Comparative phylogeography of northwest African *Natrix maura* (Serpentes: Colubridae) inferred from mtDNA sequences

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While the comparative phylogeography of European fauna is relatively well understood, with Pleistocene climatic oscillations leading to ‘southern refugia’ for many species, the equivalent pattern has not been determined for North Africa. In this context variation within North African populations of the riverine snake *Natrix maura* were assessed using mitochondrial DNA sequences. Recent literature regarding North African phylogeographic studies of reptiles was compiled, and several surprising patterns emerged. The most interesting was the extensive movement of species across the Strait of Gibraltar during the Pleistocene. Another is the general pattern of deep genetic divergences between Tunisian and Moroccan populations, often at a level implying the existence of cryptic species. *Natrix maura* has three distinct lineages in North Africa, however, it apparently did not cross the Strait of Gibraltar during the Pleistocene, but probably did so during the Messinian salinity crisis.

Key words: *Natrix maura*, mitochondrial DNA, comparative phylogeography, northwest Africa.

**INTRODUCTION**

The role of Pleistocene climatic oscillations in shaping genetic diversity is well known, with ‘southern refugia’ harbouring much of the variation (Hewitt 2000). Studies within the Iberian Peninsula have largely supported this, with deeply divergent lineages reported within many reptile species such as *Podarcis hispanica* (Pinho et al. 2006), *Blanus cinereus* (Vaconcelos et al. 2006) and *Lacerta schreiberi* (Godinho et al. 2005), leading to the hypothesis of ‘refugia within refugia’ (Gomez & Lunt 2006). In terms of phylogeography, species that occur in both the Iberian Peninsula and North Africa are of particular interest. During the Messinian period a land connection briefly linked these two areas across the Strait of Gibraltar (Duggen et al. 2003), allowing considerable faunal interchange. Since then the Mediterranean has continually separated Europe from North Africa, and most reviews of the post-glacial recolonization of Europe do not consider any role played by North African fauna (e.g. Hewitt 1999). Indeed, genetic variation across the Strait of Gibraltar has been used as a calibration for molecular clocks, assuming that diversification is a direct result of the opening of the Mediterranean, approximately 5.3 Mya (e.g. Sotiropoulos et al. 2007). Several studies of related Iberian and North African taxa do seem to support such a hypothesis, including one lineage of the *P. hispanica* complex (Pinho et al. 2006), *Acanthodactylus erythrurus* (Harris et al. 2004a) and *Salamandra* (Escoriza et al. 2006). However, other taxa crossed the narrow marine barrier, presumably by rafting, such as a different lineage of the *P. hispanica* complex (Pinho et al. 2006) and *Psammodromus algirus* (Carranza et al. 2006). More surprisingly, many other species show almost no differentiation between northwest Africa and Iberia, indicating mid-Pliocene colonizations, possibly during glacial maxima when sea levels were lower. These include chameleons (Paulo et al. 2002), tortoises (Álvarez et al. 2000), the terrapin *Mauremys* (Fritz et al. 2006) and three snakes *Macroprotodon* (Carranza et al. 2004), *Malpolon* and *Hemorrhois hippocrepis* (Carranza et al. 2006). Such studies indicate that analysis of North African taxa is necessary to better understand species distribution within Europe. They also show that widespread sampling is needed, or entire genetic lineages may go unreported. In North Africa deeply divergent genetic lineages,
possibly reflecting the existence of currently un-
recognized species, have been reported in the
lizards *Lacerta perspicillata* (Perera et al. 2007),
*Tarentola mauritanica* (Harris et al. 2004b,c), *Urom-
styx* (Harris et al. 2007), *Podarcis hispanica* (Pinho
et al. 2006, 2007) and *Agama* (Brown et al. 2002).
Deep divergences in the snakes *Macroprotodon* and
*Malpolon* have also contributed to the recognition
of multiple species within these groups in North
Africa (Carranza et al. 2004, 2006b).

The viperine snake *Natrix maura* (Linnaeus, 1758) is widespread in Europe from Portugal and
Spain through France to southwestern Switzer-
land and northwest Italy. In North Africa it occurs
in much of Morocco, northern Algeria, northern
Tunisia and northwest Libya. It is also present on
several Mediterranean islands (Arnold 2002). A
diurnal species, it is normally found close to or
within water bodies such as streams or ponds. It
has also been found in brackish water, and may
therefore be a candidate for traversing narrow
marine barriers. Guicking *et al.* (2002) reported
three genetic lineages based on mtDNA; one in
Europe, one in Morocco, and one in Tunisia and
Sardinia. In an extension of this study Guicking *et al.*
(2006) indicated that the Moroccan and European
lineages are sister taxa (96% Bayesian posterior
probability). These authors use the Strait of Gibraltar
as one of the calibration points to date a molecular
clock, assuming European and Moroccan lineages
separated 5.3 Mya.

The aim of this study was to extend the analysis
of *N. maura* across more of its North African range.
With greater sampling the chance of detecting signs of recent gene flow across the Strait, as seen in
*Malpolon* and *Macroprotodon*, is increased. By
including samples from across proposed geological
barriers, such as the Atlas Mountains and the
Moulouya river valley, the influence of these on
population structure within *N. maura* can be
assessed. Both could be barriers to gene flow in
*Natrix*, the Atlas Mountains being inappropriate
habitat except in some valleys, and the Moulouya
River valley being an expansive arid zone that
is presently too dry for *Natrix* except near the
Mediterranean coast. Additionally, samples from
Jebel Sirwah, at the extreme south of the range,
and south of the High Atlas Mountains, were
included. *Podarcis* lizards from this relatively
isolated mountain are more similar to Tunisian
than to other Moroccan populations (Harris
et al. 2002; Pinho et al. 2003, 2006). Similarly in the
terrapin *Mauremys leprosa*, specimens from south of the
Atlas Mountains resemble eastern Algerian and
Tunisian populations, but not other Moroccan
populations (Fritz *et al.* 2006). Thus the generality
of this biogeographic pattern can also be examined.

**METHODS**
Specimens were identified in the field following
Arnold (2002), digital photographs taken, and a
portion of tail removed and stored in 100% ethanol.
Several specimens were road kills. All live specimens were released immediately after sampling. Total genomic DNA was extracted from these small pieces of tissue using standard methods (Sambrook et al. 1989). Polymerase Chain Reaction primers used in both amplification and sequencing were ND4L and ND4H (Arevalo et al. 1994), which amplify a portion of the mitochondrial ND4 gene. Amplified fragments were sequenced after sampling. Total genomic DNA was extracted employing PAUP* 4.0b10 and Modeltest (Posada & Crandall 1998). Once a model of evolution was chosen using the AIC criteria, it was used to estimate a tree using ML with a 10 replicate heuristic search. The MP analysis was carried out with a 100 replicate heuristic search and TBR branch-swapping. In both cases support for nodes was estimated by bootstrapping with 1000 replicates (Felsenstein 1985). The Bayesian analysis was implemented using MrBayes version 2.01 (Huelsenbeck & Ronquist 2001) which calculates Bayesian posterior probabilities using a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) sampling approach. Bayesian analyses were conducted with random starting trees, run 1 × 10⁶ generations, and sampled every 1000 generations using the general-time reversible model of evolution with a model of among-site rate variation. Two independent replicates were conducted and inspected for consistency to check for local optima (Huelsenbeck & Bollback 2001). In both searches stationarity of the Markov chain was determined as the point when sampled log likelihood values plotted against generation time reached a stable mean equilibrium value; ‘burn-in’ data sampled from generations preceding this point were discarded. All data collected at stationarity were used to estimate posterior nodal probabilities and a summary phylogeny.

Table 1. Specimens sequenced for this analysis, with locality data.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Sample code</th>
<th>Coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ait–Ichchou, Morocco</td>
<td>1M</td>
<td>32°25′N, 3°46′W</td>
</tr>
<tr>
<td>Jebel Sirwah, Morocco</td>
<td>2M</td>
<td>30°43′N, 7°37′W</td>
</tr>
<tr>
<td>Jebel Sirwah, Morocco</td>
<td>3M</td>
<td>30°43′N, 7°37′W</td>
</tr>
<tr>
<td>Jebel Sirwah, Morocco</td>
<td>4M</td>
<td>30°43′N, 7°37′W</td>
</tr>
<tr>
<td>Taounate, Morocco</td>
<td>5M</td>
<td>34°31′N, 4°38′W</td>
</tr>
<tr>
<td>Mouth of river Moulouya, Morocco</td>
<td>6M</td>
<td>35°2′51″N, 2°35′29″W</td>
</tr>
<tr>
<td>Mouth of river Moulouya, Morocco</td>
<td>7M</td>
<td>35°2′51″N, 2°35′29″W</td>
</tr>
<tr>
<td>Mouth of river Moulouya, Morocco</td>
<td>8M</td>
<td>35°2′51″N, 2°35′29″W</td>
</tr>
<tr>
<td>Ouaimeden, High Atlas, Morocco</td>
<td>14M</td>
<td>31°12′21″N, 7°50′51″W</td>
</tr>
<tr>
<td>Zoumi, Morocco</td>
<td>18M</td>
<td>34°48′29″N, 5°21′18″W</td>
</tr>
<tr>
<td>Ketama, Morocco</td>
<td>21M</td>
<td>34°55′02″N, 4°34′64″W</td>
</tr>
<tr>
<td>Ifrane, Morocco</td>
<td>23M</td>
<td>33°32′70″N, 5°0′03″W</td>
</tr>
<tr>
<td>Sta Marta, Spain</td>
<td>12S</td>
<td>36°28′01″N, 4°59′20″W</td>
</tr>
<tr>
<td>Sta Marta, Spain</td>
<td>13S</td>
<td>36°28′01″N, 4°59′20″W</td>
</tr>
<tr>
<td>Thala, Tunisia</td>
<td>10T</td>
<td>35°34′28″N, 8°40′20″E</td>
</tr>
<tr>
<td>Thala, Tunisia</td>
<td>16T</td>
<td>35°34′28″N, 8°40′20″E</td>
</tr>
<tr>
<td>Jebel Zebla, Tunisia</td>
<td>17T</td>
<td>36°49′N, 9°16′E</td>
</tr>
<tr>
<td>Bouira, Bordj bou Arreridj, Algeria</td>
<td>24A</td>
<td>36°11′43″N, 4°23′68″E</td>
</tr>
<tr>
<td>Batna, Algeria</td>
<td>25A</td>
<td>36°34′78″N, 6°4′57″E</td>
</tr>
<tr>
<td>Batna, Biska, Algeria</td>
<td>28A</td>
<td>35°12′32″N, 6°18′09″E</td>
</tr>
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<td>Bou Saada, Sour el Ghozlane, Algeria</td>
<td>29A</td>
<td>35°12′55″N, 4°10′46″E</td>
</tr>
<tr>
<td>Faro, Algarve, Portugal</td>
<td>37P</td>
<td>37°0′08″N, 7°53′02″W</td>
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</tbody>
</table>
RESULTS & DISCUSSION

Including the outgroups, 31 mtDNA sequences were analysed. It was concluded that the TrN model with an estimated proportion of variable sites was the most appropriate model of evolution for these data. A 10 replicate heuristic search incorporating this model found one tree of –ln 1881. Maximum parsimony analysis of 78 informative characters found eight trees of 212 steps, the 50% bootstrap consensus of which was similar to the ML analysis, but differed in weakly supported nodes (Fig. 2). The Bayesian analysis produced a single estimate of relationships with an identical topology to ML.

Four genetically distinct units can be identified from these analyses, all of which are geographically coherent, although relationships between these units are not all well supported. As found in previous studies (Guicking et al. 2002, 2006), one lineage comprises all the European specimens. Within this lineage there is considerable substructuring, in particular between one sample from southern Spain (from Cadiz province) and the remaining European specimens (approximately 2.5% sequence divergence using the ML model). Such phylogeographic structuring fits with the refugia within refugia hypothesis (Gomez & Lunt 2006). In other words, not only did the Iberian Peninsula act as a glacial refugia for many taxa (Harris et al. 2007). High intraspecific variation within this region has also been reported in other reptiles, such as the lizards Acanthodactylus erythrurus (Harris et al. 2004a), A. pardalis (Fonseca et al. 2008) and Tarentola mauritanica (Harris et al. 2004b,c). Possibly in the smaller lizards, populations could survive in small, isolated patches of favourable habitat and these each evolved unique haplotypes that led to the considerable phylogeographic variation now observed. The snakes, however, required larger populations of prey to sustain viable populations and thus were limited to the very few large regions of appropriate habitat. From these they expanded rapidly after the last glacial maximum, and thus haplotypic variation is minimal.

Interestingly, M. monspessulanus, M. cucullatus and H. hippocrepis all have limited genetic differentiation between Iberian and Moroccan populations, implying a recent crossing from North Africa to Iberia. Natrix maura, however, shows no evidence of such an event. Greater sampling in southern Iberia will be needed to confirm this. Several other reptiles also show a similar phylogeographic pattern, including chameleons (Paulo et al. 2002), terrapins (Fritz et al. 2006), tortoises (Álvarez et al. 2000) and Blanus worm lizards (Vasconcelos et al. 2007).
In isolation these could be assumed to be due to recent anthropogenic introductions, but such a high proportion of diverse species requires an alternative explanation. Possibly they all crossed naturally by ‘hopping’ across the Strait of Gibraltar via temporary islands that were exposed during sea-level falls associated with the last glacial period (Carranza et al. 2006). Thus the Iberian Peninsular diversity may be the result of a mixture of European and North African lineages, as well as due to its role as a ‘southern refugium’.

Pinho et al. (2006, 2007a,b) showed that the wall lizards, *Podarcis*, from Jebel Sirwah, an isolated mountain in Southern Morocco, were more closely related to Tunisian populations than to nearby Moroccan ones. *Mauremys leprosa* also has

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**Fig. 2.** Single tree derived from a ML analysis using the model described in the text. Bootstrap values for MP and ML are given below and above the respective nodes. All nodes with bootstrap support indicated had Bayesian posterior probabilities above 95%. Codes relate to Table 1 and Fig. 1. The tree was rooted using *Natrix natrix* and *Natrix tessellata*.
haplotypes in southern Morocco that are more closely related to eastern Algerian and Tunisian haplotypes (Fritz et al. 2006). A similar pattern is seen in A. erythrurus, in that the Jebel Sirwah population is genetically distinct from Moroccan populations, and for mtDNA at least, is more closely related to Algerian populations (Fonseca et al., unpubl.). However, in N. maura such a pattern is not recovered. The higher dispersal capability of N. maura may mean that the mountain of Jebel Sirwah is less isolated for this species. Analysis of other snakes and lizards from this area will be valuable in assessing the general biogeographic pattern of a link between the mountains of the Anti-Atlas of southern Morocco with Algeria and Tunisia.

CONCLUSIONS

Although clearly not as well studied as Europe, biogeographic patterns for the northwest African reptile fauna are beginning to take shape. The most important pattern is a high ratio of species spreading across the Strait of Gibraltar during the Pleistocene. Thus, to fully understand the post-glacial recolonization of Europe, a more detailed assessment of variation within North Africa is needed. Whether or not a similar pattern also exists between North Africa and southern Italy remains to be verified. Almost every species examined in North Africa shows levels of intraspecific variation higher than expected relative to European taxa, supporting the hypothesis of a geopolitical bias affecting expectations of mtDNA variation found within species (Harris & Froufe 2005). The Mediterranean Basin is an acknowledged hotspot for biodiversity (Myers et al. 2000), but this would imply that the importance of North Africa is even greater than that of the southern European peninsulas. A general pattern of high differentiation between Moroccan and Tunisian specimens is observed in many species, but sampling is typically limited in Algeria and therefore the exact ranges of these genetic lineages often remains unknown. Further lineages may be found as sampling is extended. This is the case for N. maura, with three distinct North African lineages.

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