond to a mutation rate of 0.0025 mutations/site/million years) of the partial 12S rRNA used by Harris and colleagues (2004) also for A. erythurus was adopted. For our dataset, a relaxed molecular clock with lognormally autocorrelated rates among branches was implemented (Drummond et al., 2006). The molecular clock analyses were run with 10⁶ generations and with a burn-in of 10%, and the effective sample size (ESS) was always above 200 for the relevant statistics. The stability of each run and the convergence between runs were assured using Tracer v1.4 (Rambaut and Drummond, 2007) and Log-Combiner v1.4.7 (included in the BEAST package).

3. Results

3.1. Mitochondrial DNA sequence variation within Acanthodactylus

Sequences for partial 12S and 16S rRNA genes of 27 lizards (20 different Acanthodactylus species and three outgroup species) were aligned. The final alignment included 648 positions (303 and 345
positions of the 12S and 16S rRNA genes, respectively), of which 190 were parsimony informative. For the combined dataset, the ILD test showed no incongruence between fragments (ILD $p = 0.08$) and the $\chi^2$ test of homogeneity of base frequencies across taxa showed no significant difference ($\chi^2 = 18.66, df = 78, p = 1.00$).

For ML and BI analyses, the most appropriate model of evolution for this data set was the General Time Reversible (GTR) model with an estimate of invariable sites (0.4409) and a discrete approximation of the $\gamma$ distribution ($\alpha = 0.6076$). The different phylogenetic methods inferred trees with similar topologies, with the exception of the positions of $A.$ guineensis, $A.$ savignyi and populations within the Eastern clade (Fig. 2). Molecular analyses support monophyly of the Acanthodactylus but indicate that the $A.$ erythrurus group, as presently understood, is paraphyletic, with $A.$ guineensis and $A.$ savignyi not forming a clade with the remaining species of the $A.$ erythrurus group (i.e. $A.$ erythrurus, $A.$ blanci and $A.$ lineomaculatus). Nonetheless, a SH test showed no statistically significant difference ($p = 0.087$) between the optimal tree ($-\ln4358.32408$) and a tree with the topological constraint for monophyly of the $A.$ erythrurus group ($-\ln4370.12531$).

The phylogenetic analyses show strong support for the Eastern clade and for the $A.$ scutellatus clade (Harris and Arnold, 2000). However, the relationship between these two clades is weakly supported. A third clade, named Western clade (including $A.$ erythrurus group species, $A.$ busacki, $A.$ maculatus, $A.$ pardalis, $A.$ orientalis and $A.$ tristrami; Harris and Arnold, 2000), is not strongly supported by our analyses. In fact, this clade is not recovered by the MP method.

The mtDNA tree estimated for the genus Acanthodactylus was used to estimate the ancestral area of the $A.$ erythrurus sub-group comprising $A.$ blanci, $A.$ erythrurus and $A.$ lineomaculatus species. However, the program DIVA can only handle fully bifurcate trees. Therefore, we have tested all possible fully bifurcate trees separately, and then we summarized the results. In some cases it was possible to combine taxa in polytomies with the same distribution into a single taxon to reduce the number of arbitrary resolutions. In total, 12 equally possible topologies were tested. All the analyses hypothesized North Africa as the most parsimonious ancestral area for the $A.$ erythrurus sub-group populations.

### 3.2 Mitochondrial DNA sequence variation within the $A.$ erythrurus group

Fragments of the 12S and 16S rRNAs mtDNA ribosomal genes were sequenced from samples representative of the genetic and taxonomic variability of the $A.$ erythrurus group ($A.$ guineensis, $A.$ savignyi, $A.$ blanci, $A.$ lineomaculatus, $A.$ e. atlanticus, $A.$ e. belli and $A.$ e. erythrurus). In this dataset sequences for partial 12S (384 bp) and 16S (496 bp) rRNA genes totaling 880 bp were analyzed for 59 lizards, including the outgroup ($A.$ tristrami, $A.$ maculatus and $A.$ aureus).

The final alignment included 845 positions (380 and 465 positions of the 12S and 16S rRNA genes, respectively), of which 141 were parsimony informative. For the combined dataset, the ILD test showed no incongruence between fragments (ILD $p = 0.06$), and the $\chi^2$ test of homogeneity of base frequencies across taxa showed no significant difference ($\chi^2 = 9.82, df = 90, p = 1.00$).

The most appropriate model of evolution for the combined data set was the GTR model with an estimate of invariable sites (0.49) and a discrete approximation of the $\gamma$ distribution ($\alpha = 0.459$).

The LRT performed for the 12S rRNA dataset showed statistically significant difference between the log-likelihoods of the phylogenetic trees, with or without a molecular clock enforced ($2\delta = 43.55, df = 27, p = 0.02$).

The tree inferred by the Bayesian analysis is depicted in Fig. 3, because the ML and MP methods produced trees with similar...
topology for all the main clades. Monophyly is not recovered for *A. erythrurus* by any of the phylogenetic methods used, with *A. guineensis* and *A. savignyi* not forming a clade with the remaining species of the group (i.e. *A. erythrurus*, *A. blanci* and *A. lineomaculatus*). The latter species formed a very well supported clade; however, monophyly is not recovered for its species and subspecies. As shown by Fig. 3, (i) *A. blanci* arises inside a clade (clade A) comprising specimens of *A. e. belli* from Algeria and SW Morocco; (ii) the remaining *A. e. belli* specimens (clade B) do not group with those from clade A, making *A. e. belli* paraphyletic; (iii) the strong genetic relationship between geographically closely related specimens of *A. lineomaculatus* and *A. e. atlanticus* make them both paraphyletic; (iv) *A. e. erythrurus* specimens form two main clades (clades E1 and E2), whose status as sister taxa is uncertain.

The optimal topology obtained for this dataset was tested against constrained trees for each of the taxonomic units of the *A. erythrurus* group (see Table 2). The results clearly reject *A. lineomaculatus* and *A. erythrurus* as monophyletic species, and *A. e. atlanticus*, *A. e. belli* as monophyletic units within *A. erythrurus* species. In contrast, the monophyly of the *A. erythrurus* group and of the subspecies *A. e. erythrurus* cannot be ruled out, as the SH tests did not reject these relationships. Similarly, the species *A. blanci* may be regarded as the sister taxon of *A. erythrurus* and *A. lineomaculatus*.

3.3. Nuclear DNA sequence variation within the *A. erythrurus* group

For the β-fibint7 phylogenetic analysis, sequences were obtained from selected samples representative of the major mtDNA clades (Fig. 3, clades A–E). We could not amplify nuclear gene fragments for *A. guineensis* and *A. savignyi* species. The final alignment included 628 positions, of which 98 were parsimony informative.

The most appropriate model of evolution for this data set was the Hasegawa–Kishino–Yano (HKY) with a discreet approximation of the *c* distribution (*a* = 0.660).

All phylogenetic methods inferred similar trees. Fig. 4 shows the tree obtained for the β-fibint7 with the BI. However, the phylogeny obtained with the nuclear marker differed significantly from the one obtained with the mtDNA, as shown by the ILD test result (*p* = 0.001). The main features of the nuclear genomic tree that differ from the mtDNA tree are that (i) the samples of mtDNA clade A did not group together, with *A. e. belli* from Southwest Morocco (Aeb1) being phylogenetically distant from the others; (ii) the haplotypes from the North and Northeast Morocco and also from the Iberian Peninsula form a strongly supported clade, which includes samples of mtDNA clades B, C and E; (iii) samples of *A. e. erythrurus* (Aee1, Aee6, Aee9, Aee10 and Aee11) form a monophyletic group in all analyses (BI, ML and MP); (iv) the close relationship between Central and Southwest Moroccan haplotypes (*A. e. atlanticus*—Aea1, Aea2, Aea3—and *A. lineomaculatus*—Alin4, but not Aeb1), which includes samples of the mtDNA clades C and D; (v) specimens from North and Northeast Morocco (*A. e. belli*—Aeb5 and

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![Fig. 3. Tree derived from the Bayesian analysis of the partial 12S and 16S RNA sequences. The tree was rooted using *A. maculatus*, *A. aureus* and *A. tristrami*. Bayesian posterior probabilities and bootstrap values are given above or near the branches (BPP/ML/MP). Values under 50% are represented by “—”. Triangles, squares and circles refer to sequences of *A. erythrurus*, *A. lineomaculatus* and *A. blanci* respectively.](image-url)
Table 2
Statistical support for alternative topological hypotheses of relationships of different taxonomic units in *A. erythrurus* group (SH, Shimodaira–Hasegawa test).

<table>
<thead>
<tr>
<th>Tree</th>
<th>MtDNA</th>
<th>nDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconstrained ML tree (Fig. 2)</td>
<td>3681.83537 (best)</td>
<td>1845.20966 (best)</td>
</tr>
<tr>
<td>Constrained (<em>A. erythrurus</em> group monophyletic)</td>
<td>3686.70247 4.8671 0.708</td>
<td>1930.99807 85.788 0.000*</td>
</tr>
<tr>
<td>Constrained (<em>A. erythrurus</em> belli monophyletic)</td>
<td>3713.49423 31.65886 0.035</td>
<td>1900.49796 55.2830 0.002*</td>
</tr>
<tr>
<td>Constrained (<em>A. lineomaculatus</em> monophyletic)</td>
<td>3724.89433 43.05896 0.006*</td>
<td>1879.52930 34.1964 0.072</td>
</tr>
</tbody>
</table>

* Indicates $p < 0.05$ and suggests that the unconstrained and constrained topologies are significantly different.

Fig. 4. Tree derived from the Bayesian analysis of the $\beta$-fibint7 sequences. The tree was rooted using *L. media* and *L. lepida*. Bayesian posterior probabilities and bootstrap values are given above or near the branches (BPP/ML/MP). Values under 50% are represented by “---”. Samples followed by an asterisk (*) have different haplotypes of the nDNA marker.
Aeb6, respectively) have genetic relationships with those from Central and Southwest Morocco (A. e. atlanticus—Aea1, Aea2 and Aea3—and A. lineomaculatus—Alin4).

The optimal topology obtained for the nucDNA dataset was tested against constrained trees for each of the taxonomic units of the A. erythrurus group (see Table 2). The results are similar to those for mtDNA in rejecting A. lineomaculatus and A. erythrurus as monophyletic species and A. e. belli as a monophyletic subspecies within A. erythrurus. On the other hand, monophyly could not be rejected for A. e. atlanticus or A. blanci.

3.4. Genetic relationships and geography

Within the A. erythrurus group the genetic undistorted distances between A. guineensis and A. savignyi, and the other species were very high (between 9.4% and 12.3%) when compared to the same distances within the group, excluding A. guineensis and A. savignyi (less than 6.1%). Moreover, these two species also show high genetic distances between them (11.3%). Geographically, A. guineensis is a Sub-Saharan species, whereas A. savignyi is an endemic from the Maghreb ( Algerian coast). The later species is, therefore, geographically closer to the remaining species of the group, except for A. boueti (a Sub-Saharan species, not included in this study).

Within the other species of the A. erythrurus group, almost all clades formed geographically cohesive units but they were not coherent with taxonomy (i.e. species and subspecies were not monophyletic). Both markers (mtDNA and nDNA) showed geographic structure, but the nuclear gene analysis was more complex.

The mtDNA recovered clades corresponding to the main geographical regions Algeria/Tunisia (Ab1, Aeb3 and Aeb4—samples from clade A), North/Northeast Morocco (clade B), North/Central Morocco (clade C), West/Southwest Morocco (clade D), and the Iberian Peninsula (clades E1 and E2). All these major clades arose from a basal polytomy, with short branches giving little information about how they are related to each other.

The clades recovered by the nuclear marker were less in number, but each clade comprises bigger geographical regions: (i) Algeria/Tunisia (the same part of clade A), (ii) N + NE Morocco/Iberian Peninsula (samples from clades B, C, E1 and E2), (iii) Central + SW Morocco (samples from clades C and D). Two specimens (Aeb5 and Aeb6, mtDNA clade B) from N + NE Morocco have a genetic relationship with specimens from Central to SW Morocco (clades C and D). The mtDNA clade C is separated in the nuclear analyses with the Northern representative (Alin2) grouping with specimens from NE Morocco + Iberian Peninsula, and with the Central representatives (Aea1 and Aea2) grouping with specimens from SW Morocco. The specimens Aeb1 from SW Morocco, which groups with samples from Algeria and Tunisia in the mtDNA analyses (clade A), appears as the sister taxon to a clade comprising all other ingroup species in the nuclear analyses.

Within the Iberian Peninsula more than the two genetically distinct mtDNA clades reported in Harris et al. (2004) were found. Essentially, adding specimens from different localities increased the genetic variability. Although, specimens from the Iberian Peninsula did not group together in the mtDNA analyses, they formed a monophyletic group in the nuclear one.

3.5. Estimation of divergence times

The divergence times of the nodes were estimated based on a relaxed molecular clock approach (Drummond et al., 2006). The analyses were done for the 12S rRNA gene fragment, as described Section 2.

The oldest split of the A. erythrurus sub-group (clades A to E1–E2), which corresponds to the divergences within the species A. erythrurus, A. blanci and A. lineomaculatus, occurred 12.8 Ma. The splits were, probably, Miocene events. The range of the 95% Highest Posterior Density (HPD) interval of the divergence time ranged between 7.9 and 18.2 Ma.

Within clade A, the specimen from SW Morocco (Aeb1) appeared to diverge 9.3 Ma (HPD between 5.4 and 14.0 Ma), and the split between the NE Algerian A.e. belli (Aeb3 and Aeb4) and the Tunisian A. blanci took place 4.7 Ma (HPD between 1.9 and 8.1 Ma).

The first Iberian subspecies A. e. erythrurus split (E1 and E2) occurred around 7.3 Ma (HPD between 4.0 and 11.0 Ma) and the separations within clade E1 were Miocene–Pliocene events, with divergence time around 5.3 Ma (HPD between 2.9 and 8.2 Ma).

4. Discussion

4.1. Mitochondrial DNA sequence variation within Acanthodactylus

The molecular results of this study are congruent with the molecular results of Harris and Arnold (2000). We added mtDNA sequences for A. guineensis, A. savignyi, A. lineomaculatus and A. blanci to their dataset. The main difference is that, in our analyses, the support for the Western clade is very low. Therefore, the inclusion of novel molecular data added complexity to the results and this was, mainly, caused by the A. guineensis and A. savignyi mtDNA sequences.

In fact, the novel molecular data used in this study did not clarify the relationships within the A. erythrurus group. The phylogenetic analyses did not group A. guineensis and A. savignyi with the remaining species of the group. Nonetheless, monophyly of the A. erythrurus group could not be rejected.

In the Acanthodactylus analyses, both A. guineensis and A. savignyi have basal positions in the Western clade and show distinctive high genetic p-distances between them and between other species of the group. This was expected for A. guineensis, because it is separated from all species of the group (except A. boueti) by the Sahara desert. However, it was surprising to observe similar high genetic distances in A. savignyi because it is a North-African endemic and coexists with A. erythrurus belli in North Algeria. Although morphologically, A. guineensis and A. savignyi are similar to the other species of the group, each species has distinctive characters (Arnold, 1983). Nevertheless, morphological distinctiveness of this group, as well as other Acanthodactylus groups, is not conclusive (see Salvador, 1982; Arnold, 1983; Squalli-Houssani, 1991; Bons and Geniez, 1995).

On one hand, the phylogenetic results might suggest that the A. guineensis and A. savignyi mtDNA lineages are quite old or that they evolved extremely fast within the A. erythrurus group. On the other hand, ancient hybridization events of these two species with a non-A. erythrurus group species could not be excluded. The inclusion of a nuclear marker would help resolve this issue too.

Based on the genetic relationships within Acanthodactylus, the ancestral distribution for the A. erythrurus group was North Africa. Consequently, the Iberian Peninsula was colonized from North Africa and not the reverse. This result is consistent with the fact that Acanthodactylus has most representatives in Asia and North Africa and reaches the edge of its distribution in the Iberian Peninsula, and by the non-basal position of the A. e. erythrurus subspecies in the estimated phylogeny of the genus (Fig. 2). Lower protein electrophoretic polymorphism in Iberian populations than in Moroccan ones is likewise consistent with the hypothesis that Iberian populations were derived from a North-African source (Bussack, 1986).
4.2. DNA sequence variation within the *A. erythrurus* group

The new sequences included in the mtDNA analyses of the *A. erythrurus* group showed that this group has high genetic variability, and confirmed the genetic distinctiveness of *A. guineensis* and *A. savignyi*. The position of these two species on the trees, suggests a very old split between them and the other species of the group. Moreover, the morphological characters also support the species status of *A. guineensis* and *A. savignyi*. In the former species, the fronsalas is undivided, the subocular scale is never separated from the lip and it has the medial side of the hemipenis and armature absent and a peculiar arrangement of nasal scales (Arnold, 1983). The latter species has a unique distinctive character, blue coloration of the tail.

Although we do not have genetic data for *A. boueti*, also a member of the *A. erythrurus* group, we hypothesize that this species should be closely related to *A. guineensis*: (i) it is morphologically similar and (ii) it is also a Sub-Saharan species. Additionally, we expect that the Saharan desert could be acting as a biogeographic barrier to the distribution of *A. boueti* in the same way that it does for *A. guineensis*.

The results for the remaining members of the group (*A. blanci, A. lineomaculatus* and *A. erythrurus*) show a phylogeny structured with geography. In both markers (mtDNA and nuDNA), most well supported clades correspond to geographic regions with a few exceptions.

4.2.1. Maghreb region

Clade A, which is the most complex, includes *A. e. belli* from SW Morocco, NE and NW Algeria and *A. blanci* from Tunisia. The clade is not highly supported in the mtDNA analyses (BI-83; ML-60; MP < 50) and it is not recovered in the nuclear analyses. However, inside this clade there is a sub-group well supported by both markers, which correspond to the most Eastern samples included in this study (NE Algeria–Aeb3, Aeb4— and Tunisia—Ab1). This divergence between Moroccan and Algerian/Tunisian populations is not novel and it was also reported for other North-African taxa, including reptiles and amphibians (Mateo et al., 1996; Álvarez et al., 2000; Harris et al., 2002; Mendonça and Harris, 2007). Nonetheless, an adequate sampling mainly in Algeria is needed for a deeper understanding of a possible geographic barrier in this region.

Both markers indicate that *A. blanci* had recent genetic contact with *A. e. belli* from SE Algeria. In addition, the positions of *A. blanci* in the *A. erythrurus* group are not clear in terms of morphological characters (see Salvador, 1982; Arnold, 1983).

The phylogenetic position of the other two specimens of clade A, both *A. e. belli* (Aeb1 and Aeb2), are not well supported, but raise interesting questions: (i) How genetically variable is this species in Algeria? (ii) Why are Aeb1 and Aeb2 more genetically distant from specimens that are geographically closer? Taking into account the high genetic variability within the *A. erythrurus* group, we hypothesize that the Algerian specimens will also show high genetic variability. We also hypothesize that the Algerian specimen Aeb2 did not group with specimens from Morocco, which are geographically closer, because the Moulouya Valley could be acting as a biogeographic barrier in the same way proposed for other species (Alvarez et al., 2000). On the other hand, the specimen from the Anti-Atlas Mountains (Aeb1) appears to be separated from other *A. e. belli* from Morocco by the Atlas Mountains, which could be acting, in some degree, as a biogeographic barrier to the distribution of *A. erythrurus*. This pattern was also found in agamid lizards (Brown et al., 2002) and in Mauremys leprosa turtles (Fritz et al., 2005).

The fact that this specimen (Aeb1) from Jebel Sirwah is more closely related to specimens from Algeria than to all other specimens from Morocco is consistent with other taxa such as the Wall Lizards *Podarcis* (Pinho et al., 2006; Pinho et al., 2008), or Mauremys freshwater turtles (Fritz et al., 2006). This common pattern could suggest that the past history of these species was strongly affected by specific historical biogeographic events that occurred in this region. Interestingly, the estimated divergence date (9.3 Ma; HPD between 5.4 and 14.0 Ma) for the clade A, which includes *A. e. belli* from the South of the Atlas, is coincident with the timing of uplift of the Moroccan Atlas (even taking into account the confidence intervals). The main period of uplift of these mountains began during the mid-late Miocene as a consequence of the compression of the European and Eurasian plates (Gómez et al., 2000). The divergence between North and South Atlas populations could be caused by the formation of these mountains. This biogeographical event was suggested to be responsible for the differentiation in other reptiles (Schleich et al., 1996; Brown et al., 2002).

Clade B is not well supported in the mtDNA analyses (BI-66; ML-58; MP < 50). In the nuclear analyses it forms a well supported group with the other samples from Northern Morocco and from the Iberian Peninsula (BI-100; ML-90; MP-91), but it also has haplotypes related to those from samples of Central and Southwestern Morocco. This scenario might suggest that these populations were recently in contact as a result of climatic changes and consequent habitat expansions/contractions leading to periods of contact/isolation between populations (Fonseca et al., 2008).

Both clades C and D are well supported in the mtDNA analyses and include two different species (*A. lineomaculatus* and *A. e. atlanticus*). Paraphyly of *A. erythrurus* is also present in the nuclear analyses. Based on the SH tests, monophyly of *A. e. atlanticus* could not be rejected by the nDNA marker and all the remaining tests suggest that there is no support for the specific and subspecific status of *A. lineomaculatus* and *A. e. belli*, respectively. Therefore, our results do not suggest that *A. lineomaculatus* was isolated on the Atlantic coast for a long period of time, as Bons and Geniez (1995) suggested. Moreover, the gene flow observed between *A. lineomaculatus* and *A. e. belli* may suggest that these different “species” are, in fact, single ecotypal adaptations to different habitats.

Curiously, Salvador (1982) and Arnold (1983) do not retain the subspecies *A. e. atlanticus* or the species *A. lineomaculatus*. These two revisers considered the latter species as a subspecies of *A. erythrurus*. In addition, Squalli-Houssani (1991) suggested that the different subspecies of *A. erythrurus* occurring in Morocco should not be recognized taxonomically, as morphological variability is just reflection of their distribution. A more recent study, which analyzed morphologically 87 specimens of *A. erythrurus* from different localities from the region of Marrakech (Morocco), detected individuals with intermediate characteristics between *A. e. lineomaculatus* and *A. e. atlanticus*, suggesting that these two subspecies can hybridize and that there is perhaps no reproductive isolation between them (Slimani and Roux, 1994). Nonetheless, Bons and Geniez (1995) retain the taxonomic status of *A. e. atlanticus* and of *A. lineomaculatus* based on the morphological analyses of 496 Moroccan individuals from 22 localities.

4.2.2. Iberian Peninsula

We found within the Iberian Peninsula (mtDNA clades E1 and E2) more genetically distinct lineages than the two reported in Harris et al. (2004). Essentially, adding specimens from different localities increased the genetic variability. In fact, specimens from the Iberian Peninsula do not form a monophyletic group in the mtDNA analyses. Two specimens from Southern Spain, one from Cadiz (Aee11) and one from Malaga (Aee10), formed a different clade. This could suggest independent colonization events from North Africa to the Iberia Peninsula or colonization from multiple populations.

Nevertheless, the monophyly of *A. e. erythrurus* could not be rejected based on the SH test of the mtDNA tree. In addition, in the
nuclear phylogenetic analyses the Iberian group was monophyletic and nested within the N–NE Moroccan clade (mtDNA clades B and C). These results suggest that the European lineages share their recent common ancestral with the geographically closest Northern Morocco mainland lineages.

The estimated divergence time between North-African and European lineages (5.3 Ma) is coincident with the reopening of the Strait of Gibraltar at the end of the Messinian Salinity Crisis. This estimation suggests that the reopening of the Strait of Gibraltar at the end of the Messinian Salinity Crisis was the main event causing the divergence between European and North-African populations. Harris and colleagues (2004) already suggested this hypothesis, but we must take into account our confidence intervals of the estimated date. The lowest value of HPD is down to 2.9 Ma and the highest HPD value is up to 8.2 Ma. Consequently, we cannot exclude pre or post Messinian Salinity Crisis vicariance hypotheses between North-African and European lineages. The inclusion of other genes (e.g. cytochrome b), already used for calibration dates in similar studies (Carranza et al., 2006; Paulo et al., 2008), would probably clarify some uncertainties of the divergence times.

Our results show subclades inside the Iberian Peninsula with geographical structure (Fig. 3). The mtDNA tree is better resolved than the nDNA one, as expected from the higher mtDNA mutation rate. Interestingly, the higher genetic variability found in the South, which is concordant with the fact that this species is typically Mediterranean, suggests that A. e. erythrurus was isolated in a Southern refugium inside the Iberian Peninsula, probably near or in the Southern Betic Ranges (Gómez and Lunt, 2007).

4.3 Connecting biogeography, phylogeny and taxonomy of the A. erythrurus group

The fringe-toed lizards of the A. erythrurus group occur in different bioclimatic regions in North Africa and Iberia but avoid hyper-arid areas (sensu Le Houérou, 1996). In the past climatic history of North Africa, four pluvial and arid periods alternated between the Miocene arid climate and the Pleistocene (~1.6 Ma). However, the climate changed again to arid at the Last Glacial Maximum (Sarnthein, 1978). After that, humidity increased again, reaching a maximum between 9000 and 8000 years ago, but then it was followed by a short arid phase.

The complex micro-evolution of this group could be a consequence of these “fast” climate changes associated with contraction/expansion phases of the habitats of the Acanthodactylus lizards (Fonseca et al., 2008). Furthermore, North Africa was much less affected by the last ice ages, so that while European fauna were restricted to glacial refugia and thus lost much genetic variability, North-African species retained more diversity (Pinho et al., 2007).

The molecular complexity resulting from alternating short intervals of isolation and differentiation with intervals of connectivity and admixture associated with dispersal of A. erythrurus group lizards makes it difficult to estimate the phylogeny of the group and to identify species boundaries within the group, especially between A. erythrurus, A. blanci and A. lineomaculatus. A similar scenario was described for the genus with the A. pardalis group, and it was suggested that typical binomial classification of species did not fit well in this species complex (Fonseca et al., 2008). To resolve the taxonomy of these three species we suggest two alternative hypotheses: (i) all species are reduced to one with very high genetic variability and a wider distribution; or (ii) these species are merged and termed the “A. erythrurus species complex” pending further investigation. No solution is entirely satisfactory, but we suggest the latter. Nonetheless, deeper investigations, including contact zones between the clades and analyses of other species subject to similar biogeographic histories (e.g. Acanthodactylus boskianus), may help to clarify these complex evolutionary scenarios.

4.4 Conservation issues

Although biodiversity conservation is a global concern, the feasibility of conservation actions requires priorities. Myers et al. (2000) contributed in this respect by identifying biodiversity hotspots characterized by high levels of endemism affected by an exceptional degree of habitat loss. One of the suggestions highlighted by Myers et al. (2000) was that the conservation support should focus on the Mediterranean basin. Within the Mediterranean basin there are 355 species of reptiles, of which 170 (48%) are endemic. Endemism is especially high in the Lacertidae family. Although the species richness of reptiles is highest in the Eastern part of the region, North Africa also has considerable numbers of reptile species. Furthermore, according to Cox et al. (2006) there is a concentration of threatened reptile species in North Africa, especially in Northern Morocco and North-eastern Algeria. As expected, habitat loss and degradation are the major threats to the survival of both threatened and non-threatened species of reptiles.

Our study (and also Fonseca et al., 2008) may change the conservation status of some Acanthodactylus species, particularly, if A. blanci (A. erythrurus group), an endangered species (IUCN Red List of Threatened Species, v.3.1, 2001), is considered a population of A. erythrurus, listed as least concern (IUCN, 2001), and if A. mechriguensis (A. pardalis group), a critically endangered species (IUCN, 2001), is synonymised with A. maculatus; listed as least concern (IUCN, 2001).

Conservation actions will fall, firstly, upon endemic species with restricted spatial distribution. Therefore, it is urgent to solve the taxonomic incongruence of the genus Acanthodactylus, because if taxonomy within the group changes, then conservation priorities should also be reassessed.

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