Phylogeographic patterns of the Moroccan lizard-fingered gecko *Saurodactylus brosseti*

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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ABSTRACT

Various assessments of phylogeographic patterns have been extensively performed throughout Europe, and general patterns discerned however similar studies remain insufficient for other regions such as North Africa. Morocco has multiple geographical barriers, such as the Moulouya River basin, the Rif Mountains and the Atlas Mountains. The latter divides this country into two bioclimatic regions, which in turn is responsible for the high levels of endemism. It has been reported that genetic subdivision in several species is coincident with the orogeny of the Atlas Mountains around 9 Mya, for example in *Natrix maura*, *Agama impalearis* and *Myotis nettereri*.

One very old genus of gecko endemic to Morocco is *Saurodactylus*, comprising three species: *S. mauritanicus* (Duméril and Bibron, 1836); *S. fasciatus* Werner, 1931 and *S. brosseti* Bons and Pasteur, 1957. A previous study demonstrated high variation within S. brosseti, with 11.4% of genetic diversity for ND4, implying complex phylogeographic patterns. This raises several questions, such as if this might represent a species complex, and how diversity may be divided by the known geological barriers in the region. However, sampling was insufficient to address these issues.

In order to assess if phylogenetic and geographical patterns of S. brosseti are related to the orogeny of the Atlas Mountains, additional sampling was needed, and therefore two trips to Morocco were carried out in 2013 and 2014. The field surveys resulted in a short note of new range expansions for some species, and in particular *Bufo spinosus, Trapelus boehmei, Tropiocolotes algericus, Acanthodactylus erythrurus, Chalcides polylepis* and *Scutophis moilensis*. Accurate species distribution maps are necessary for any conservation efforts, and this highlights the need for more prospection in the region.

For the target species, *S. brosseti*, two different approaches were combined. For a phylogeographic assessment within the species two mitochondrial and three nuclear genes were sequenced and analyzed. Four main lineages can be differentiated with a level of diversity typically observed between species. The orogeny of the Atlas Mountains occurred at about the same time as these lineages split, and therefore may well have been the barrier that led to the differentiation of these lineages. However, species delimitation approaches were not completely effective at identifying each lineage as a distinct “species”, possibly due to the limited number of specimens included for two of the four lineages. The second approach was to employ a species distribution model to try to identify regions of appropriate habitat were the species may occur. The model identified a large patch of suitable habitat where future sampling effort should be directed. Such studies are crucial for conservation issues; as

regardless of whether these “lineages” are recognized as species or not, considerable diversity occurs in some small populations to the East of the Atlas that could easily be lost to habitat destruction. The study demonstrates the value of combining fieldwork, molecular analyses and modelling approaches to gain new insights into the evolutionary history of a species.

KEYWORDS

Phylogeography, *Saurodactylus brosseti*, Species probability of occurrence, Vicariance, Atlas Mountains

RESUMO

Padrões filogeográficos têm sido extensivamente estudados na Europa. No entanto, estudos semelhantes são limitados noutras regiões, tal como o Norte de África. Marrocos apresenta várias barreiras geográficas, tais como a bacia do Rio Moulouya, as Montanhas Rif e as Montanhas Atlas. Estas últimas dividem o país em duas regiões bioclimáticas, o que por sua vez é responsável pelos altos níveis de endemismo do país. A subdivisão genética de várias espécies, como por exemplo, *Natrix maura*, *Agama impalearis* e *Myotis nettereri* é coincidente com a orogenia do Atlas há cerca de 9 milhões de anos.

Saurodactylus é um género de osga muito antigo endémico de Marrocos, incluindo três espécies: *S. mauritanicus* Duméril and Bibron, 1836; *S. fasciatus* Werner, 1931 e *S. brosseti* Bons and Pasteur, 1957. Um estudo realizado demonstrou que *S. brosseti* apresenta uma grande variação intraespecífica, com 11.4% de diversidade genética para o marcador ND4, o que implica padrões filogeográficos complexos. Este facto suscita várias questões, tais como a existência de um complexo de espécies e de que forma a diversidade observada pode estar dividida pelas barreiras geológicas da região. No entanto, a amostragem mostrou-se insuficiente para responder a estas questões.

De modo a compreender a relação dos padrões filogenéticos e geográficos de *S. brosseti* com a orogénese das Montanhas Atlas, seria necessária amostragem adicional. Como tal, duas viagens de campo a Marrocos, em 2013 e 2014, foram realizadas. As observações feitas resultaram numa anotação científica com a descrição de expansões de distribuição para algumas espécies, em particular *Bufo spinosus, Trapelus boehmei, Tropiocolotes algericus, Acanthodactylus erythrurus, Chalcides polylepis* e *Scutophis moilensis*. Para processos de conservação, mapas de distribuição de espécies com maior precisão são necessários realçando a necessidade de uma maior prospeção da região.

Duas diferentes abordagens foram combinadas para a espécie em estudo, *S. brosseti*. Para uma avaliação filogeográfica, dois genes mitocondriais e três nucleares foram sequenciados e analizados, podendo ser distinguidas quatro linhagens com um nível de diversidade tipicamente observado entre espécies. A orogénese do Atlas ocorreu na mesma altura que a separação destas linhagens, indicando a possibilidade de esta ter constituído a barreira que levou à sua diferenciação. Contudo, a abordagem de delimitação de espécies não foi completamente eficaz na identificação de cada linhagem como “espécies” diferentes, possivelmente devido ao número limitado de indivíduos em duas das quatro linhagens.

A segunda abordagem consistiu na modelação de distribuição de espécies com o objectivo de tentar identificar locais onde o habitat seja adequado à ocorrência de espécies. O modelo identificou um grande fragmento onde amostragem futura deverá ser realizada. Este tipo de estudos é crucial em termos de conservação, independentemente destas linhagens serem reconhecidas como espécies ou não. Pequenas populações a Este do Atlas possuem uma diversidade considerável, podendo estar facilmente sujeitas à destruição de habitat. Esta análise realça o valor de combinar trabalho de campo, análises moleculares e modelação, de forma a obter melhores perspectivas acerca da história evolutiva de uma espécie.

PALAVRAS-CHAVE

Filogeografia, *Saurodactylus brosseti*, Probabilidade de ocorrência de espécies, Vicariância, Montanhas Atlas

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

LIST OF ABBREVIATIONS

Mya	Million years ago
DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial Deoxyribonucleic acid
NE	Northeast
SW	Southwest
MRCA	Most recent common ancestor
RFLP	Restriction fragment length polymorphism
UPGMA	Unweighted pair group method with arithmetic mean
NJ	Neighbor joining
MP	Maximum parsimony
ML	Maximum likelihood
MCMC	Markov chain monte carlo
ENM	Ecological niche model
GBIF	Global biodiversity information facility
GPS	Global positioning system
WGS	World geodetic system
SVL	Snout vent length
PCR	Polymerase chain reaction
UK	United Kingdom
dNTP	Deoxynucleotide
$MgCl_2$	Magnesium chloride
NCBI	National center of biotechnology information
AUC	Area under the curve

GENERAL INTRODUCTION

Genus Saurodactylus

The genus *Saurodactylus* (Family Gekkonidae) is an old taxon dating back almost 100 million years (Gamble *et al*., 2011) and is endemic to Maghreb region, North Africa (Bons and Geniez, 1996; Schleich *et al.* 1996). It currently comprises three species; *Saurodactylus mauritanicus* Duméril and Bibron 1836, *Saurodactylus fasciatus* Werner 1931 and *Saurodactylus brosseti* Bons and Pasteur 1957 (Bons and Geniez, 1996; Schleich *et al.* 1996) (Fig. 1). *Saurodactylus brosseti* was initially considered a subspecies of *Saurodactylus mauritanicus,* however, morphological differences in pholidosis, (mainly its sub-tail scales), head shape and coloration (Bons and Geniez, 1996) along with recent phylogenetic analysis support the full species status of each form (Rato and Harris, 2008; Gamble *et al.*, 2011).

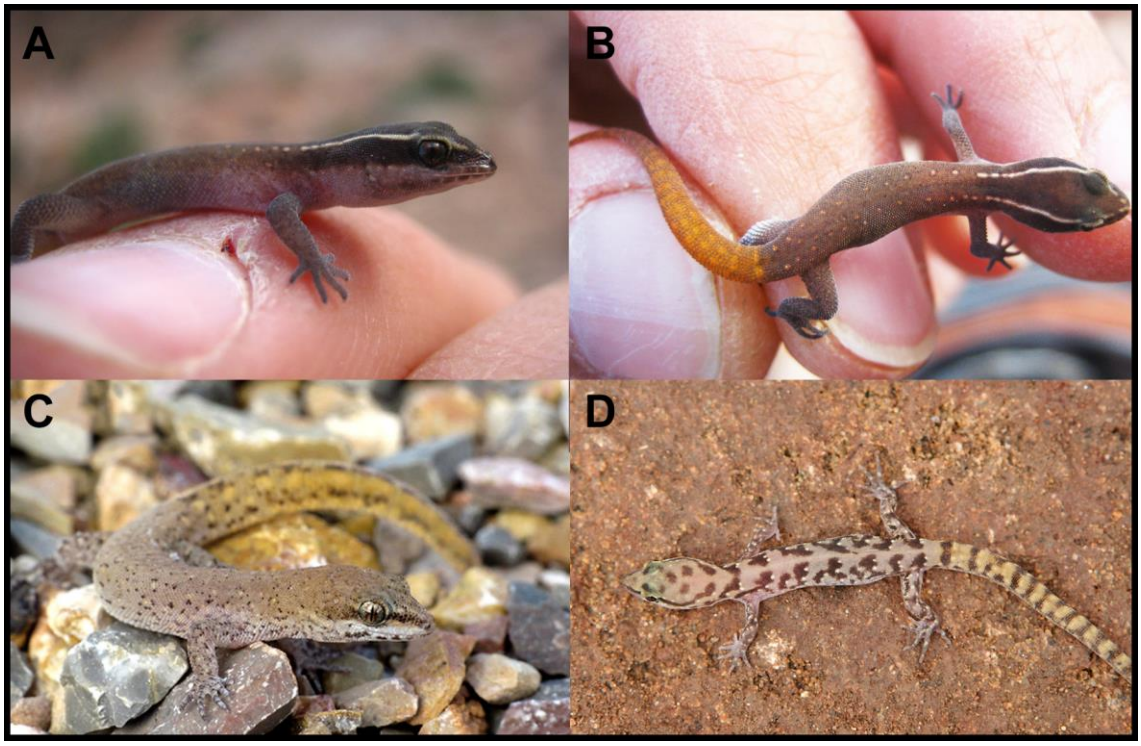

Fig.1. – A: *Saurodactylus brosseti*, lateral view; B: *Saurodactylus brosseti*, dorsal view; C: *Saurodactylus mauritanicus*; D: *Saurodactylus fasciatus*. Photos of *S. brosseti* were taken by Daniele Salvi; photos of *S. mauritanicus* and *S. fasciatus* were downloaded from www.moroccoherps.com (accessed on May 2013).

Although in (Rato and Harris, 2008) the genus *Saurodactylus* was found to be paraphyletic with respect to *Teratoscincus przewalskii*, in (Gamble *et al*., 2011) there is support for the monophyly of the genus. Diversification of the three lineages within *Saurodactylus* occurred between approximately 93 and 25 million years ago (Mya).

According to Bons and Geniez (1996) *Saurodactylus brosseti* is endemic to Morocco with a wide range from Beni Mellal, central Morocco, to the depths of Western Sahara in the south (Fig. 2.). The southernmost specimens were reported in Gueltat Zemmour, and specimens have been recorded from both sides of the Atlas Mountains (Bons and Geniez, 1996). *Saurodactylus mauritanicus* inhabits northeast Morocco and western Algeria (Bons and Geniez, 1996) and is allopatric with *Saurodactylus brosseti*, whilst *Saurodactylus fasciatus*, also an endemic species to Morocco, is distributed in the north and west of the High Atlas and in the southeast of the Rif (Bons and Geniez, 1996) (Fig. 2.). In some localities the range of *S. fasciatus* overlaps with the *Saurodactylus brosseti* distributions (Fig. 2.). The three species inhabit arid and semiarid bioclimates, with *Saurodactylus brosseti* also occurring in the Saharan floor (Bons and Geniez, 1996).

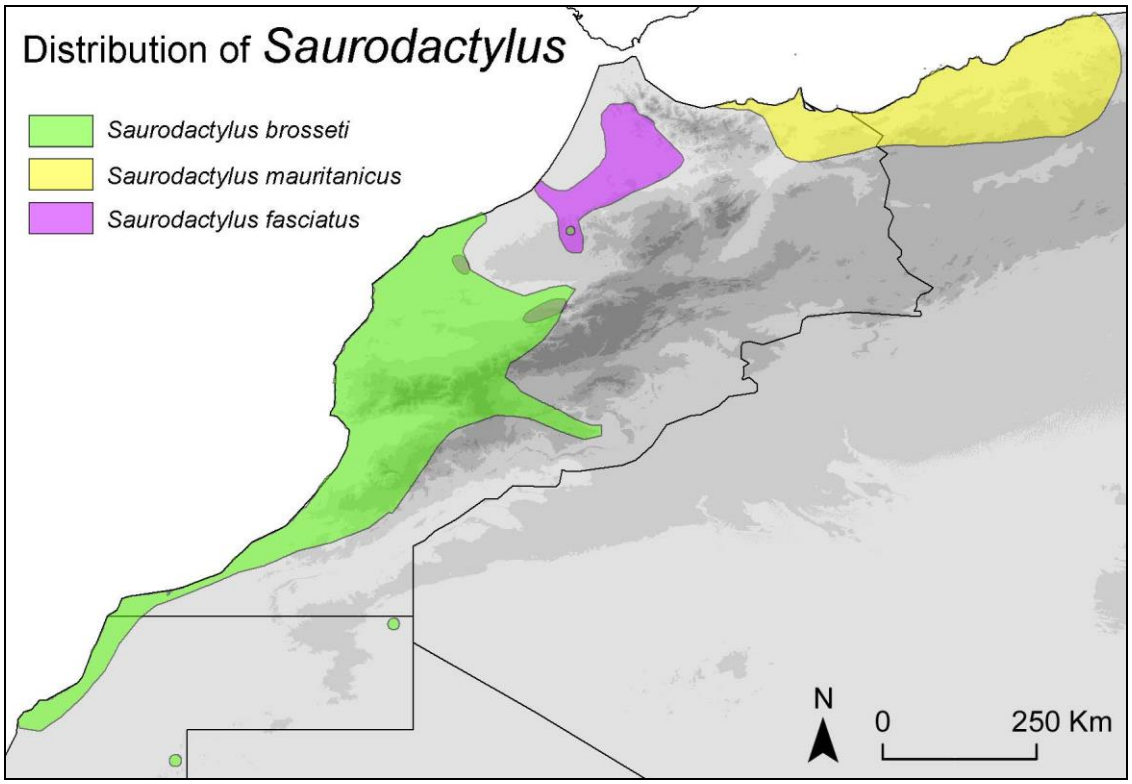

Fig. 2. Distribution of all three species of *Saurodactylus*. Shapefiles downloaded from IUCN website.

Since *Saurodactylus* is a very old genus there has been considerable time for diversity to accumulate. The only study regarding the phylogeography of the genus was conducted by (Rato and Harris, 2008) who analysed variation within *Saurodactylus* and assessed relationships of the genus with respect to the remaining Gekkota members using two mitochondrial (12S and ND4) and two nuclear markers (RAG1 and C-*mos*). They concluded that *Saurodactylus brosseti* differed from *Saurodactylus mauritanicus*

by 21% and that these two had a 25% of divergence from *Saurodactylus fasciatus*, which is higher than levels of divergences typically observed between reptiles of the same genus (Harris, 2002).

Saurodactylus brosseti

This study aims to unravel the evolutionary history of *Saurodactylus brosseti*. Being a widely distributed gecko species in Morocco, this study gives us insight into interesting phylogeographic and speciation events and in an area in which molecular evolutionary studies remain sparse. The ecology of *Saurodactylus brosseti*, similarly to the other two species of the genus, includes the occupancy of arid areas with reduced slope in which dispersed stones and rocks provide refuge where the animals spend most of their time (Bons and Geniez, 1996). *Saurodactylus brosseti* is considered a Mediterranean species that adopts a sit-and-wait predation strategy emerging only during dusk and/or at night. (Meek, 2008) reports how *S. brosseti* occasionally actively forages during the day (Meek, 2008). An ecophysiological study (Meek, 2008) reported that thermoregulation in *Saurodactylus brosseti* is associated with microhabitat selection, i.e., selecting rocks or barks as a retreat site and subtly moving under them, a behaviour also observed in other geckos (reviewed in Meek, 2008). Due to their small size, geckos often have a high surface area to volume ratio. By seeking refuge in suitable microhabitats, *S. brosseti* minimises the effects of extreme body temperatures and dehydration as well as reducing predation vulnerability (Meek, 2008). No other thermoregulation methods, for example basking and above ground activity, have been recorded in this species.

Despite the limited geographical sampling of the study by (Rato and Harris, 2008), *Saurodactylus brosseti* showed variation within forms with maximal divergence for ND4 of 11.4% which is indicative of a complex phylogeographic pattern (e.g. Pinho *et al.*, 2008). On the other hand, high levels of intraspecific mitochondrial DNA variation are often reported for geckos (e.g. Perera and Harris, 2010), and thus it is not certain whether this is only a mitochondrial DNA artefact which may not be representative of overall genetic diversity (Jesus *et al.*, 2006). Furthermore, this study (Rato and Harris, 2008) did not include samples from either the populations from the extreme South of the range, or from the East of the Atlas Mountains. Therefore a more thorough study on the phylogeographic patterns of this species was needed.

Geographic region – Morocco

The Maghreb region (formed by Morocco, Western-Sahara, Algeria and Tunisia) exhibits different geology, topography, climate, flora and fauna compared to the rest of Africa, making it a unique region in the continent (Bons and Geniez, 1996).

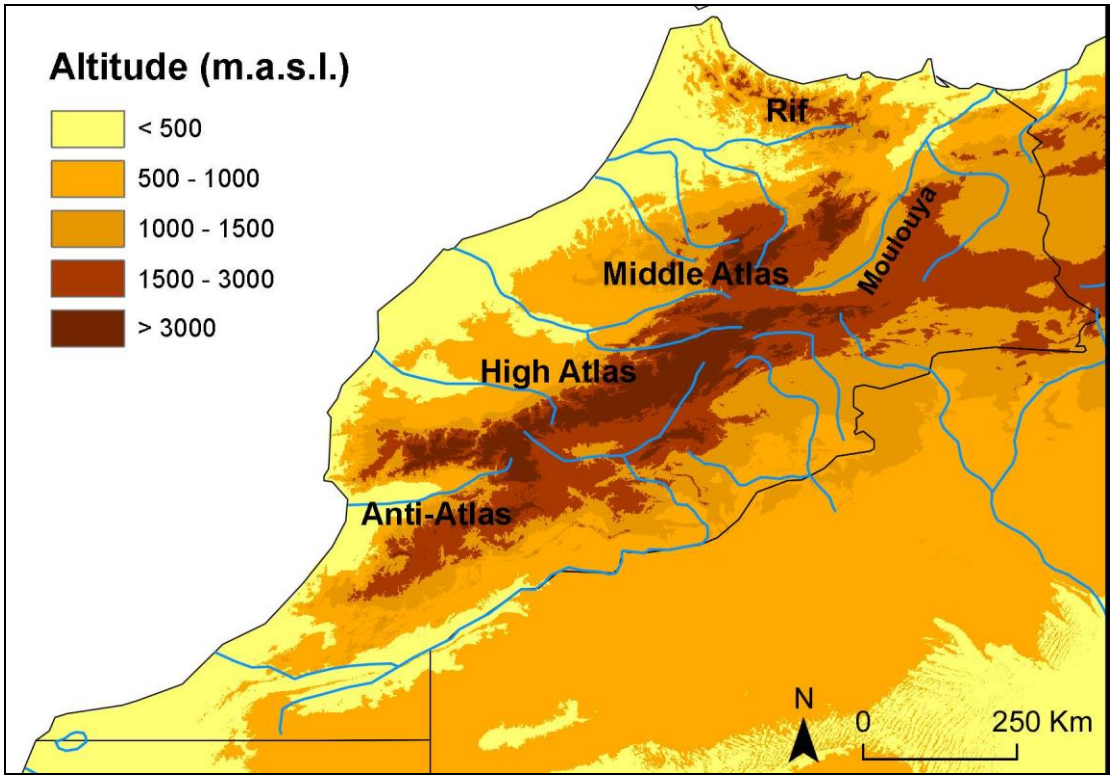

Fig. 3. – Main geographic barriers of Morocco. Moutains: Rif Mountains and the Atlas Mountains (Middle Atlas, High Atlas and Anti-Atlas). Rivers: Moulouya River.

Morocco is part of the Maghreb region and is the only country in Africa that has a maritime front with both the Atlantic and Mediterranean oceans. It has four big mountain chains, including the Rif in the North, the Middle Atlas, the High Atlas and the Anti-Atlas (Fig. 3.). The latter three form the Atlas Mountains in a NE/SW direction (Bons and Geniez, 1996). The most elevated regions in Morocco are situated in the Atlas Mountains (Missenard *et al.*, 2006).

The Atlas Mountain range covers an area of 2500 Km^2 located within Morocco, Algeria and Tunisia and form an important barrier that partitions Morocco into two different climatic and bioclimatic regions (Bons and Geniez, 1996); in the North and West of Morocco there is a Mediterranean climate with dry and hot summers and precipitation occurring irregularly during mild winters, categorising the region as sub-humid and

semiarid global bioclimates (Bons and Geniez, 1996). In the South of the Atlas the climate is Saharan and an arid bioclimate occurs in almost every region in the east of the Atlas and almost to the shores of the Mediterranean. Here, the summers are characterized for being dry and torrid with cooler winter, with the exception of the oceanic coast where cloudiness is a factor. Precipitation is extremely rare and occurs mostly during the winter; this feature is responsible for the north Sahara climate being included in the Mediterranean region (Bons and Geniez, 1996).

Morocco is also known for being quite windy throughout the year, especially during spring. Minimum temperatures at high altitudes can fall below zero degrees in winter, except along coastal strips that have milder conditions. Maximum temperatures in the summer can reach up to 60º C in Saharan regions and 46º C in the remaining territory except in the coast where the climate is more temperate due to the maritime influence. Precipitation generates particular humid biotopes and snowfall has never been recorded in the Saharan regions; in contrast it does snow in the High Atlas, Middle Atlas and highest parts of the Rif Mountains, leaving these areas covered in snow during winter and spring, which are subsequently an important reservoir of water (Bons and Geniez, 1996). The Moulouya, Sebou, Oum-er-Rbia and Tennsitt are the most important rivers originating from these mountains (Schleich *et al.*, 1996).

The multitude of bioclimatic regions within Morocco has influenced the dispersion of several Mediterranean species from the North, and some species from the Sahara. This is mainly due to the Atlas Mountains, which also cause the existence of high levels of endemic species of reptiles and amphibians in Morocco (Bons and Geniez, 1996). Indeed, Morocco is included in the Mediterranean Basin, a recognized hotspot of biodiversity (Myers *et al.*, 2000). From the western Mediterranean region, Morocco has one of the richest and most diverse herpetofauna, along with Algeria, with 104 described species of reptiles and amphibians, of which 22 are endemic (Bons and Geniez, 1996). It is well documented how endemism is especially high among amphibians, tortoises and the lizard families Lacertidae and Scincidae (e.g. Cox *et al.*, 2006). In Morocco, endemism is less seen in amphibians, which is not surprising considering the arid and semi-arid habitats predominant in large parts of the region (Cox *et al.*, 2006).

The country itself is isolated by several geographical barriers – in the North by the Mediterranean Sea, in the West by the Atlantic Ocean, in the South by the Sahara Desert and in the East by the Moulouya River Valley (Bons and Geniez, 1996). Thus, Morocco presents an ample diversity of geographic barriers and calibration points can be assessed by considering their formation age, in order to try to date phylogeographic breaks. Regarding the Moulouya River Valley, it is sometimes proposed to be a barrier

preventing dispersal and gene flow of, for example, *Testudo graeca* (Álvarez *et al.*, 2000), *Natrix maura* (Barata *et al.*, 2008) and *Gerbillus campestris* (Nicolas *et al.*, 2014). However, sampling is key – in a study with more extensive sampling of *Natrix maura* it has been shown that genetic subdivision is not completely coinciding with the Moulouya River Valley (Harris *et al.*, 2003). Evidence of the Atlas Mountain Chain acting as a barrier causing genetic subdivision has been shown in many species, such as *Agama impalearis* (Brown *et al.*, 2002; Gonçalves *et al.*, 2012), the *Acanthodactylus erythrurus* group (Fonseca *et al.*, 2009), the freshwater turtle *Mauremys leprosa* (Fritz *et al.*, 2005 and Fritz *et al.*, 2006), scorpions of the genus *Buthus* (Habel *et al.*, 2012; Husemann *et al.*, 2012), the scorpion *Androctonus mauritanicus* (Coelho *et al.*, 2014) and the bat complex *Myotis nattereri* (Salicini *et al.*, 2013).

Formation of the Atlas Mountains

The formation of the Atlas Mountains derived from three events – tectonic deformation, extension of the Earth's crust and tectonic convergence (Seber and Barazangi, 1996). In the Paleozoic Period, around 300 Mya, occurred the first event where tectonic deformations lead to the formation of the Anti-Atlas; Africa was part of the Gondwana and North America comprising Euroamerica. The Anti-Atlas is a result of the collision between the African and American plates (that formed Pangea), and was initially formed as part of the Alleghanian orogeny (Hatcher, 2008). The second event, an extension of the Earth's crust, took place 65 Mya during the Mesozoic period, which separated the African and American continents through rock deposition in the ocean that today forms the High-Atlas. The Middle Atlas is trending northeast-southwest while the High-Atlas trend is west southwest-east northeast corresponding to inverted Mesozoic intracontinental basins; both directions were inherited from initial Triassic rifting (Missenard *et al.*, 2006). Around 35 Mya during the Tertiary period, tectonic convergence of the African and European landmasses occurred in today's Strait of Gibraltar area, uplifting the mountain chains that now form the Atlas Mountains.

The orogeny of the Atlas is estimated to have occurred around 9 Mya in the late Miocene (Hsu, 1978). Contrarily, there is some evidence based on scattered direct surface suggesting that the uplift of the Middle-Atlas and High-Atlas took place around 7.1 to 5.3 Mya in the post-Miocene (Ayarza *et al.*, 2005).

Phylogeography

Phylogeography is a discipline that relates phylogenies with geographic distributions of species, allowing the assessment of how genes and their history in time affect the current distribution of species in space (Avise, 1998). Early studies using mitochondrial DNA revealed a link between genealogies and geography, which later was also demonstrated for nuclear DNA, becoming the obvious field for microevolution studies (Avise, 2009). The first practical phylogeographic study of a species was conducted using mtDNA in 1979 (Avise *et al.*, 1979), and phylogeography was subsequently recognized as a discipline. The first human study regarding mtDNA variation was conducted a year later (Brown, 1980), and some years after that a phylogeographic study involving several codistributed species was published (Bermingham and Avise, 1986).

Some phylogeographic general patterns have been identified and one simple case is species with low dispersal ability. Frequently, populations of these species are highly genealogically different, but with only a few mtDNA haplotypes, while most of the haplotypes are similar and geographically localized (Avise, 2009). Differentiation of these geographically localized haplotypes is usually coincident with historical events preventing dispersal; for example, events such as climatic oscillations in the Quaternary that forced species to refugia, which is known to have led to a lack of gene flow and thus haplotype divergence (e.g. Hewitt, 2004). Even species with high dispersal capabilities can display divergence due to historical events such as the formation of geological barriers (e.g.Jaramillo-Correa *et al.*, 2010; Mirams *et al.*, 2011; Lessios and Robertson, 2013). Dispersal barriers have an impact on the genealogical patterns of lineages or species and split events usually coincide or are more recent than the emergence of the barrier (Avise, 2009). Species with high dispersal ability can also show high genetic differentiation due to philopatry, which is a loyalty behavioral predisposition to particular locations (e.g. Wenink *et al.*, 1996; Karl *et al.,* 2011). On the other hand, not all species present such a defined phylogeographic structure. For instance, recent population expansions are generally defined by a prevalent single haplotype and many other rare haplotypes that are probably derived from a common haplotype enduring separate mutations in time. The widespread haplotype is geographically the source of the expansion (e.g. Watson *et al.*, 1997; de Jong *et al.*, 2011). Should several species have the same ecological or habitat requirements, they tend to show similar genealogical structure. This implies a broader impact of the forces shaping genes and several cases have been documented in Europe (Avise, 2009). With all of this, sometimes history repeats itself and phylogeographic patterns also

show that. The most common history repetition example is when species are forced to "southern refugia" during glaciations/deglaciations in the Pleistocene, or to multiple refugia (e.g. Hewitt, 2000; Miraldo *et al.*, 2012).

Although there are no specific natural rules dictating how genetic lineages are spatially distributed, phylogeography is currently a growing field of study (e.g. Taberlet *et al.*, 1998; Hahn *et al.*, 2014).

Coalescent Theory

Coalescent allows knowledge of historical population processes and was first described by Kingman (Kigman, 1982), although it has been discovered individually by several authors (reviewed in Rosenberg and Nordborg, 2002; Salemi and Vandamme, 2003). Coalescent is mathematically calculated and traces back the time to ancestry of a genealogy until all lineages coalesce into the most recent common ancestor (MRCA). The absence of selection is typically assumed and the rate at which lineages coalesce depends on the size of the population (Salemi and Vandamme, 2003). Should only genetic drift be a factor, smaller populations will have a faster rate of coalescent lineages; while larger populations will present a slower rate of drift and, consequently, of coalescent lineages. When comparing similarity between more than one population, historical processes can be assessed by the increased or decreased rate of genetic diversity (Salemi and Vandamme, 2003). Selection is, however, always present, and that means genetic diversity is not as random some genotypes have a higher fitness than others (Rosenberg and Nordborg, 2002).

When studying a species tree, the branch length represents the time that an ancestral form took to split into its descendants and is measured by the number of mutations that accumulated between that split. The mutations have a rate, which is logically defined by the number of mutations that are expected in each generation (Salemi and Vandamme, 2003).

Coalescent methods identify lineages that are evolving independently, testing several alternative hypotheses of divergence by using multilocus data, and can help to explain species diversity (Fujita *et al.*, 2012).

Phylogenetic data: mitochondrial and nuclear DNA

Linnaeus (1758) started using morphology (the study of the physical aspects an organism) as a way to formulate differences between species which today is still the core of taxonomy. However, modern day micro evolutionary studies are often based on DNA sequence data that allows additional information to be obtained, and is

particularly useful for assessing the relationships between taxa. Biodiversity as we observe today exists because of variations that have accumulated through diverse mechanisms (mutations, duplications of genes, reorganization of genomes, genetic exchanges (e.g. Faria *et al.*, 2011), allowing organisms to evolve. Species that are more closely related to each other typically have fewer point mutations (or substitutions) between themselves than two species that are not closely related (Salemi and Vandamme, 2003). Since some genes are highly conserved, while others are much more variable, suitable genes can be chosen that are likely to show a suitable level of variation for a variety of different questions (Salemi and Vandamme, 2003).

Although there are differences between mitochondrial and nuclear DNA, both provide valuable information and are employed to build phylogenies to understand phylogeography (e.g. Perera and Harris, 2010). Animal mitochondrial DNA (mtDNA) is a circular molecule with typically 37 genes and approximately 17,000 nucleotide base pairs with no introns or large non-coding regions. In higher animals it has a high mutation rate and is usually transmitted maternally. MtDNA is a major tool in phylogeography and it is widely used to study the evolution of species and populations (William *et al.*, 2004). The first utilization of mtDNA variation was conducted with RFLPs (Avise *et al.*, 1979; Brown and Wright, 1979) and later with sequences (Kocher *et al.*, 1989). More recently, it has been concluded by some researchers that studies using mtDNA are not sufficient if more compound questions about the history of populations are to be studied (Godinho *et al.*, 2008). This is because mtDNA provides knowledge of a single locus and it allows researchers to see only a small part of the story; consequently there is an underestimation of diversity and an oversimplification of the evolutionary history (Zhang and Hewitt, 2003). Nuclear DNA has 3 billion base pairs, almost 180,000 more than mtDNA. It also differs in the degree of recombination, ploydy and mutation rate, which is usually slower (William *et al.*, 2004). The rate of substitution in single copy nuclear polymorphic sequences depends on the genic region (Zhang and Hewitt, 2003). However, using nuclear DNA for phylogeography can also bring difficulties related to recombination, heterozygosity, rate variation and also amplification and sequencing (Zhang and Hewitt, 2003). Many studies now include nuclear DNA (Perera and Harris, 2010; Rato *et al.*, 2010), although most available data is still mitochondrial. Nuclear DNA is not going to replace mtDNA in phylogeography, but it will instead help disentangle different aspects of evolutionary history (Zhang and Hewitt, 2003).

Phylogenetic Analysis Methods

Inferring phylogenies is not an easy procedure and several approaches are accessible, although there is no perfect method and getting to the "true" phylogeny is never certain. The most common analysis methods include UPGMA (Sneath and Sokal, 1973), Neighbor-joining (NJ) (Saitou and Nei, 1987), Maximum parsimony (MP) (Fitch, 1971), Maximum likelihood (ML) (Felsenstein, 1981) and Bayesian inference (Huelsenbeck and Ronquist, 2001).

The construction of a phylogenetic tree can be separated according to the data they use (character state or distance matrix) or according to the underlying strategy (tree-evaluation or clustering) (Salemi and Vandamme, 2003). There are assumptions in each method that need to be carefully taken into consideration, as they may not be valid for the data being used. The most widely used methods are Maximum likelihood and Bayesian inference.

Maximum likelihood (ML) uses character state data type and a tree-evaluation method, using a tree topology and a model of evolution to estimate the highest probability. The likelihood is determined by adding all possible nucleotide states in the internal nodes and is optimized with the best combination of evolutionary parameters and branch length possible. This is also done by a given number of trees and the algorithm choses the tree topology that has the maximum likelihood. This process requires time and is computationally challenging. Furthermore, to assess support for actual nodes requires additional analyses, the most common of which is to apply a bootstrap approach, or to use likelihood ratio testes to compare alternative tree topologies.

Bayesian methods are in the same group as ML, however they do not search for the best tree. Instead, they try to infer several tree topologies that explain the data, which is called posterior distribution of trees, and has a confidence estimate. The method requires that a prior belief, or prior distribution is given i.e. before the analysis begins, it needs to know what the tree topology is, branch lengths and substitutions model parameters. A technique called Markov chain Monte Carlo (MCMC) is then used to acquire posterior probabilities of the tree. It calculates the likelihood in every "step" and it moves forward and if the likelihood is better than before, until it reaches the maximum value. However, it can also occasionally move backwards, allowing the methodology to overcome local optima. The analysis is typically run multiple times and with a random starting point, again so it does not stop at a local optimum. In the end, the worst trees are discarded as burn-in and posterior probabilities are the posterior values that will be represented in the Bayesian tree (Huelsenbeck and Ronquist, 2001). Bayesian

inference methods take phylogenetic uncertainty into account, but are still computationally demanding progress.

These methods can be performed on separate genes, but the data are usually combined, employing a concatenated approach. However, increase of available molecular data has made it evident that gene trees are not always coincident with species trees (Fujita *et al.*, 2012). This was first thought about when studying the disagreement of gene trees (e.g. Satta *et al.*, 2000) with the realization that is an expected feature, and concatenation approach may lead to an incorrect species tree. One other approach is a coalescent species delimitation approach which allow discordance of the gene tree under genetic drift (Fujita *et al.*, 2012). This approach can also use either ML or Bayesian; each node of the species tree represents a speciation event and each branch is an independent evolutionary lineage (Fujita *et al.*, 2012).

Biogeography

Biogeography is the science that tries to understand and describe spatial patterns of biodiversity. It studies the past and present distributions of organisms and their associated variation patterns on earth (Brown and Lomolino, 1998). Additionally, it attempts to answer questions such as 1) why does a species or family has such a range; 2) what are the characteristics that allow a species to live in such areas and not others?; 3) what is the role of climate and topography and how do these interact with the species?; 4) how do species change as the environment changes in space?; 5) where was the ancestor of a species found?; and 6) how do historical events shape species distributions? (Brown and Lomolino, 1998). The central question is related to how organisms are distributed and their history on earth.

Biogeography is intimately connected to ecology and phylogenetic history (Brown and Lomolino, 1998). It differs from other biological disciplines as it deals with scales of space and time at which experimental manipulations is difficult, serving as a comparative observational science (Brown and Lomolino, 1998). Historical biogeography is important in ecology, and large-scale biogeographical events are also the outcome of ecological processes. Historical biogeography helps to explain large-scale patterns of species richness. The ecology of the species is extremely important to better understand patterns of diversity (Wiens and Donoghue, 2004). Also, biogeographical processes determine the composition of the regional species pool (Ricklefs and Schluter, 1993; McPeek and Brown, 2000; Webb *et al.*, 2002), which has a strong effect on the composition of local-scale communities (Morin, 1999). The absence of a certain ecological association from a local community might not be

described solely by ecology or phylogenetic history of the species, but also by biogeographical patterns of the group or groups comprising the absent ecological association (Ricklefs and Schluter, 1993; Stephens and Wiens, 2004).

Geographic distribution

The geographical distribution of species is influenced by different factors according to the scale. At a macro-scale, the weather plays a predominant role in influencing geographical distributions of species. Topography, hydrology, geology and weather have an influence at a regional scale; type of soil and hydrology influence at a local scale; and herbivory, micro-disturbances and intraspecific competition have the largest influence at a micro-scale (Brown and Lomolino, 1998). The individual distribution pattern of species depends of the temporal scale of the analysis because the limits of occurrence vary with extinction or recolonization and distribution and abundance expand and contract with environmental variations and anthropogenic activities (Brown and Lomolino, 1998).

A fundamental key for biogeography is that each species has a unique geographic range and the ecological processes coupled with historical events provide essential contributions for shaping these ranges. Firstly, how can we measure and define geographic ranges? The easiest and most direct way is with range maps; they are easy to organize and can be later used by other researchers (Brown and Lomolino, 1998). There are three kinds of maps: outline maps, dot maps and contour maps, each reflecting a different aspect. Outline maps depict the supposed limit of the known species distribution as an irregular area (Brown and Lomolino, 1998). It is very prone to inaccuracies, especially if the distribution is not very well understood and if the author has made assumptions based on his/her knowledge. Dot maps are often part of a taxonomic study and plot points on a map correspond to localities where a species has been recorded (Brown and Lomolino, 1998). They can also depict places where verified museum species have been collected and they are, usually, more accurate. However, they can also represent an infinitesimal fraction of the actual distribution, for instance, in the case of sightings of birds, and they do not extrapolate beyond the relatively few sampled locations (Brown and Lomolino, 1998). Contour maps use contour lines to show variation in density, being more informative than the previous types of maps (Brown and Lomolino, 1998). The disadvantage of this method is the lack of available information relating to abundance; despite this, there is a statistical procedure, termed kriging, which interpolates between data points and produces three-dimensional landscape depicting variation in abundance within the range (Brown and

Lomolino, 1998). Databases are also a powerful tool for mapping geographical distributions, although they may contain errors on the spatial location of a species or in species identification (e.g. Lozier *et al.*, 2009).

The distribution of species and/or populations is a spatial reflection of their niche. A species will occur when the environmental conditions are most fitting and will be absent in areas where one or more essential resources are missing. Also carrying capacities for a species – maximum population size given the environmental conditions - frequently changes with environmental variation.

Ecological Niche

There are many definitions of a 'niche', but all similarly point towards an environment which allows a population or species to survive differing only on the emphasis they put on the key points (Soberón and Nakamura, 2009).The fundamental ecological niche of a species is defined by its biological critical characteristics, including feeding ecology, physiology and reproductive behaviour (Hutchinson, 1957) and describes the abiotic conditions in which it can persist and maintain viable populations (Hutchinson, 1957). However, species are commonly forced to occupy a niche that is a contraction of the fundamental niche, as an effect of pressure from, and interactions with other organisms (Hutchinson, 1957). Also, environmental conditions are not always appropriate for a species in its entire occurrence (Brown and Lomolino, 1998). The fundamental niche is the species' niche in the absence of any disturbance, such as interspecific competition, and is determined by the physiological capabilities of the species. The realized niche is where the species is competitively superior as well as more physiologically adapted than competing species (Roughgarden, 1974). Together, organisms occupy a widespread range of environmental conditions but almost every species or lineage inhabit only a limited subset of such conditions, which is determined by intrinsic traits of each organism that are preserved over long evolutionary timescales. Some groups of animals have a wide range across specific regions, for example in the tropics, but fail to colonize other biomes, even if the opportunity to do so has persisted for hundreds of millions of years.

Natural populations are exposed to different biotic and abiotic factors including competition, predation, climate and food resources variations (Rundle and Nosil, 2005; Schluter, 2009). This can lead to divergent evolutionary responses and patterns of climatic tolerances (reviewed in Parmesan, 2006), and subsequently evolution of organisms to fulfil new habitats; a process termed niche divergence (e.g. Kozak and Wiens, 2007; Cadena *et al.*, 2012). In contrast, the preservation of ecological similarity

among populations over time is niche conservatism (Wiens and Graham, 2005; Peterson, 2011), and it can limit adaptation to ecological conditions and promote isolation (e.g. Wiens, 2004; Kozak and Wiens, 2007; Cadena *et al.*, 2012). Although it has been attempted to test conservative evolution before (Bartlein *et al.*, 1989; Ricklefs and Latham, 1992; Peterson and Vargas-Barajas, 1993), ecological niche models have only been tested quantitatively and (Peterson *et al.* 1999). It was predicted that fundamental niches of species could slowly change under natural selection. Using genetic models it was predicted that niche conservatism rates of adaptation in environments outside the fundamental niche should be frequently slower than extinction process (Houston and McNamara, 1992; Kawecki and Stearns, 1993). The results of a previous study to (Peterson *et al.*, 1999) indicated that ecological niches evolve little at or around the time of a speciation event and that the differences in the ecological niche seem to develop and accumulate later, over the time scale of familial relationships (Peterson, 2011). The types of speciation involved can also be inferred by the conservatism of ecological niches across moderate periods of evolutionary time; for example geographic isolation leads to vicariance. Until recently, evidence for niche conservatism was mixed but a study has shown some structure after setting all evidence in a time-scale (Peterson, 2011). According to this, recent and short-term events, such as distributional shifts at the end of the Pleistocene period, or species invasions present some trend towards conservatism. However, long-term events including differentiation across phylogenies show increasing conservatism breakdown. This means that niche conservatism breaks down over time but at a rate that is still questionable. On timescales in which speciation events are involved, almost all lineages show overall niche conservatism, although no ecological signal associated with speciation is obvious (Peterson, 2011). Some studies have reported niche shifts associated with invasion events in the European plant *Centaurea maculosa* (Broennimann *et al.*, 2007), the South American fire ant *Solenopsis invicta* (Fitzpatrick *et al.*, 2007) and the mosquito *Aedes albopictus* (Medley, 2010), but they may be all a consequence of methodological artefact and not a biological reality (Warren *et al.,* 2008). This difference of opinion also reflects the immature nature of this field and one of the ways forward is a more detailed analysis of niche characteristics and their change through time (Peterson, 2011). Frequent and marked niche change is only seen in phylogenetic history of older groups, and thus estimates of phylogeny give the opportunity to understand the rate of niche change, its speed and how these change events are correlated (Peterson, 2011). During speciation events niche differentiation is expected to be rare, and demonstrations of ecological innovation during this time have to be cautiously studied in order to avoid incorrect conclusion. Moreover, clear

consideration of temporal dimensions and interpretations of niche characteristics and their similarity among lineages should be contemplated to prevent vulnerability of conclusions and biases, especially when dealing with models. Niche conservatism implications should be fully incorporated into evolutionary biology and biogeography (Knowles *et al.*, 2007; Waltari and Guralnick, 2009) to allow many features of the potential geography of species to be recreated with more confidence (Peterson, 2009). It is essential to assess whether the ecological niche of natural populations has evolved in a conservative (Peterson *et al.*, 1999; Wiens, 2004; Wiens and Graham, 2005; Peterson, 2011) or divergent way (Rundle and Nosil, 2005; Schluter, 2009; Schluter and Conte, 2009), enabling the evaluation of the possibility that an ecological speciation event has occurred.

Ecological niche models (ENMs)

Ecological niche modelling (ENM) has recently received greater consideration with more researchers using, developing and improving methods for use. ENM use a population's environmental necessities and its observed presences and/or absences to recognize which places are fit for the species survival (Elith *et al.*, 2006). Furthermore, models estimate areas of potential distribution or sets of favourable habitats. Essentially, species distribution models try to deliver detailed predictions of distributions by linking presence or abundance of a species with environmental predictions (Elith *et al.*, 2006). There is little agreement in terminology and concepts related to ENMs and also methodological issues that need to be thoroughly considered.

Species distribution

Many applications in evolution, ecology and/or conservation are starting to use predictions of species' distribution as a fundamental component. In the light of conservation planning and forecasting, to understand which ecological and evolutionary traits best define biodiversity spatial patterns, it has been seen that meticulous knowledge of species' geographic and ecological distributions is crucial (Brown and Lomolino, 1998; Ferrier, 2002; Funk and Richardson, 2002; Elith *et al.*, 2006). There are several processes that maintain species distributions that have ecological characters at small scales, such as physical factor limitations, metapopulation dynamics, ecological interactions and barriers; other processes change species distributions and are not only of ecological but also historical importance at

small and big scales, such as dispersion/expansion/corridors, paleo-geographic change and climatic change (Brown and Lomolino, 1998).

Speciation is the evolutionary process by which new biological species arise (Cook, 1908). There are two processes of evolution: anagenesis that is the phyletic modification in time, and cladogenesis or speciation, i.e., origin of new species through splitting of pre-existing ones (Singh, 2012). Darwin (1859) first proposed natural selection to explain evolutionary changes, which was later defined as transformation of species in time (Romanes, 1897) and phyletic evolution (Simpson, 1944). It is possible to have evolutionary change without species multiplication, and also through true speciation or what was once called 'multiplication of species in space' (Romanes, 1897). Mayr (1966) defined speciation as the splitting of an originally uniform species into several daughter species, which is also seen as one of the key ways by which organisms adapt in order to exploit the diverse environments available to them (White, 1978). Some evolutionary biologists have proposed different possible modes of speciation. Huxley (1942) suggested geographical, ecological and genetic types of speciation. Mayr (1942) classified speciation into geographic, semi-geographic and non-geographic (or sympatric). Six modes of speciation were stated by Mayr and Ashlock (1991): polyploidy, sympatric, parapatric, two types of allopatry – dichopatric and peripatric – and speciation in time. White (1978) divided speciation processes in three sets of variables: genetic mechanisms generating genetic variability; genetic isolating mechanisms leading to the origin of the reproductive isolation; and geographic component ranging from complete (allopatry) to absent (sympatry) that are the origin for 7 models of speciation. The models are: strict allopatry without a narrow population bottleneck; strict allopatry with a narrow population bottleneck (founder effect); extinction of intermediate populations in a chain of races; clinal speciation; area-effect speciation (primarily genic); stasipatric speciation (primarily chromosomal); and sympatric speciation (White, 1978; Singh, 2012). Speciation is mainly originated by natural selection (Schluter and Conte, 2009) through two mechanisms: mutation-order and ecological speciation (Mani and Clarke, 1990; Schluter, 2009; Schluter and Conte, 2009). A mutation-order speciation occurs when two distinct beneficial or neutral mutations are fixed between different entities, even though they are exposed to the same selective pressures and ecological conditions (Mani and Clarke, 1990; Schluter, 2009). The fitness of the hybrids decreases over evolutionary time scales with the fixation of these mutations when they are a result of vicariance or drift (Coyne and Orr, 2004). The other type of speciation is natural selection, termed ecological speciation, which takes place with the evolution of reproductive isolation mechanisms between entities through differential adaptation to dissimilar ecological or environmental

conditions (Schluter, 2001; Rundle and Nosil, 2005; Funk, 2009). Here, natural selection is a divergence mechanism that leads to the fixation of distinct advantageous mutations in each of the different environments (Schluter, 2009; Schluter and Conte, 2009). It is difficult to understand in practice by observing the ecological traits of lineages in a given phylogeny if ecological divergence is the crucial force leading to speciation or if it acts secondarily during interruption of gene flow due to other causes occurs (Schluter, 2009). If the ecological divergence is assessed, then it can work as a proxy to support species delimitation and understand which factors were ecologically associated to speciation (e.g. Raxworthy *et al.*, 2007; Ahmadzadeh *et al.*, 2013).

Models of Species Distribution

Different types of methods use different data input, which in turn varies how they model response distributions, select pertinent environmental features, define variable functions, weight each variable influence and allow or not for interactions and forecast geographic patterns of occurrence, producing different outputs (Guisan and Zimmerman, 2000; Wintle and Bardos, 2006). There are two types of models that predict the distribution of a species – correlative models and mechanistic models (Peterson, 2011, but see Sillero, 2011). Mechanistic models use the biophysical properties of the entities to link functional traits with environmental conditions and determine areas where species may exist (Kearney and Porter, 2009; Dormann *et al.*, 2012). They have limited use due to the detailed information and sampling coordinates they require (Alvarado-Serrano and Knowles, 2014). Correlative methods identify statistical associations between a species or population distribution and environmental conditions, using distribution data and environmental layers (Alvarado-Serrano and Knowles, 2014).

Since distribution modelling is now a common tool for ecological studies, and given the data accessibility and numerous methods available for the purpose, a synthetic analysis with accurate results is needed in order to correctly predict species' distribution with presence-only data. The first efforts to analyse this kind of data used calculation of envelopes or distance-based measures and were developed specifically for that purpose (Rapoport, 1982; Silverman, 1986). Subsequently, the attempt was to adjust presence-absence methods for presence-only data, by using random points of the background environment designated as "non-use" or "pseudo-absence" areas (e.g. Stockwell and Peters, 1999; Boyce *et al.*, 2002). In recent years, new methods use information based on the presence of other similar species of a community, which perform better with noisy data, something that is particularly important in the case of

rare species, helped in distribution modelling (Elith *et al.*, 2006). In general, new modelling methods of species distribution outperform established methods, especially because they are originated in other disciplines and deal with much wider ranges of ecological applications thus delivering new future research prospects (Elith *et al.*, 2006). Specifically, methods that characterize the background environment and weight variables in a differential manner are better than methods that use presence-only data, such as BIOCLIM, LIVES or DOMAIN (Elith *et al.*, 2006). Most of the studies comparing methods use the same data set that was used to develop them and/or are focused on one single geographic region and/or small number of species (e.g. Ferrier and Watson, 1997; Moisen and Frescino, 2002; Segurando and Araújo, 2004; Phillips *et al.*, 2006). Because of this, these studies do not allow the correct generalization of the results or the ability to discern if models are accurate or are simply overfitting. One shared characteristic of the best performing models is a great level of flexibility in fitting complex responses, i.e. they are effective tools for modelling relations among variables, even though each model achieves it in different ways (Elith *et al.*, 2006). Nevertheless, this ability needs to be balanced with the condition of the model to be ecologically realistic (Austin, 2002). So far, there is no single model able to replace detailed field collection including abundance, interactions, demography and accurate species distribution data (Guisan and Thuiller, 2005). However, there are some methods that try to incorporate this kind of information, such as Bayesian approaches (Gelfand *et al.*, 2006), investigations of competitors (Leathwick and Austin, 2001; Anderson *et al.*, 2002), and studies of connectivity (Moilanen *et al.*, 2005).

The ideal way of modelling is by iterative cycles that incorporate the uses desired for the model, considering the ecological rationality of the modelled responses and accurately exploring errors made in predictions (Burgman *et al.*, 2005; Barry and Elith, 2006).

Input Occurrence data

Information about species occurrence is vast and electronically available for almost every known species; mainly records from museums and herbariums (Graham *et al.*, 2004, Huettmann, 2005). Distribution data can be gathered from primary surveys, natural history collections, published species ranges (e.g. Barnes and Wagner, 2004) or public databases (e.g. Natureserve, GBIF). However, for the majority of species, information is scarce and consists mostly in presence-only records, so that it is hard to adequately compile data for usage as input on the numerous approaches for modelling distributions (Elith *et al.*, 2006), especially when so many errors can be made in

sampling and/or uploading records to databases. Collection information is often unknown, making presences and particularly absence records uncertain, and furthermore these may include bias and errors associated with sampling. There is an accumulation of information available that is frequently incorrect or inconsistent (Hijmans *et al.*, 2000, Reese *et al.*, 2005). Accuracy and precision of distributional data is strongly associated to their source, and format and should be scrutinized (Alvarado-Serrano and Knowles, 2014). Elith *et al.* (2006) states that having absence data is important and is a good alternative to random background samples when modelling single species. This is not so easy to put into practice when studying small species because they often require very specific ecological requirements or vary in seasonal activity. Assuming that a species does not exist in a particular geographic region at a specific time because it was not seen at that time and space can be erroneous, and may mislead the model. This implies an intensive and extensive sampling effort, which is not always logistically and/or humanly possible especially in remote areas. All the data must be carefully studied before being used and each species is a different case requiring different considerations according to its ecology and the questions we want to answer. It is also important to remove redundant data (points that fall within the same grid cell of environmental data) to avoid artificial bias in model predictions. The distribution data is normally mapped into a predefined map coordinate system that matches the one from the environmental data.

Input Environmental Variables

Environmental data is presented in the form of digital grid cells and can be derived from field data, interpolated surfaces (e.g. climatic data from WorldClim) or remote sensing (e.g. landcover from MODIS). When variables relevant to the test in question are selected it is necessary to process it, i.e., transform the environmental data into raster grids with the same coordinate system and, preferably, with the same software (Bolstad, 2008). The variables must not be correlated with each other and some techniques such as orthogonal transformation or elimination of the variables according to the goal of the study are used. Environmental data can also be used to understand what their relative contributions to the model are.

Accuracy of the output

When evaluating the output accuracy, one of the major problems faced is that the exact species distribution is not known and the best way for evaluation is by predictive performance (Elith *et al.*, 2006). Predictive performance uses some sample points of

the species occurrence that are withdrawn from the input dataset, and in the end accuracy is evaluated based on how well the model has predicted those occurrences (Boyce *et al.*, 2002). The withdrawal of the sample points can be performed in different ways, such as splitting the data set, k-fold partitioning, or bootstrapping (Fielding and Bell, 1997; Araújo *et al.*, 2005). Predictive performance is not the most accurate, as we know that occurrence records have biases in environmental and geographic space (Bojorquez-Tapia, 1995; Hijmans *et al.*, 2000; Soberón *et al.*, 2000; Kadmon *et al.*, 2004) and that those biases will continue no matter the resampling design.

OBJECTIVES

The main aim of this thesis was to determine phylogeographic patterns of variation within *S. brosseti*. In particular we hoped to determine a) if mtDNA lineages previously identified were also recovered with nuclear markers b) to try to date the origin of these lineages and associate them with known geological events, especially the orogenesis of the Atlas Mountains c) to use species delimitation approaches to assess if *S. brosseti* may actually correspond to a species complex and d) to extend sampling across the range to assess if additional but previously unsampled lineages might occur. The second aim was to asses which environmental variables might limit the occurrences of *S. brosseti* in the region, and then use this to predict possible areas where the species might occur but not be recorded. These potential distribution maps should also help to identify possible geographical barriers that shape the distribution of the species.

Since these primary aims involved considerable fieldwork, a side aim was to record all the herpetofauna idenfied during the fieldwork, including a DNA barcoding approach when necessary, and to use this to determine possible range extensions to poduce better distribution maps of the herpetofauna of this region.

MATERIAL AND METHODS

In this chapter, a more detailed description of the materials and methods used is offered.

Phylogenetic Analysis

Sampling

Over the years, several fieldtrips have been carried out, where specimens where sampled. Two fieldtrips (May 2013 and May 2014) were conducted and I had the opportunity to be included in both of them, resulting in a sampling of 53 species from 138 localities (see Manuscript II), from which 36 were *Saurodactylus brosseti* specimens (see Appendix 1 and Fig. 4.).

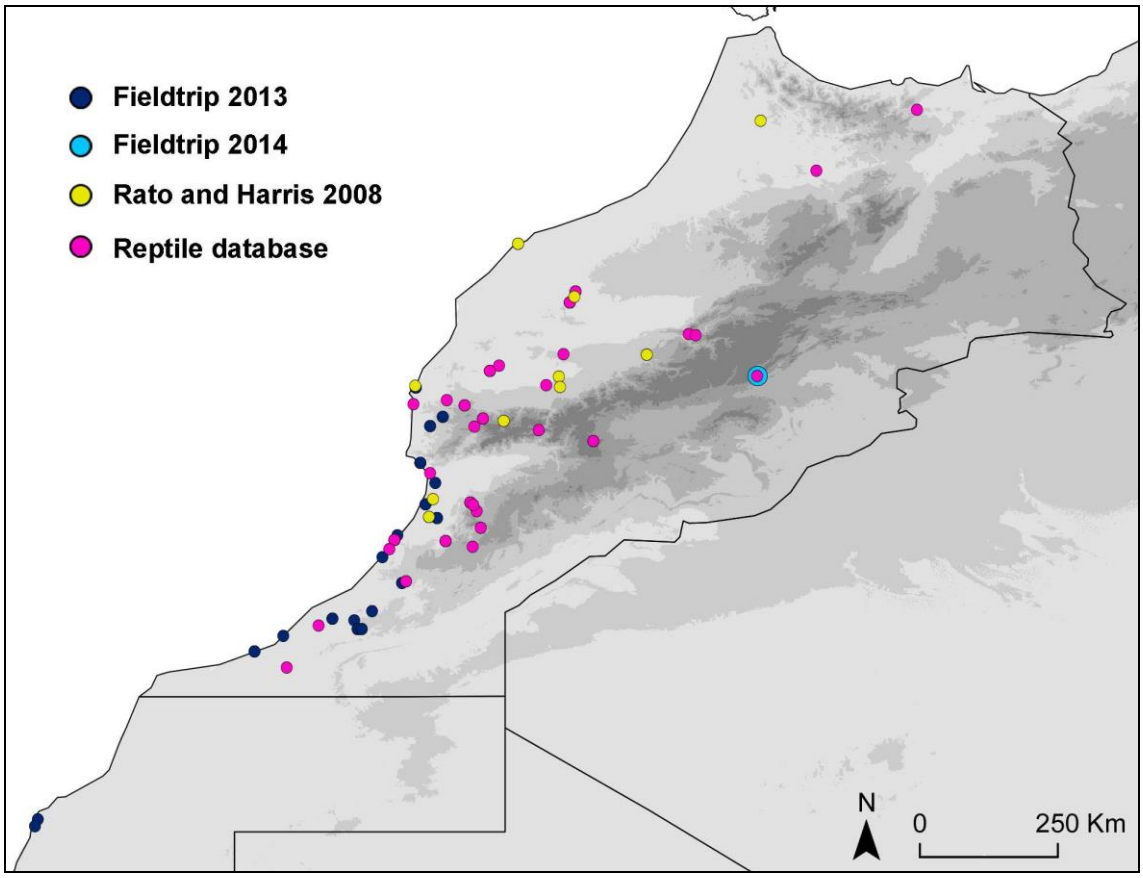

Fig. 4. – Samples used for phylogenetic analyses.

The sampling localities included the southernmost known locations in Western Sahara, near Boujdour. Samples were collected by hand and data recorded at the site with GPS and annotations of the basic information about the specimen – DB number (to be included in the reptile database), species, subspecies, date, locality, latitude, longitude,

altitude, sex, age, snout-vent length (SVL) and, when applicable, pellet, slide, blood, parasites, photo information and observations (e.g. pregnant female). All sample information is available in the reptile database of CIBIO. Additionally, a small piece of each specimen's tail tip was collected from live animals and kept in 96% ethanol and photos taken before releasing the animal. *Saurodactylus mauritanicus* and *Saurodactylus fasciatus* were used as outgroups, for which samples were already available in the CIBIO database.

DNA extraction, amplification and sequencing

In the CIBIO laboratory, DNA was extracted from tail tips tissue using a High Salt method (Sambrook *et al.*, 1989). DNA amplification was performed through PCR for two mitochondrial fragments – 12S and ND4 – two nuclear fragments – ACM4 and MC1R – and one intron – BZW1. Mitochondrial genes were chosen so that data produced could be directly compared to an earlier study (Rato and Harris, 2008). ACM4 and MC1R markers were chosen due to its wide use in phylogeographic studies in reptiles (e.g. Rato *et al.*, 2010) and the intron was chosen because they normally present higher mutation rate and consequently higher variation and BZW1 have been used in other members of the Gekkota family (e.g. Fujita *et al.*, 2010). PCR conditions and primers are described on Table 1.

Table 1. – Primers names and amplification conditions.

Gene	12S			ND4			ACM4			BZW1			MC1R		
Step	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X
Initial Denaturation	95º	1'	1	94º	3'	1	94º	5'	1	94º	3'	1	92º	2'	1
Denaturation	95º	15''	35	94º	3'	35	94º	5'	1	94º	3'	37	92º	2'	35
Annealing	48º	15''		94º	30''		94º	30''	32	94º	30''		92º	1'	
Extension	72º	10''		48º	30''		55º	45''		62º	45''		55º	45''	
Final Extension	72º	10'	1	72º	40''	1	72º	1'	1	72º	1'		72º	1'	1
Primer Forward	12S L			ND4			Tg-F			Tar1			MC1R F		
Primer Reverse	12S H			LEU			Tg-R			Tar2			MC1R R		
Citation	Kocher *et al.* 1989			Arévalo *et al.* 1994			Gamble *et al.* 2008			Fujita *et al.* 2010			Pinho *et al.* 2010		

Polymerase Chain Reaction (PCR) amplification was performed with a final 25 µL volume for each sample, according to MyTaq™ protocol (UK) – 15.8 µL of purified water, 5 µL of MyTaq buffer, 1 µL of Forward Primer, 1µL of Reverse Primer, 0.2 µL of Taq, and 2 µL of DNA extraction product. MyTaq™ buffer contains dNTPs, $MgCl_2$, stabilizers and enhancers. PCR were run in a Biometra TProfessional thermal cycler. For each run, two controls were added – a positive control, which is a sample that previously amplified for the gene in question to certify that PCR reaction was effective; and a negative control, which has all reagents except DNA to check for contaminations. To check the success of amplification, 2 µL of PCR product from each sample ran in 2% agarose gel with GelRed Nucleic Acid stain, visualized in an ultraviolet transilluminator. Pictures of the each gel run were taken and saved. Amplification products were sent to Beckman Coulter Genomics (UK) for purification and Sanger sequencing with same primers used in amplification.

Phylogenetic analysis

Sequences were blasted to the NCBI database on GenBank to confirm the species. Chromatographs were checked and sequences were aligned for posterior phylogenetic analysis using Geneious v 5.6 (Drummond *et al.*, 2012). Alignment was performed using default settings of MAFFT. Nuclear genes were sequenced in both directions to ensure identification of heterozygotes. 12S and ND4 sequences of *Saurodactylus brosseti* from a previous study (Rato and Harris, 2008) available on GenBank (accession numbers in Appendix 1) were also included in the alignment. jModelTest v2.1.4 (Darriba et al. 2012 – 201) was used to infer which model best fit each data under the Akaike Information Criterion (Cavanaugh 2007 – 205) for separate and concatenated genes in order to decrease the error (Brandley et al. 2005 - 227). Nuclear genes were phased using Seqphase (Flot 2010 - 231) and PHASE (Stephens et al. 2005 – 230) with a threshold of 0.6 and default for all the other parameters. Phylogenetic accuracy can be higher when data sets from different genes are combined into a single phylogenetic analysis (Rokas et al. 2013 - 226). Maximum Likelihood analysis was performed using RAxML v3.0 (Stamatakis, A. 2014 - 229) with 1000 bootstrap replicates for concatenated genes. Bayesian analyses were performed with best fitting models applied to each gene with MrBayes v3.2.2 (Huelsenbeck and Ronquist 2001 – 192) for a concatenated approach (one partition) and *BEAST v1.8.0 (Drummond et al. 2012 – 204) for a coalescent approach (five partitions, all parameters unlinked across partitions, except for 12S and ND4, for which trees were linked into “mitochondrial DNA”). MrBayes analysis began with random starting tree, ran for 10

million generations and was sampled every 100 generations. 25.000 (25%) of burn-in trees were discarded and the remaining were used to assess posterior probability values. *BEAST ran three times for 150 million generations with an uncorrelated lognormal relaxed clock with a rate of 0.00701 for 12S (Metallinou et al. 2012 - 225) as calibration point to estimate divergence times. In order to have a species tree, sequences were grouped into four groups, according to the four major clades assessed by MrBayes and RAxML. All runs were combined with LogCombiner v1.8.0 (package of *BEAST) with a burn-in of 10% for each run. Mean genetic distances between the four major clades (given the results from MrBayes and RAxML) were calculated with MEGA6 (Tamura et al. 2013 – 203) for ND4. Consensus trees were visualized in Figtree (v. 1.3.1) and posterior modifications, such as insertion of posterior values and colouring of branches, were performed with Inkscape (v. 0.48).

Modelling

Occurrence Data and Environmental Variables

The input occurrence data for modelling was obtained from geographic coordinates of known occurrence localities (Bons and Geniez, 1996) and the reptile database of CIBIO, in which GPS coordinates were recorded during fieldwork expeditions. In total, 246 points were depicted using ArcMap v 9.3 under the WGS 1984 Datum geographic coordinate system. Twenty current environmental variables were downloaded from WorldClim – Global Climate Data database (Hijmans *et al.*, 2005) (Table 2.). All variables were in 30-arc seconds (approximately 1 Km) resolution tiles: tile 15 and tile 25 in order to comprise all of Morocco and Western Sahara area. Since correlation of environmental variables vary according to its extension, all five variables had to be cut giving *Saurodactylus brosseti* known distribution range, using ArcMap v9.3. Covariance and correlation matrix were computed using ArcMap with a threshold of 75% - all variables with correlation higher than 0.75 were considered correlated and one of each two correlated variables was withdrawn. Choice of environmental variables was not entirely random, as variables were chosen according to the known limiting factors of the species (Meek, 2008). In the end, five environmental variables were chosen: Annual mean temperature (BIO1), Minimum temperature of coldest month (BIO6), Temperature annual range (BIO7), Precipitation of driest month (BIO14), and Annual precipitation (BIO12). These five variables were also cut according to the distribution of *Saurodactylus mauritanicus* and *Saurodactylus fasciatus*. As the distribution of *S. brosseti* can overlap the distribution of the other two species of the genus, this was

done to ensure that localities with high probability of occurrence for *S. brosseti* were not coincident of the localities from either of the other two species.

Table 2. – Environmental variables downloaded from WordClim.

Code	Variable
ALT	Altitude
BIO1	Annual mean temperature
BIO2	Mean diurnal range
BIO3	Isothermality
BIO4	Temperature seasonality
BIO5	Maximum temperature of warmest month
BIO6	Minimum temperature of coldest month
BIO7	Temperature annual range
BIO8	Mean temperature of wettest quarter
BIO9	Mean temperature of driest quarter
BIO10	Mean temperature of warmest quarter
BIO11	Mean temperature of coldest quarter
BIO12	Annual precipitation
BIO13	Precipitation of wettest month
BIO14	Precipitation of driest month
BIO15	Precipitation seasonality
BIO16	Precipitation of wettest quarter
BIO17	Precipitation of driest quarter
BIO18	Precipitation of warmest quarter
BIO19	Precipitation of coldest quarter

Species Probability of Occurrence

To estimate the presence probability of the three species of *Saurodactylus* the software Maxent (Phillips and Dudik, 2008) was used. All duplicate presence records from the known occurrence data were removed before the software run to prevent data

overfitting. All runs were performed with 25 random test percentage, 20 bootstrap replicates and in order to create response curves with jacknife to measure variable importance. The area under the curve (AUC) was used to assess model accuracy. Even though it is not as reliable as it would be expected (Lobo et al. 2007 - 217) this is still the best method. Since Maxent presents its results in a gradient “probability of occurrence” ranging from 0 to 1, a threshold was defined in order to build maps of “suitable or unsuitable” habitat. This was done using the average of the 10 percentile training presence logistic threshold of the 20 replicates for each species. Values above the threshold were considered as “suitable habitat” and values below threshold were considered as “unsuitable habitat”.

MANUSCRIPTS

Manuscript I

Moroccan herpetofauna: distribution updates including a DNA barcoding approach.

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The Kingdom of Morocco contains some of the richest diversity of herpetofauna in the Maghreb and Western Europe. This fact is clearly related with the considerable topological variation of the region, with the Rif and Atlas Mountains dividing the country into climatically different zones. The first step towards any ecological, conservation or modeling approaches concerning this rich diversity is to develop accurate distribution datasets. Bons & Geniez (1996) presented a detailed assessment of the known diversity at that time. However, various researchers have since then presented data indicating range extension for many species (e.g. Guzman et al., 2007, Harris et al., 2008, Harris et al. 2010, Barnestein et al., 2010; Barata et al., 2011, Beukema et al., 2013, Damas-Moreira et al., 2014), and it is clear that current records are still limited, especially in the Eastern region (Barata et al., 2011, Beukema et al., 2013). At the same time modeling approaches have been employed, which may help to highlight regions in need of further prospection (de Pous et al., 2010). In particular, in an extensive review of the distribution and biogeography of Moroccan amphibians (Beukema et al., 2013), models indicating regions of high probability of occurrence were presented for all species along with greatly improved distribution maps. New records for amphibians can therefore both be compared to these models and can be informative in determining whether distribution maps are stabilizing for such well-studied groups. Additionally, they can also be used to draw a parallel scenario in groups known to be much harder to locate, such as fossorial species or snakes.

The accuracy of distribution maps relies on species being correctly identified. This is not always simple – many forms of Moroccan herpetofauna have recently been identified as species complexes (e.g. Rato et al., 2012), and several of these are "cryptic" implying that identification is made using molecular markers. This includes forms of the *Podarcis vaucheri* complex (Pinho et al., 2007), or lineages within various geckos including *Quedenfeldtia* (Barata et al., 2012), *Ptyodactylus* (Perera & Harris, 2010) and *Stenodactylus* (Metallinou et al., 2012). Other species can be difficult to identify when only juveniles or tadpoles are collected. Additionally many groups such as snakes are often widely sampled as road-killed animals, and in some cases identifying remains to the species level is again difficult. In these cases the use of a DNA "barcoding" approach (Hebert & Gregory, 2005) can be extremely useful.

In the present study the authors compile records of two expeditions to Morocco over a combined 5 week period in Spring 2013 and 2014. Sampling covers a wide range of Southern and Eastern Morocco, and was chosen to be complementary to a recent survey of Northern and Central regions (Damas-Moreira et al., 2014). In total 138 localities were sampled and 53 species recorded. GPS coordinates and a detailed list of species per locality are given in Table 1. Photos of most animals are available on request from the authors. Distribution data was compared to published records, and interesting new localities are discussed in the text that follows. In cases where a species diagnosis based on morphological characters could not be made with certainty, a DNA barcoding approach was used. DNA was extracted using standard high-salt methods (Sambrook et al., 1989). PCR was used to amplify a region of the 12S rRNA, using published protocols (Harris et al., 1998). This gene was chosen rather than the classic barcoding COI region since comparative published data for 12S was already available for most of the presumed species. For example 12S rRNA sequences are available for most toad species from Morocco (e.g. Harris & Perera, 2009; de Pous et al., 2013), so this gene could be used to confirm the species diagnosis of tadpoles of this group. All species for which this approach was used are highlighted in Table 1, and are discussed in the text when relevant. New sequences are published in GenBank (accession numbers xxxxx to xxxxx).

Overall our findings indicate that even for better-known groups the current distribution maps are imprecise. Several new distribution points and range extensions were found in the Eastern region, where models predicated that more species would be expected (Beukema et al., 2013), or coincided with high probability areas proposed for specific species. This further demonstrates the value of these models, which can be used to guide future prospection in Morocco and other regions.

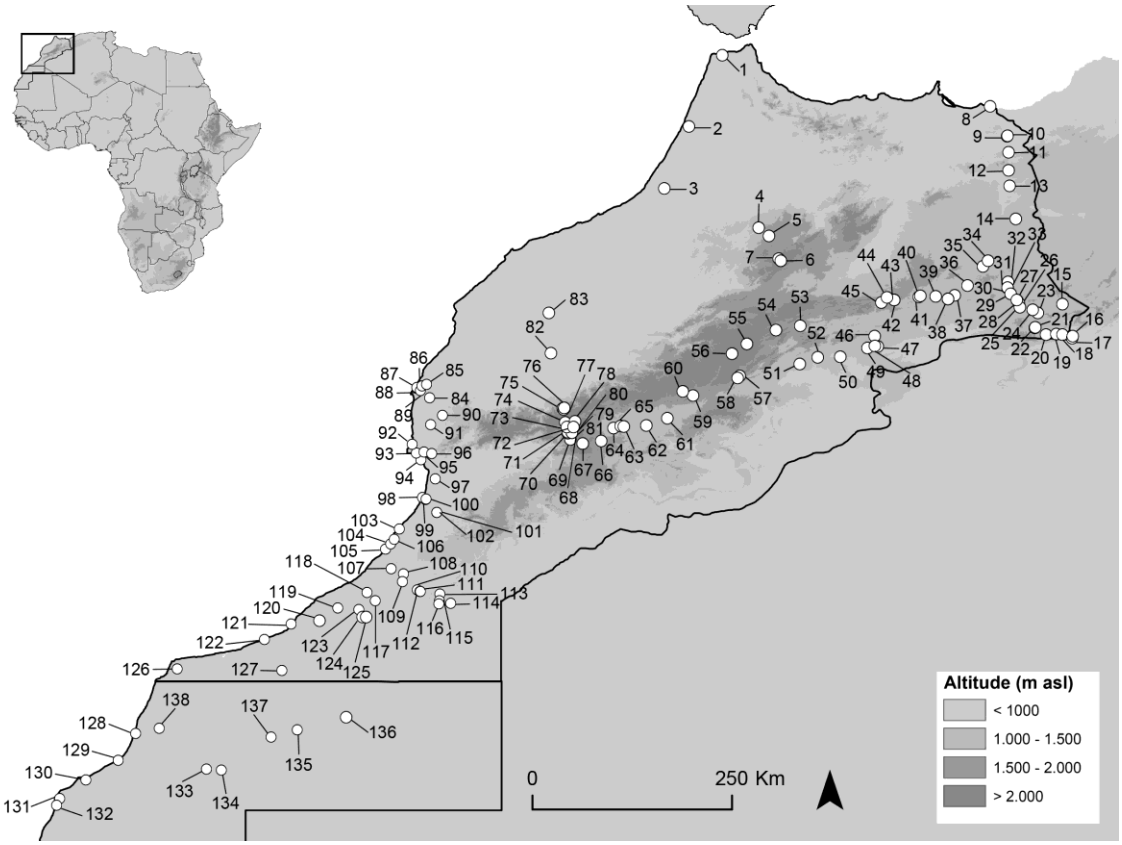

Figure 1 – Sampling points of both expeditions to Morocco.

Bufonidae – *Bufo spinosus* (Localities 13, 60, 67). Despite the considerable recent advances in distribution records for amphibians in Morocco (Beukema et al., 2013), one of our new records (locality 13, Fig. 2A) represents a considerable range extension in North Eastern Morocco. Also for *Barbarophryne brongersmai* (Localities 90 and 107), locality 90 represents a new locality in a region where distribution models predicted the occurrence of the species with high probability (Beukema et al., 2013). These samples were tadpoles, and their identity was confirmed by DNA sequencing.

Testudinidae - *Testudo graeca* (Localities 35, 87, 88, 101). Locality 35 is a new record in the high plateau region of northeastern Morocco, where only a few isolated records of this species are known.

Agamidae - *Trapelus boehmei* (Localities 21, 27, 29, 34, 37, 40, 49, 50, 51, 52, 79, 113, 127, 133). Many new records were identified in the northeastern high plateau region (Localities 21, 27, 29, 34, 37 and 40). This is a region where models suggest that more surveys are needed to complete distribution maps (Beukema et al., 2013),

and this large number of new records for a fairly common and conspicuous species confirms it. Probably *T. boehmei* actually occurs throughout this whole region. One other locality, at a point North of Jebel Sirwah (Locality 79), is extremely interesting as it is both a new altitude record for the species (2273 a.s.l.; 1500 previously known maximum altitude following Bons & Geniez, 1996), and also extends the species distribution considerably into this region between the High and Anti Atlas Mountains. This sample was collected from a roadkill (Fig. 2B), and species identity was confirmed using DNA sequencing.

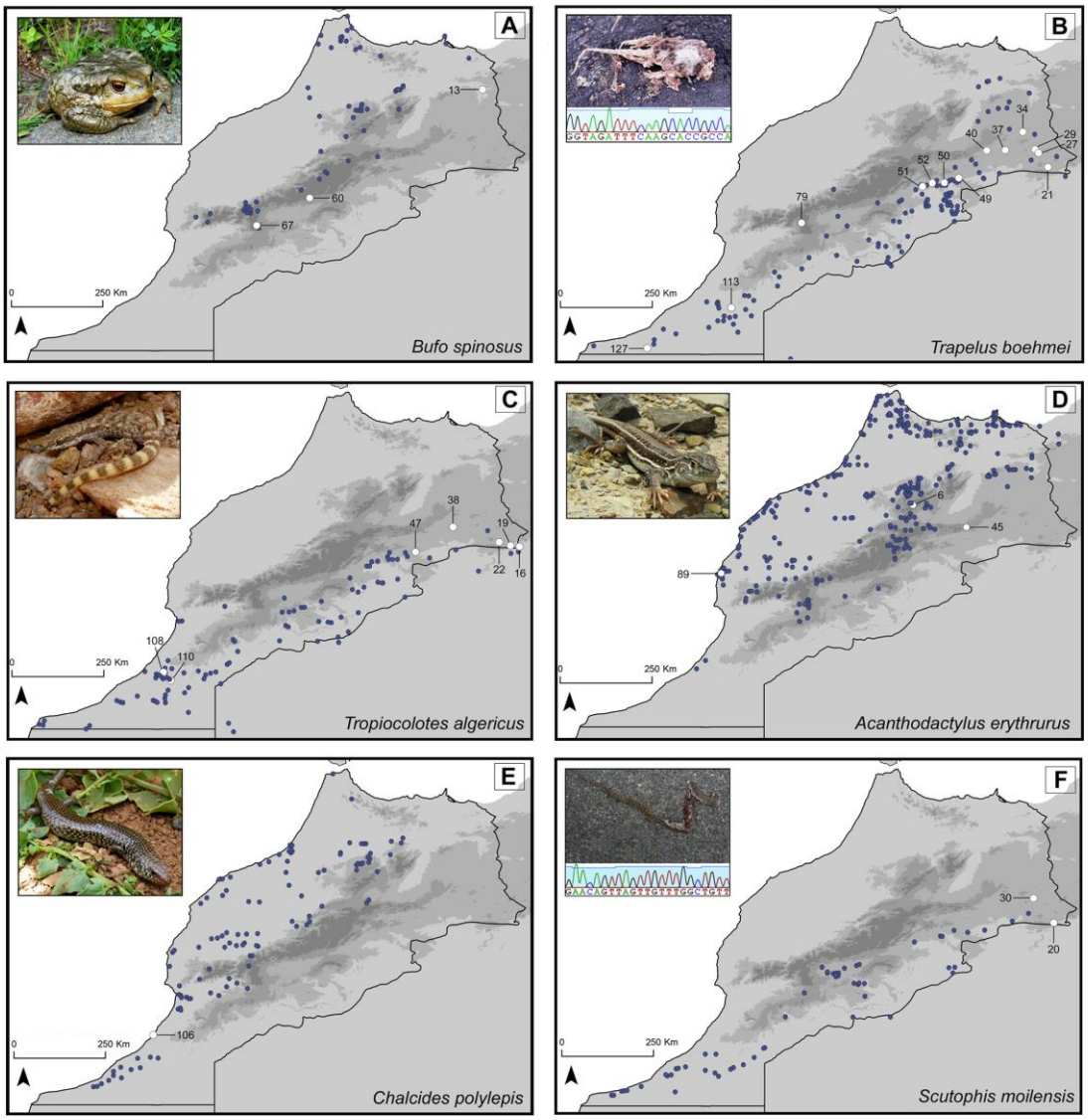

Figure 2 – Maps of species occurrence for the most significant species range expansions. Blue dots represent published data records and white dots represent new distribution points detected in this study. All photographs were taken by Daniele Salvi. *Scutophis moilensis* and *Trapelus boehmei* pictures include a part of the 12S chromatograms used to confirm genetically the species.

Phyllodactylidae - *Tarentola deserti* (Localities 15, 17, 24, 26, 32, 38). This species has a distribution restricted to the south of the Oriental and Meknes-Tafilalet provinces. Combined with other recent new records in the region (e.g. Damas-Moreira et al., 2014), these additional localities show a fairly continuous distribution near the Algerian border for *T. deserti*.

Gekkonidae - *Tropiocolotes algericus* (Localities 16, 19, 22, 38, 47, 108, 110, 132, 134, 135). Again, several new records for this underexplored Northeastern region were found (Fig. 2C), in particular locality 38 represents a new northern record for the species in Morocco, and is quite isolated from currently known localities.

Lacertidae - *Acanthodactylus erythrurus* (Localities 6, 45 and 89). Locality 45 is a considerable range extension for this species towards the eastern side of the Atlas Mountains (Fig. 2D). Interestingly other more “Mediterranean” species are also known from this region, including *P. vaucheri* and *Scelarcis perspicillata perspicillata* (Bons & Geniez, 1996). The new localities are quite isolated from the other populations of the species and may thus be interesting from a phylogeographic viewpoint. Further prospection in the region for other typical Mediterranean species is clearly warranted. *Atlantolacerta andreanskyi* (Localities 55, 56, 68, 80) may actually represent a species complex (Barata et al., 2012), with highly genetically distinct lineages occurring in populations separated by less than 50 Km. Therefore these localities, although not far from other known localities in the Middle and High Atlas, are extremely important as further assessment may help to delimit taxonomic units within the complex. *Scelarcis perspicillata* (localities 44, 60, 67, 78). Locality 44 confirms the isolated population in this Eastern region. Locality 60 had the *S. perspicillata perspicillata* morphotype, but was in an area where other morphotypes are distributed. *Mesalina olivieri* (Localities 13 and 45). Locality 45 is also a considerable range extension for this species, further highlighting the lack of records for this area.

Scincidae - *Chalcides montanus* – (Localities 57, 76). With the recognition of *C. lanzai* as a full species (Carranza et al., 2008), there are very few records of *C. montanus* in the Middle and High Atlas. Locality 57 is a new locality along with only other 5 or 6 known from the Middle Atlas. *Chalcides polylepis* (locality 106, Fig. 2E). Previously the known distribution of this species had a considerable gap of over 100 km between the northern populations and those South of the Anti-Atlas. Locality 106, a sandy coastal area, lies almost in the middle of this gap and may therefore indicate that the distribution is actually continuous.

Colubridae/Psammophiidae - *Coronella girondica* and *Malpolon insignitus* (locality 45). Another small range extension for both these species, and again an example of “Mediterranean” species found in the area along with *A. erythrurus*. *Psammophis*

schokari (localities 26, 31, 37, 39, 44, 97, 114, 120). New records from the Northeastern region (localities 31, 37, 39 44) confirm that this species is widespread in the region. *Spalerosophis dolichospilus* (Locality 22). (Damas-Moreira et al., 2014) reported new localities in the Northeastern region, where it had not previously been known (Bons & Geniez, 1996). The additional locality here (22) further increases the range to close to the Algerian border in this region. *Scutophis moilensis* (localities 20, 30 and 138). Previously considered as *Malpolon moilensis*, Bons & Geniez (1996) suggested this species was "represented in all regions with a Saharan climate except for a zone between Boudenib and Figuig". Locality 20 is exactly in this region (Fig. 2F), while locality 30 also extends the range in the region and is the most northern record for the species in Morocco. Roadkilled samples were confirmed through DNA sequencing.

Table 3 – Details of the sampled localities, including latitude, longitude and species found. Specimens that were identified using DNA barcoding approach are indicated with *.

Point	Latitude	Longitude	Species sampled
1	35.771	-5.787	*Discoglossus scovazzi*
2	34.853	-6.224	*Tarentola mauritanica*
3	34.046	-6.548	*Psammodromus algirus*
4	33.544	-5.318	*Psammodromus algirus, Tarentola mauritanica*
5	33.434	-5.180	*Bufotes boulengeri, Tarentola mauritanica*
6	33.112	-5.028	*Acanthodactylus erythrurus, Podarcis vaucheri, Timon tangitanus*
7	33.142	-5.051	*Podarcis vaucheri*
8	35.111	-2.300	*Acanthodactylus boskianus*
9	34.726	-2.078	*Chalcides ocellatus*
10	34.732	-2.075	*Agama impalearis, Tarentola mauritanica*
11	34.520	-2.058	*Agama impalearis, Chalcides ocellatus*
12	34.286	-2.057	*Acanthodactylus boskianus, Agama impalearis, Psammodromus algirus, Trogonophis wiegmanni*
13	34.080	-2.047	*Bufo spinosus, Mesalina olivieri*
14	33.654	-1.963	*Stenodactylus mauritanicus*
15	32.552	-1.358	*Agama impalearis, Ptyodactylus oudrii, Tarentola deserti, Uromastyx nigriventris*
16	32.135	-1.223	*Acanthodactylus scutellatus, Tropiocolotes algericus*
17	32.107	-1.226	*Tarentola deserti**
18	32.159	-1.357	*Uromastyx nigriventris*
19	32.165	-1.438	*Cerastes cerastes, Tropiocolotes algericus*
20	32.158	-1.572	*Scutophis moilensis**
21	32.248	-1.714	*Trapelus boehmei*
22	32.241	-1.717	*Agama impalearis, Spalerosophis dolichospilus, Stenodactylus mauritanicus, Tropiocolotes algericus*
23	32.436	-1.679	*Uromastyx nigriventris*
24	32.479	-1.748	*Tarentola deserti*
25	32.510	-1.913	*Uromastyx nigriventris*

26	32.566	-1.924	*Agama impalearis, Psammophis schokari, Tarentola deserti*
27	32.597	-1.943	*Trapelus boehmei*
28	32.607	-1.955	*Acanthodactylus boskianus*
29	32.685	-2.031	*Trapelus boehmei*
30	32.759	-2.071	*Scutophis moilensis**
31	32.760	-2.070	*Psammophis schokari*
32	32.763	-2.070	*Bufotes boulengeri, Chalcides ocellatus, Tarentola deserti**
33	32.838	-2.066	*Acanthodactylus boskianus, Chalcides ocellatus, Stenodactylus mauritanicus*
34	33.110	-2.328	*Acanthodactylus boskianus, Trapelus boehmei*
35	33.038	-2.392	*Acanthodactylus boskianus, Testudo graeca*
36	32.795	-2.594	*Acanthodactylus boskianus, Mesalina guttulata**
37	32.665	-2.761	*Psammophis schokari, Trapelus boehmei*
38	32.615	-2.846	*Tarentola deserti, Tropiocolotes algericus, Uromastyx nigriventris*
39	32.653	-3.008	*Psammophis schokari*
40	32.656	-3.211	*Ptyodactylus oudrii, Trapelus boehmei*
41	32.645	-3.227	*Ptyodactylus oudrii, Uromastyx nigriventris*
42	32.602	-3.551	*Amietophrynus mauritanicus*
43	32.602	-3.553	*Pelophylax saharicus, Ptyodactylus oudrii*
44	32.636	-3.641	*Agama impalearis, Psammophis schokari, Ptyodactylus oudrii, Scelarcis perspicillata, Timon tangitanus*
45	32.569	-3.719	*Acanthodactylus erythrurus, Coronella girondica, Malpolon insignitus, Mesalina olivieri*
46	32.133	-3.801	*Amietophrynus mauritanicus, Mauremys leprosa*
47	32.005	-3.765	*Acanthodactylus boskianus, Mesalina guttulata, Stenodactylus mauritanicus, Tropiocolotes algericus*
48	31.997	-3.801	*Psammophis schokari**
49	31.982	-3.900	*Trapelus boehmei*
50	31.869	-4.251	*Trapelus boehmei*
51	31.774	-4.781	*Trapelus boehmei*
52	31.863	-4.546	*Trapelus boehmei*
53	32.267	-4.777	*Acanthodactylus boskianus, Agama impalearis*
54	32.210	-5.092	*Timon tangitanus*
55	32.036	-5.466	*Atlantolacerta andreanskyi, Discoglossus scovazzi, Podarcis vaucheri*
56	31.912	-5.663	*Atlantolacerta andreanskyi*
57	31.621	-5.560	*Chalcides montanus, Mesalina guttulata, Ptyodactylus oudrii, Quedenfeldtia moerens, Saurodactylus brosseti, Tarentola mauritanica*
58	31.595	-5.593	*Tarentola mauritanica*
59	31.365	-6.172	*Agama impalearis, Amietophrynus mauritanicus, Pelophylax saharicus*
60	31.421	-6.304	*Bufo spinosus, Pelophylax saharicus, Scelarcis perspicillata*
61	31.075	-6.505	*Acanthodactylus boskianus, Uromastyx nigriventris*
62	30.978	-6.779	*Uromastyx nigriventris*
63	30.969	-7.071	*Uromastyx nigriventris*
64	30.944	-7.210	*Acanthodactylus pardalis, Amietophrynus mauritanicus, Mauremys leprosa, Mesalina guttulata, Pelophylax saharicus*
65	30.972	-7.111	*Uromastyx nigriventris*
66	30.779	-7.371	*Acanthodactylus boskianus, Agama impalearis*, Lytorhynchus diadema, Ptyodactylus oudrii*

67	30.744	-7.610	*Acanthodactylus boskianus, Amietophrynus mauritanicus, Bufo spinosus, Hyla meridionalis, Natrix maura, Podarcis vaucheri, Psammodromus algirus, Quedenfeldtia trachyblepharus, Scelarcis perspicillata, Timon tangitanus*
68	30.885	-7.748	*Atlantolacerta andreanskyi, Quedenfeldtia trachyblepharus**
69	30.802	-7.767	*Agama impalearis, Pelophylax saharicus, Psammodromus algirus, Timon tangitanus,*
70	30.877	-7.802	*Acanthodactylus boskianus*
71	30.891	-7.802	*Acanthodactylus boskianus, Agama impalearis, Ptyodactylus oudrii, Quedenfeldtia moerens, Timon tangitanus*
72	30.960	-7.728	*Timon tangitanus*
73	30.956	-7.817	*Hemorrhois hippocrepis*
74	31.017	-7.837	*Chamaeleo chamaeleon*
75	31.200	-7.870	*Quedenfeldtia moerens*
76	31.208	-7.851	*Chalcides montanus, Natrix maura, Quedenfeldtia moerens*
77	31.190	-7.854	*Podarcis vaucheri*
78	31.035	-7.709	*Acanthodactylus boskianus, Podarcis vaucheri, Quedenfeldtia trachyblepharus, Scelarcis perspicillata, Timon tangitanus*
79	30.886	-7.746	*Trapelus boehmei**
80	30.886	-7.746	*Atlantolacerta andreanskyi*
81	30.944	-7.741	*Quedenfeldtia trachyblepharus, Timon tangitanus*
82	31.919	-8.025	*Tarentola mauritanica*
83	32.441	-8.049	*Amietophrynus mauritanicus, Natrix maura*
84	31.345	-9.594	*Tarentola mauritanica*
85	31.520	-9.634	*Eumeces algeriensis, Tarentola mauritanica*
86	31.502	-9.697	*Chalcides mionecton*
87	31.481	-9.760	*Saurodactylus brosseti, Testudo graeca*
88	31.466	-9.759	*Chalcides mionecton, Testudo graeca*
89	31.442	-9.718	*Acanthodactylus erythrurus, Chalcides mionecton, Trogonophis wiegmanni*
90	31.122	-9.430	*Barbarophryne brongersmai*, Saurodactylus brosseti*
91	30.998	-9.583	*Saurodactylus brosseti*
92	30.746	-9.824	*Agama impalearis*
93	30.633	-9.768	*Tarentola mauritanica*
94	30.544	-9.706	*Saurodactylus brosseti*
95	30.646	-9.671	*Agama impalearis, Tarentola mauritanica*
96	30.625	-9.565	*Timon tangitanus*
97	30.299	-9.521	*Psammophis schokari, Saurodactylus brosseti*
98	30.057	-9.687	*Acanthodactylus aureus*
99	30.031	-9.645	*Amietophrynus mauritanicus*, Mauremys leprosa*
100	30.038	-9.639	*Eumeces algeriensis, Saurodactylus brosseti*
101	29.865	-9.499	*Testudo graeca*
102	29.865	-9.500	*Saurodactylus brosseti*
103	29.651	-9.990	*Saurodactylus brosseti*
104	29.514	-10.058	*Agama impalearis*
105	29.454	-10.104	*Tarentola mauritanica*
106	29.387	-10.172	*Chalcides mionecton, Chalcides polylepis, Saurodactylus brosseti*
107	29.137	-10.098	*Barbarophryne brongersmai*, Natrix maura, Pelophylax saharicus*
108	29.064	-9.933	*Saurodactylus brosseti, Tropiocolotes algericus*
109	28.968	-9.952	*Acanthodactylus boskianus*

110	28.863	-9.755	*Tarentola mauritanica, Tropiocolotes algericus*
111	28.839	-9.712	*Uromastyx nigriventris*
112	28.838	-9.721	*Uromastyx nigriventris*
113	28.809	-9.464	*Trapelus boehmei*
114	28.688	-9.318	*Psammophis schokari, Uromastyx nigriventris*
115	28.718	-9.466	*Uromastyx nigriventris*
116	28.677	-9.472	*Uromastyx nigriventris*
117	28.719	-10.302	*Saurodactylus brosseti, Tarentola mauritanica*
118	28.829	-10.412	*Tarentola mauritanica*
119	28.628	-10.791	*Saurodactylus brosseti*
120	28.455	-11.038	*Psammophis schokari**
121	28.416	-11.398	*Saurodactylus brosseti*
122	28.221	-11.750	*Saurodactylus brosseti*
123	28.607	-10.519	*Saurodactylus brosseti*
124	28.498	-10.478	*Ptyodactylus oudrii, Saurodactylus brosseti, Tarentola mauritanica*
125	28.499	-10.428	*Saurodactylus brosseti*
126	27.835	-12.884	*Hemorrhois algirus*
127	27.820	-11.522	*Trapelus boehmei*
128	27.004	-13.428	*Tarentola chazaliae*
129	26.652	-13.652	*Tarentola chazaliae*
130	26.400	-14.075	*Acanthodactylus aureus*, Tarentola chazaliae*
131	26.155	-14.418	*Acanthodactylus busacki, Saurodactylus brosseti*
132	26.075	-14.457	*Acanthodactylus busacki, Saurodactylus brosseti, Tropiocolotes algericus*
133	26.541	-12.506	*Trapelus boehmei*
134	26.529	-12.307	*Sphenops sphenopsiformis, Tropiocolotes algericus*
135	27.051	-11.322	*Stenodactylus mauritanicus, Tropiocolotes algericus*
136	27.199	-10.694	*Acanthodactylus boskianus*
137	26.955	-11.664	*Tarentola annularis*
138	27.067	-13.118	*Scutophis moilensis**

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Manuscript II

Atlas Mountains: fuel for speciation? Phylogeographic patterns of *Saurodactylus brosseti*

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Abstract

Phylogeographic assessments in regions such as North Africa are sparse when compared to Europe. However, various geographical barriers of Morocco, especially the Atlas Mountains have been associated with genetic subdivision in several species. *Saurodactylus brosseti* is a widely distributed species endemic to Morocco and a previous study demonstrated divergence within forms of up to 11.4% for a region of the mitochondrial ND4 gene. This was suggested to be indicative of a possible species complex, although sampling was limited and only mtDNA was employed, which may have unusually high rates of variation in geckos. In order to address these shortcomings, phylogeographic patterns within the species were assessed using two mitochondrial (12S and ND4) and three nuclear markers (ACM4, MC1R and BZW1), and with a greater coverage including apparently isolated southern and eastern populations. Results show four main lineages with high genetic diversity and which split at approximately the same time as the orogenesis of the Atlas Mountains. Species distribution modeling was also employed, identifying a large patch of suitable habitat where future sampling could be directed. The species delimitation approach employed was not completely concordant with the hypothesis that each of the four lineages could correspond to a distinct species. However this may have been due to limited sampling within two lineages. Further assessment, possibly including morphological data, would be valuable prior to any revision of the taxonomy, but the highly distinct lineage in the East clearly is of conservation concern.

Keywords: Phylogeography, *Saurodactylus brosseti*, mitochondrial DNA, nuclear DNA, Species probability of occurrence, Vicariance, Atlas Mountains

Introduction

Assessment of genetic diversity within various species in Europe has led to a strong consensus regarding typical phylogeographic patterns and in particular the role of "southern refugia" during Pleistocene glaciations (Hewitt, 1999). However, similar assessments within North Africa are much scarcer (Barrientos *et al.,* 2014), and have often focused on relationships between North Africa and Europe (reviewed in Husemann *et al.,* 2014). Yet various climatic and geological forces are likely to play a role in structuring diversity in North Africa, and particularly in the Northeastern region that is present day Morocco. For example, how successive expansions and contractions of the Sahara may have affected species is still poorly understood (reviewed in Brito *et al.,* 2013) Further back in time, the genesis of the mountain chains in the region, the Rif and the Atlas Mountains, are expected to play a role as important geological barriers. In particular the orogeny of the Atlas Mountains is estimated to have occurred around 9 million years ago (Mya) in the late Miocene (Hsu, 1978), and separates the region climatically with more Mediterranean conditions to the West and more arid environments to the East. Various studies have indicated a vicariant role for these mountains, causing genetic subdivision in *Agama impalearis* (Brown *et al.*, 2002; Gonçalves *et al.*, 2012), the *Acanthodactylus erythrurus* group (Fonseca *et al.*, 2009), the freshwater turtle *Mauremys leprosa* (Fritz *et al.*, 2005; Fritz *et al.*, 2006), scorpions of the genus *Buthus* (Habel *et al.*, 2012; Husemann *et al.*, 2012), the scorpion *Androctonus mauritanicus* (Coelho *et al.*, 2014), and the bat complex *Myotis natteri* (Salicini *et al.*, 2013). However, although in these cases the Atlas mountains played a role in separating populations, at a smaller scale quite different patterns were observed, with some groups such as scorpions showing many microrefugia (Husemann *et al.*, 2012), and others such as the agamas showing much simpler subdivisions into just two major lineages (Brown *et al.*, 2002). There is therefore a clear need to assess diversity within additional species from the region to try to develop an overall phylogeographic scenario for the region.

Saurodactylus is a genus of geckos endemic to the Maghreb region (Bons and Geniez, 1996). It is an extremely old taxon that is estimated to have separated from its sister group almost 100 million years ago (Gamble *et al.*, 2011). It currently comprises three species: *Saurodactylus mauritanicus* Duméril and Bibron 1836, *Saurodactylus fasciatus* Werner 1931 and *Saurodactylus brosseti* Bons and Pasteur 1957. *Saurodactylus brosseti* is endemic to Morocco, known to occur from Beni Mellal, central Morocco, to the depths of Western Sahara in the south (Figure 1). An assessment of phylogenetic relationships between the three species indicated that each was monophyletic, but that *S. brosseti* harbored a very high level of mitochondrial diversity, up to 11.4% for the ND4 gene region analyzed (Rato and Harris, 2008). Such a level of diversity is much higher than between many recently described lizard species (e.g. Ahmadzadeh *et al.,* 2013), and was considered to be indicative of a possible species complex. However, sampling in this earlier study was limited, and in particular samples from the known localities of *S. brosseti* in the very arid regions in Western Sahara and to the East of the Atlas Mountains were not included.

Just as molecular data, and particularly mitochondrial DNA sequencing, revolutionized the assessment of shifting distributions with Pleistocene climatic fluctuations, so the current tendency to combine molecular data with ecological niche modelling is transforming our predictions of current and future distributions. With improving data for various climatic variables it is possible to predict species distributions, both under current conditions and for expected future ones. This is particularly valuable in regions like North Africa and for groups such as reptiles were distribution data is still being refined (e.g. Damas-Moreira *et al.*, 2014). Furthermore, this tool has been demonstrated to be effective in the Wall lizards *Podarcis*, with targeted fieldwork recording specimens in the expected but previously unreported localities (Kaliontzopoulou *et al.*, 2008).

In this study a phylogeographic assessment of variation within *S. brosseti* was augmented with an ecological niche modelling approach and a relaxed molecular clock to determine both patterns and timing of subdivisions within the species. This was then used to assess the influence of the different geological events on the evolutionary history of *S. brosseti*. In particular the aim was to a) determine if the Atlas mountains had caused separation between lineages, b) to assess colonization pathways across the different occupied habitats, c) to determine if the isolated populations were genetically distinct, or were the result of recent fragmentation, possibly associated with climatic changes, and d) to identify other possible regions with high probability of the species occurring. Finally, a species delimitation approach was used to infer if taxonomic changes might be needed, that is if *S. brosseti* is actually a species complex.

Material and methods

Phylogenetic Analysis

Sampling

Two fieldtrips (May 2013 and June 2014) were carried out, from which 36 samples of *Saurodactylus brosseti* were collected. Samples were collected by hand and data recorded with GPS and annotations of relevant information. In addition, a small piece of the animal tail tip was collected and stored in 96% ethanol and photos were taken before the release of the animal. Available data from previous fieldwork was also used from *Saurodactylus brosseti* but also *S. mauritanicus* and *S. fasciatus* to be used as outgroups.

DNA extraction, amplification and sequencing

DNA was extracted from tail tips tissue using High Salt method (Sambrook *et al.*, 1989). DNA amplification was performed through PCR for mitochondrial genes (12S and ND4), nuclear genes (ACM4 and MC1R) and one intron (BZW1). PCR conditions and primers were according to the references (Table 1). PCR products were checked with electrophoresis and positive amplification products were sent to Beckman Coulter Genomics (UK) for purification and Sanger sequencing.

Table 1 – Primers names and amplification conditions.

Gene	12S			ND4			ACM4			BZW1			MC1R		
Step	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X	T (ºC)	Time	X
Initial Denaturation	95º	1'	1	94º	3'	1	94º	5'	1	94º	3'	1	92º	2'	1
Denaturation	95º	15''	35	94º	3'	35	94º	5'	1	94º	3'	37	92º	2'	35
Annealing	48º	15''		94º	30''		94º	30''	32	94º	30''		92º	1'	
Extension	72º	10''		48º	30''		55º	45''		62º	45''		55º	45''	
Final Extension	72º	10'	1	72º	40''	1	72º	1'	1	72º	1'		72º	1'	1
Primer Forward	12S L			ND4			Tg-F			Tar1			MC1R F		
Primer Reverse	12S H			LEU			Tg-R			Tar2			MC1R R		
Citation	Kocher *et al.* 1989			Arévalo *et al.* 1994			Gamble *et al.* 2008			Fuijta *et al.* 2010			Pinho *et al.* 2010		

Phylogenetic analysis

Confirmation of species was done blasting sequences to the NCBI database on GenBank. Chromatographs were checked and sequences aligned with Geneious v5.6 for posterior phylogenetic analysis. 12S and ND4 sequences of *Saurodactylus brosseti* from a previously published study (Rato and Harris, 2008) available on GenBank were also included in the alignment. jModelTest v2.1.4 (Darriba *et al.,* 2012 – 201) was used to infer which model best fit each data under the Akaike Information Criterion (Cavanaugh, 2007 – 205) for separate and concatenated genes in order to decrease the error (Brandley *et al*., 2005 - 227). Nuclear genes were phased using Seqphase (Flot, 2010 - 231) and PHASE (Stephens *et al*., 2005 – 230) with a threshold of 0.6 and default for all the other parameters. Phylogenetic accuracy can be higher when data sets from different genes are combined into a single phylogenetic analysis (Rokas *et al.,* 2013 - 226). Maximum Likelihood analysis was performed using RAxML v3.0 (Stamatakis, 2014 - 229) with 1000 replicates for concatenated genes. Bayesian analyses were performed with best fitting models applied to each gene with MrBayes v3.2.2 (Huelsenbeck and Ronquist, 2001 – 192) for a concatenated approach (one partition) and *BEAST v1.8.0 (Drummond *et al*., 2012 – 204) for a coalescent approach (five partitions, all parameters unlinked across partitions, except for 12S and ND4, for which trees were linked into "mitochondrial DNA"). MrBayes analysis began with random starting tree, ran for 10 million generations and was sampled every 100 generations. 25.000 (25%) of burn-in trees were discarded and the remaining were used to assess posterior probability values. *BEAST ran three times for 150 million generations with an uncorrelated lognormal relaxed clock with a rate of 0.00701 for 12S (Metallinou *et al.,* 2012 - 225) as calibration point to estimate divergence times. In order to have a species tree, sequences were grouped into four groups, according to the four major clades assessed by MrBayes and RAxML. All runs were combined with LogCombiner v1.8.0 (package of *BEAST) with a burn-in of 10% for each run. Mean genetic distances between the four major clades (given the results from MrBayes and RAxML) were calculated with MEGA6 (Tamura *et al*., 2013 – 203) for ND4.

Species probability of occurrence

Input occurrence data was obtained from geographic coordinates of known occurrence localities (Bons and Geniez, 1996) and CIBIO's reptile database. In total, 246 points were depicted using ArcMap v9.3 under the WGS 1984 Datum geographic coordinate system. Five environmental variables (Table 2) were chosen according to the species known limiting factors and downloaded from WorldClim – Global Climate Data database (Hijmans *et al*., 2005 – 147) in 30-arc seconds resolution tiles. Since correlation of environmental variables varies according to its extension, all five variables were cut giving *Saurodactylus brosseti* known distribution range, using ArcMap v9.3. Distribution of *S. brosseti* can overlap distribution of the other two species of the genus (Bons and Geniez, 1996), and so variables were also cut according to their distribution ranges, ensuring that localities with high probability of occurrence for one species are not coincident of the localities from neither the two other species. To estimate the presence probability of the three species of *Saurodactylus* the software Maxent (Phillips and Dudik, 2008 – 206) was used. All duplicate presence records from the known occurrence data were removed before the software run to prevent data overfitting. All runs were performed with 25 random test percentage, 20 bootstrap replicates and in order to create response curves with jacknife to measure variable importance. Area under the curve (AUC) was used to assess model accuracy. Even though it is not as reliable as it would be expected (Lobo *et al.,* 2007 - 217) it still the best method. Since Maxent presents its results in a gradient "probability of occurrence" ranging from 0 to 1, a threshold was defined in order to build maps of "suitable or unsuitable" habitat. This

was done using the average of the 10 percentile training presence logistic threshold of the 20 replicates for each species. Values above the threshold were considered as “suitable habitat” and values below threshold were considered as “unsuitable habitat”.

Table 2 – Environmental variables used in the assessment of species distribution probability.

Code	Variable
BIO1	Annual mean temperature
BIO6	Minimum temperature of coldest month
BIO7	Temperature annual range
BIO12	Annual precipitation
BIO14	Precipitation of driest month

Results

From the Bayesian and Maximum Likelihood, four major groups can be distinguished: North, South, Northeast and Anti-Atlas. Northeast and South form a polytomy (Fig. 3), meaning that evolutionary relationships between themselves are not fully resolved. Some of the branches do not have high support. For example, South lineage has little support (posterior value = 0.65) and also in the split into Anti-Atlas and North lineages (posterior value = 0.85). On the other hand, Northeast, Anti-Atlas and North lineages are highly supported (posterior values = 0.99, 1 and 0.98 respectively; bootstrap values = 100 Northeast and 92 for Anti-Atlas lineages).

After different groups within the main lineages are considered and spatially plot into the map (Fig. 3, different colors within the main clades), a more or less clear geographical distribution can be seen. South lineage has a geographical distribution partitioned from center of Morocco to the south most geographic known location (color gradient within South lineage); North lineage does not present such perfect division but it is also clear that some geographical structure is present (color gradient within North lineage). Either looking to the phylogeny or to the map into more detail, the region of the Draa Valey between North and South lineages presents lot of variation (Fig. 3); even the Anti-Atlas lineage is covered by that zone.

Considering the species tree (Fig. 4), the four lineages are more clearly split, even though most branches do not present high support. Even though, it is evident that divergence occurred at the same time to the North and South of Morocco. Part of the population expanded to the South and has an obvious separation within the lineage that goes further into the south (Fig. 3); the specimens in southernmost locations of Morocco and the ones in Western Sahara are closely related, despite the gap between them. Divergence of the species into its lineages started at roughly the same time, which was in the Miocene around 10 Mya. Atlas Mountains are thought to have started their orogeny around 9 Mya (Hsu 1978, – 22), which then would have had influence on the split of the species. North and Anti-Atlas lineages are estimated to have split around 6 Mya and these two from the Northeast lineage at around 8 Mya. Genetic distances for ND4 show 6.9% of variation between North and Anti-Atlas lineages, 8% of variation between Anti-Atlas and Northeast lineages, and 8.1% divergence between North and Northeast lineages. Higher genetic variation is seen between South and Northeast (11%), North and South (11.3%) and Anti-Atlas and South lineages (11.5%).

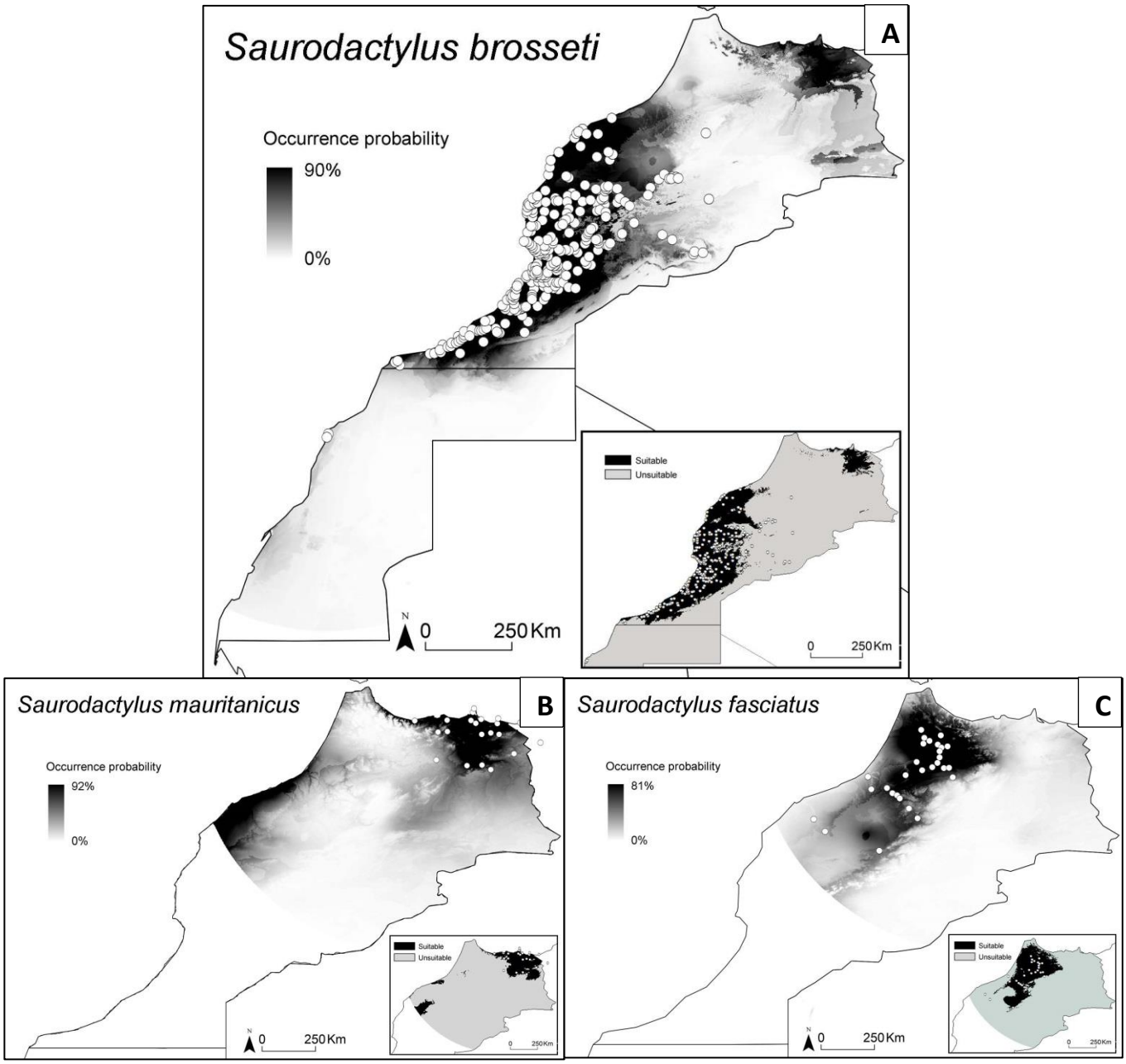

Fig. 1 – Modeling results of species probability of occurrence and habitat suitability for (A) *Saurodactylus brosseti*, (B) *Saurodactylus mauritanicus*, and (C) *Saurodactylus fasciatus*.

Modeling has predicted new patches of probability of occurrence for *Saurodactylus brosseti* (Fig. 1). The largest part is in the north of the country, but since it corresponds to the distribution range of *Saurodactylus mauritanicus* it is not very likely that *S. brosseti* will occur in that area. In the model prediction for *S. mauritanicus* there is also areas of suitable habitat for this species in which *S. brosseti* occur. Other interesting patch of high occurrence probability for *S. brosseti* is the south most border area of oriental region, which is not of high probability of occurrence for *S. mauritanicus*, especially by looking at suitable/unsuitable map (Fig. 1). Western Saharan areas present very low probability of occurrence and do not appear as suitable for *S. brosseti*. Regarding *S. fasciatus* results, there are no major changes to the actual known distribution range (Fig. 1). AUC showed good fit of the model for the three analyses: 0.955 for *S. brosseti*, 0.935 for *S. mauritanicus* and 0.925 for *S. fasciatus*

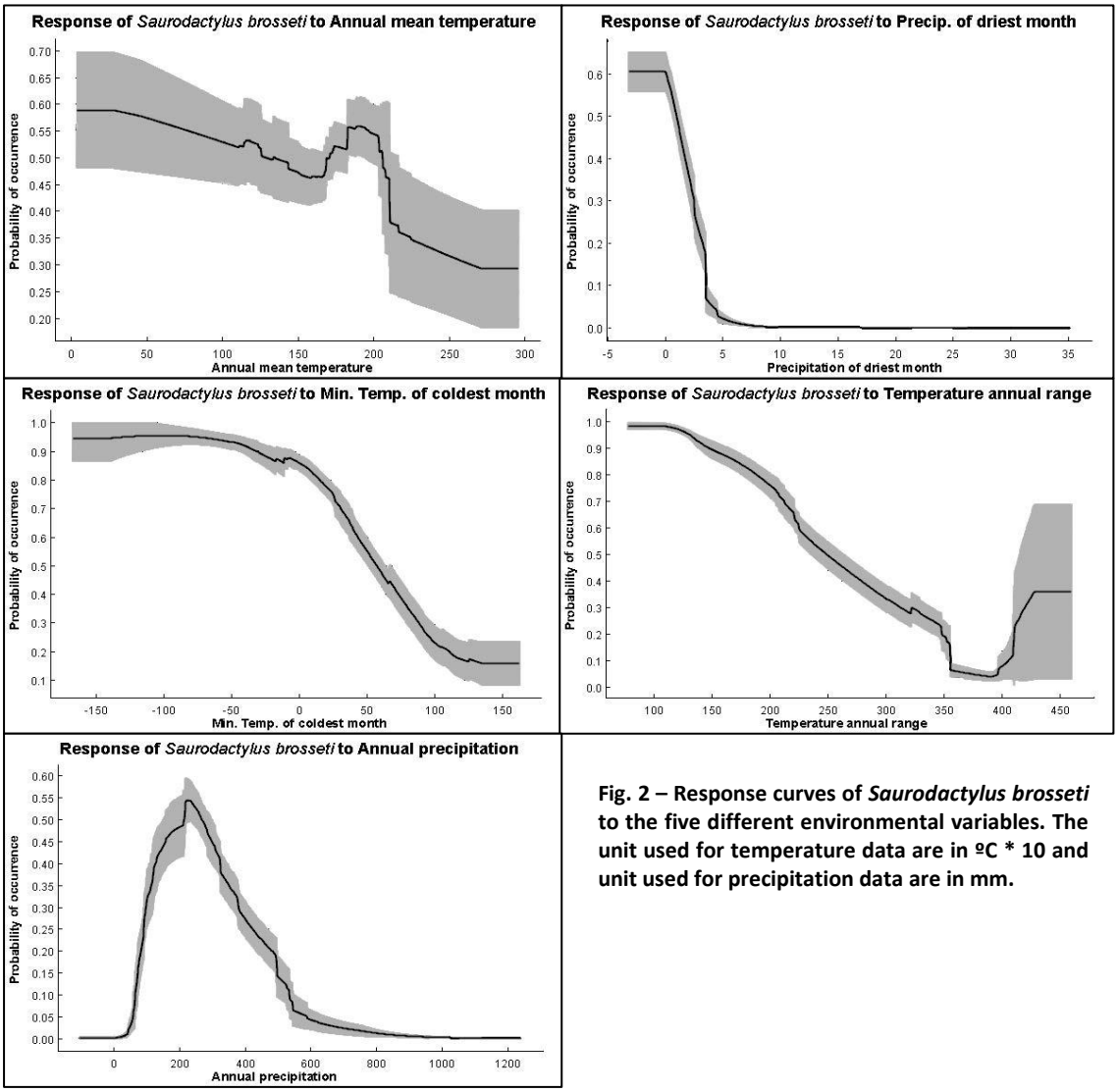

Fig. 2 – Response curves of *Saurodactylus brosseti* to the five different environmental variables. The unit used for temperature data are in ºC * 10 and unit used for precipitation data are in mm.

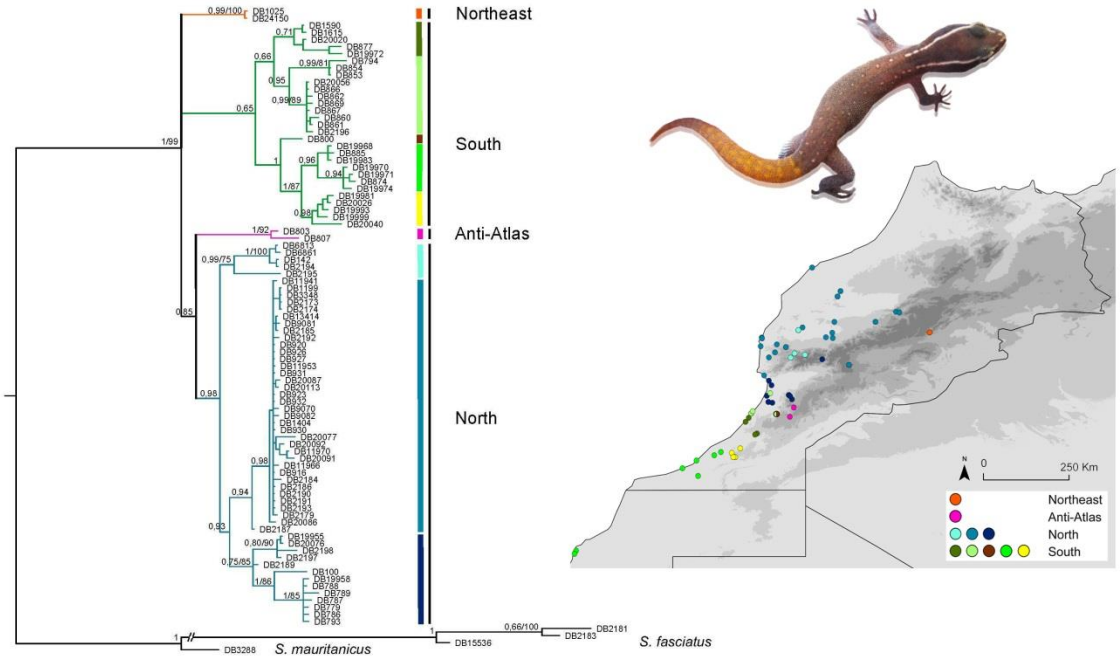

Fig. 3 – Left: tree derived from Bayesian analysis with MrBayes (5 concatenated genes). Posterior values higher than 0.95 (95%) and bootstrap values higher than 75% (RAxML analysis) are given in the branches. Right: corresponding distribution map with color correspondence to each of the major groups within lineages.

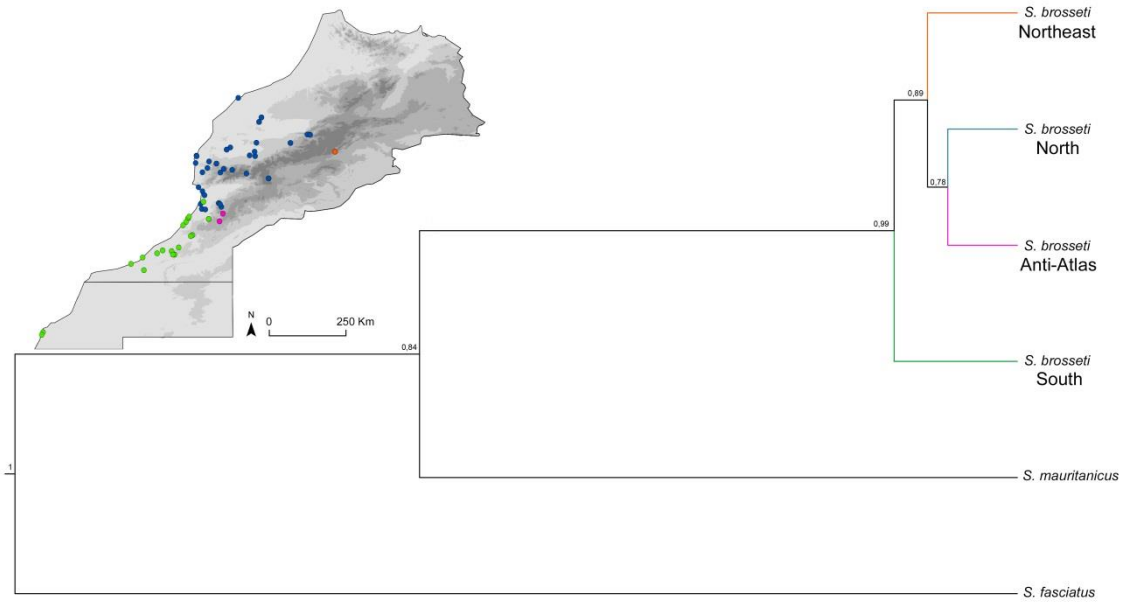

Fig. 4 – Tree derived from Bayesian analysis (species delimitation approach) with *BEAST. Posterior values higher than 0.95 (95%) are given in the branches. Map shows the distribution with color correspondence to colored branches.

All environmental features decrease, at some point, the probability of occurrence of *S. brosseti* (Fig. 2), except for Annual mean temperature that presents a high peak of probability of occurrence between 18ºC and 22ºC but with a weaker variation. Precipitation of the driest month causes an abrupt decrease in the species probability of occurrence between 0 and 5 mm. Minimum temperature of the coldest months also presents a decrease, but not so rapid. Higher values of temperature annual range cause a decrease in the species probability of occurrence until 35ºC. Lastly, annual precipitation causes a peak in the probability of occurrence at around 200 mm.

Discussion

From both the Bayesian and Maximum Likelihood analysis it is clear that there is lot of geographically structured variation within *Saurodactylus brosseti* (Fig. 3). Four lineages were identified, with two widespread ones (North and South) and two from small areas (Northeast and Anti-Atlas). The polytomy between these can be a “hard” polytomy which means that the ancestor split actually forming the different lineages at the same time; or it might be a “soft” polytomy, that is a consequence of lack of phylogenetic information. Only two mitochondrial and three nuclear genes were sequenced and more genes should be added in order to resolve the phylogenetic relationships seen in the phylogeny, especially between Northeast and South clades.

The low support for the South lineage (posterior value = 0.65) is most likely due to a high level of divergence between specimens. Northeast lineage high support (posterior value = 0.99; bootstrap value = 100) is due to the fact that it is comprised only by two sequences that are so similar. There is a more or less clear geographical distribution pattern within South and North lineages (Fig. 3). It is even possible that South lineage could be considered as two geographically separated lineages. There is one specimen (DB800) in one of the clades that occupies the same geographic space as specimens belonging to the other main clade within the South lineage. This strengthens the conclusion that South clade is so divergent. The specimens from the isolated population in Western Sahara were not genetically very different from other specimens in the South lineage. The geographical gap between southernmost specimens can be because species does not occur there or simple because it was never seen in any of the fieldtrips. In fact, by looking at Fig. 1 it is possible to see that modeling did not predict that area as suitable and it has a very low species probability of occurrence. This lack of differentiation and currently suitable habitat means that the separation between the populations is recent, and related to habitat changes. Most likely, increasing aridity during the Pleistocene has had a strong recent impact on the distribution of the species, with many areas to the south and east of the current range becoming unsuitable so that only a few isolated populations remain.

It is clear that there is particularly high variation in the region of the Draa Valley (Fig. 3) both in terms of lineages (3 of the 4 occur here), and for variation within the South lineage. This is therefore the ideal region for further studies of potential gene flow between major lineages, as a transect across the Anti-Atlas could be performed, in which all three lineages would be included.

Regardless, the 95% confidence interval of the divergence dating estimations is somewhat wide, enough to be affirmative about the influence of the Atlas Mountains on the split of *Saurodactylus brosseti* lineages. Furthermore, climatic oscillations in the Miocene, Pliocene and Pleistocene coupled with the prolongation of the rising of the Atlas Mountains continued to have an effect on the species distribution. The limited variation in much of the range of the Northern lineage could be associated with a recent range expansion, while the southern lineage has simultaneous reduced. The model predicts patches of habitats that are not suitable for the species (Fig. 1), but where it is known to occur or has been recorded. In such cases,

conservation priorities should be thought, especially if the species is considered cryptic. Northeast lineage is particularly in danger and more sampling in the area is needed to fully understand the situation. For example, *Saurodactylus brosseti* has been recorded (Bons and Geniez, 1996) around the area of Zagora, but the authors have more recently done fieldwork in the area without detecting any specimen. The old localities for the species are now in highly disturbed habitats along rivers surrounded by completely unsuitable desert habitat This means that habitat destruction is likely to have an impact on the species distribution, particularly in such area where conditions are not the most suitable, and fortifies the idea of conservation priorities in such areas, especially given how genetically distinct the only sampled population to the East of the Atlas mountains is.

The low branch support of the species tree (Fig. 4) can be again a result of only few genes have been used or not enough sampling, especially in the Northeast and Anti-Atlas lineages. There is a lot of disagreement in species delineation and specific rules by which one can consider a lineage or population to be a different species are not certain (Fujita *et al.,* 2012 - 186). In such case as the one of *Saurodactylus brosseti*, given the phylogenetic analysis (Fig. 3 and Fig. 4), it is easy to lean towards speciation between lineages. Furthermore, such high level of divergence of ND4 between lineages is an indication of cryptic speciation – many currently described species of reptiles have lower levels of variation than this between species (eg Ahmadzhadeh *et al.* 2013). Species delimitation is thought to be easier when several methodologies are implemented such as morphology and phylogeography based on different approaches (Fujita *et al,.* 2012 - 186). However, morphological differences assessment of *S. brosseti* is an arduous task given the small size of the species. Therefore, will it seems that the Atlas mountains are fuelling the diversity within *S. brosseti*, there is still currently not enough evidence to be sure whether it is fuelling speciation or just intraspecific diversity.

Modeling results show overlap of suitable habitats between *S. brosseti* and *S.mauritanicus*, which is most likely due to the fact that both species are so similar; they were even previously considered only one species (Bons and Geniez, 1996). They have been both elevated to their current species status but they still present very similar ecological requirements, which is why model present such results, almost joining both species ranges together as suitable habitat. The patch of suitable habitat in the south most border area of oriental region for *S. brosseti* is of high interest and sampling should be focused on that area, especially because *S. mauritanicus* does not present that area as suitable (Fig. 1). Indeed, models seem to predict that the distribution range and habitat suitability of all three species has a tendency to decrease.

Various other phylogeographic studies within Morocco and North Africa have showed high levels of divergence within species (Barata *et al.,* 2012), but one very well known problem of the region is sampling effort, especially northeast of Morocco (Beukema *et al.,* 2013 - 241). Further sampling is thus needed for this and other species with unresolved phylogenies; also conservation efforts need to be considered in such cases as the one of *S. brosseti* that clearly presents geographically structured high divergence between and within lineages.

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GENERAL DISCUSSION

There are not as many phylogeographic studies of organisms from North Africa compared to Europe, but there are reports of species phylogenies being influenced by geographical barriers, such as the Moulouya River (e.g. Álvarez *et al.*, 2000) or the Atlas Mountains (e.g. Fonseca *et al.*, 2009). Also, climatic fluctuations, especially in the Pleistocene have had an impact in species distribution ranges (Hewitt, 2000). Around 9 Mya the formation of the Atlas Mountains began (Hsu, 1978) and it has had an impact, mainly of a vicariant nature, on several species. In *Agama impalearis* a simple subdivision into two lineages clearly formed at around 8.5 to 9.4 Mya was reported (Brown *et al.*, 2002). It is suggested that the Atlas Mountains and also the reopening of the Strait of Gibraltar split the *Acanthodactylus erythrurs* group (Fonseca *et al.*, 2012). *Mauremys leprosa* (Fritz *et al.*, 2005; Fritz *et al.*, 2006), *Myotis natteri* (Salicini *et al.*, 2013), *Androctonus mauritanicus* (Coelho *et al.*, 2014) and various *Buthus* species (Habel *et al.*, 2012; Husemann *et al.*, 2012) were also biogeographically substructured by the Atlas Mountains, although the latter displays many microrefugia (Husemann *et al.*, 2012). Thus the Atlas Mountains are known to act as both a simple barrier, and a complex region in which multiple endemic forms have evolved, particularly those specialising as high-montane species such as *Quedenfeldtia* species (Barata *et al.*, 2011) or *Atlantolacerta andreanskyi* (Barata *et al.*, 2012). The Atlas Mountains have also had an impact and divide the climate of Morocco, which thus presents a much diversified topology, characteristics that together make the kingdom one of the richest in herptofauna diversity. For the reasons presented above, there is a need to assess phylogeographic patterns of the many other species of Morocco. Hewitt extensively studied the effects of the climatic oscillations during the Pleistocene and showed that diversity across many taxa was heavily influenced by those oscillations (Hewitt, 2000). Africa, and specifically the Sahara desert, was not an exception in experiencing climatic variations in the Quaternary Period, suffering from humid and drier periods (Brito *et al.*, 2014); this also had an impact in the flora and fauna of the region.

There is an additional need to accurately complete range distribution datasets, mainly in the Eastern region of the country (Beukema *et al*., 2013). This is the first step towards a complete phylogeographic study and only with a good knowledge of a species distribution range is it possible to truly understand evolutionary history and patterns of diversity within a species.

After two fieldtrips to Morocco, a total of 53 different species in 138 localities were sampled. After DNA barcoding techniques were used for species for which identification was not possible in the field, various new range expansions were unveiled

(Manuscript I). These range extensions, especially for *Bufo spinosus*, *Trapelus boehmei*, *Tropiocolotes algericus*, *Acanthodactylus erythrurus*, *Chalcides polylepis* and *Scutophis moilensis* show that even species that are thought to be well known in terms of their distribution, sometimes are not.

Specifically regarding *Saurodactylus* brosseti, the main focus of this thesis, a previous phylogenetic study, despite its limited sampling, had shown that *Saurodactylus brosseti* presents high levels of intraspecific variation, up to 11.4% for ND4 (Rato and Harris, 2008). Some recently described species do not present such high variation (e.g. Ahmadzadeh *et al.*, 2013) and so, it represented a possible species complex. The results presented in the previous section again emphasize such diversity, given that results show that *S. brosseti* presents lot of variation (from 6.9% to 11.5%) between four distinct lineages, which are also geographically structured (Manuscript II). This also demonstrates that by increasing sampling effort it is possible to see more clearly the history of a species. In Rato and Harris (2008), two lineages were shown and that raised questions about the phylogeny of *Saurodactylus brosseti*; however only after a greater effort to analyse more specimens and with more genes was it possible to understand a new level of structuring in the species. This is very important, meaning that the higher and more accurate the effort is, the better researches can understand biogeographic patterns of species.

A plausible explanation for such high differences between lineages is the rise of the Atlas Mountains. Estimated time of divergences showed that the time of the split of the four lineages coincides with the time for the orogeny of the Atlas Mountains. It seems like species expanded North and South at roughly the same time and started to diverge separately. However, the weak support of the tree branches turns makes it difficult to exactly interpret the history of the split. The Northeast lineage could have been an expansion from the South lineages through the east side of the Anti-Atlas or it was originally more related to the North lineage and was after split by the High-Atlas.

Furthermore, there is still considerable variation within Northern and Southern lineages (Manuscript II) that is probably due to climatic oscillations during the Pleistocene coupled with the continuity of the Atlas Mountains rising since 9 Mya and onwards. Low support of some branches can be related to high diversity in some lineages, such as the Southern lineage, coupled with low sampling number in other lineages – Northeast and Anti-Atlas.

Considering modelling of species distribution, it has been shown to be a useful tool to understand ecological requirements of species and also for directing specific sampling effort. For example, in the *Podarcis* lizards, modelling was demonstrated to be highly effective, highlighting patches were the species had not been but was later recorded

(Kaliontzopoulou *et al.*, 2008). In this previous study, the area predicted was small and thus easier to do targeted fieldwork on; the area predicted for *Saurodactylus brosseti* is much bigger and thus more difficult to prospect extensively. Environmental modelling is a growing and powerful area in which past, present and future historical events can be predicted, making correspondence between environmental conditions and species distribution. Once again, accurate sampling is of great importance in order to get better predictions and model predictions help direct sampling efforts.

Hence, the study of niche evolution has gained a major importance in the last few years, focusing on techniques to estimate niche overlap in realized niches between taxa in an explicit spatial context (Broennimann *et al.,* 2012), either based on ordination methods (Hof *et al.* 2010; Thuiller *et al.* 2005) or on the output of species distribution models (SDMs) (Guisan and Thuiller, 2005; Warren *et al.* 2008). More recently, based on the methodology proposed by Evans *et al.* (2009), SDMs are being combined with calibrated phylogenies in order to study the evolution of climatic niches by reconstructing the ancestral environmental tolerances among lineages (e.g. Ahmadzadeh *et al.* 2013; Jakob *et al.* 2010; Smith and Donoghue, 2010). This would also be an interesting future work that could help understand differences in the ecological requirements of the different lineages.

Speciation and species delimitation is extremely difficult and it is clear that in order for a lineage or population to be considered a species, integrative frameworks should be used including not only different phylogeny approaches but also morphology (Fujita *et al.*, 2012). *Saurodactylus brosseti* is a small gecko and morphological assessment is challenging but it could be done in order to support phylogenetic findings. This thesis comprises a phylogeographic analysis from which is clear that the levels of genetic variation within the species is very high. Since only two mitochondrial and three nuclear genes were used and due to small sampling of Northeast and Anti-Atlas lineages, the relations between all four lineages are still not fully understood. A higher number of genes could be sequenced in the future, in order to better estimate the phylogeographic patterns of the species, combined with morphological characters assessment, comprising a full species analysis. This is of extreme importance, since it is also clear that at least Northeast lineage appears to be effectively small and spatially separated from the other lineages, possibly needing conservation assessment.

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APPENDIX

Origin of sample	Species	Lat	Long	Accession numbers (12S/ND4/ACM4/BZW1/MC1R)
Fieldtrip 2013	*Saurodactylus brosseti*	30.30	-9.52	
Fieldtrip 2013	*Saurodactylus brosseti*	29.86	-9.50	
Fieldtrip 2013	*Saurodactylus brosseti*	29.86	-9.50	
Fieldtrip 2013	*Saurodactylus brosseti*	29.86	-9.50	
Fieldtrip 2013	*Saurodactylus brosseti*	29.06	-9.93	
Fieldtrip 2013	*Saurodactylus brosseti*	28.63	-10.79	
Fieldtrip 2013	*Saurodactylus brosseti*	28.63	-10.79	
Fieldtrip 2013	*Saurodactylus brosseti*	28.42	-11.40	
Fieldtrip 2013	*Saurodactylus brosseti*	28.22	-11.75	
Fieldtrip 2013	*Saurodactylus brosseti*	28.22	-11.75	
Fieldtrip 2013	*Saurodactylus brosseti*	26.16	-14.42	
Fieldtrip 2013	*Saurodactylus brosseti*	26.07	-14.46	
Fieldtrip 2013	*Saurodactylus brosseti*	28.61	-10.52	
Fieldtrip 2013	*Saurodactylus brosseti*	28.50	-10.48	
Fieldtrip 2013	*Saurodactylus brosseti*	28.50	-10.48	
Fieldtrip 2013	*Saurodactylus brosseti*	28.50	-10.48	
Fieldtrip 2013	*Saurodactylus brosseti*	28.50	-10.43	
Fieldtrip 2013	*Saurodactylus brosseti*	28.50	-10.43	
Fieldtrip 2013	*Saurodactylus brosseti*	28.72	-10.30	
Fieldtrip 2013	*Saurodactylus brosseti*	29.39	-10.17	
Fieldtrip 2013	*Saurodactylus brosseti*	29.65	-9.99	
Fieldtrip 2013	*Saurodactylus brosseti*	30.04	-9.64	

Fieldtrip 2013	*Saurodactylus brosseti*	30.04	-9.64	
Fieldtrip 2013	*Saurodactylus brosseti*	30.54	-9.71	
Fieldtrip 2013	*Saurodactylus brosseti*	30.54	-9.71	
Fieldtrip 2013	*Saurodactylus brosseti*	30.54	-9.71	
Fieldtrip 2013	*Saurodactylus brosseti*	31.00	-9.58	
Fieldtrip 2013	*Saurodactylus brosseti*	31.00	-9.58	
Fieldtrip 2013	*Saurodactylus brosseti*	31.12	-9.43	
Fieldtrip 2013	*Saurodactylus brosseti*	31.12	-9.43	
Fieldtrip 2013	*Saurodactylus brosseti*	31.48	-9.76	
Fieldtrip 2013	*Saurodactylus brosseti*	31.48	-9.76	
Fieldtrip 2013	*Saurodactylus brosseti*	31.48	-9.76	
Fieldtrip 2013	*Saurodactylus brosseti*	31.48	-9.76	
Fieldtrip 2013	*Saurodactylus brosseti*	31.48	-9.76	
Fieldtrip 2014	*Saurodactylus brosseti*	31.62	-5.56	
Published in Rato and Harris 2008	*Saurodactylus brosseti*	33.25	-8.50	EU014300/EU014325/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.62	-8.00	EU014301/EU014326/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.50	-9.77	EU014311/EU014336/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.68	-8.85	EU014302/EU014327/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.68	-8.85	EU014312/EU014337/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.68	-8.85	EU014304/EU014329/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.68	-8.85	EU014303/EU014328/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.07	-8.68	EU014305/EU014330/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	30.10	-9.55	EU014306/EU014331/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	29.88	-9.60	EU014307/EU014332/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	29.88	-9.60	EU014313/EU014338/-/-/-

Published in Rato and Harris 2008	*Saurodactylus brosseti*	33.25	-8.50	EU014314/EU014339/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.89	-6.91	EU014308/EU014333/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.89	-6.91	EU014309/EU014334/-/-/-
Published in Rato and Harris 2008	*Saurodactylus brosseti*	31.49	-7.98	EU014310/EU014335/-/-/-
Reptile DB	*Saurodactylus brosseti*	30.95	-8.25	
Reptile DB	*Saurodactylus brosseti*	30.99	-9.04	
Reptile DB	*Saurodactylus brosseti*	30.06	-9.09	
Reptile DB	*Saurodactylus brosseti*	30.06	-9.09	
Reptile DB	*Saurodactylus brosseti*	30.06	-9.09	
Reptile DB	*Saurodactylus brosseti*	30.06	-9.09	
Reptile DB	*Saurodactylus brosseti*	29.95	-9.01	
Reptile DB	*Saurodactylus brosseti*	30.03	-9.05	
Reptile DB	*Saurodactylus brosseti*	29.58	-9.40	
Reptile DB	*Saurodactylus brosseti*	29.58	-9.40	
Reptile DB	*Saurodactylus brosseti*	29.51	-9.06	
Reptile DB	*Saurodactylus brosseti*	29.74	-8.96	
Reptile DB	*Saurodactylus brosseti*	29.58	-9.40	
Reptile DB	*Saurodactylus brosseti*	29.58	-9.40	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	29.60	-10.03	
Reptile DB	*Saurodactylus brosseti*	28.03	-11.36	

Reptile DB	*Saurodactylus brosseti*	29.48	-10.09
Reptile DB	*Saurodactylus brosseti*	28.54	-10.96
Reptile DB	*Saurodactylus brosseti*	32.53	-7.86
Reptile DB	*Saurodactylus brosseti*	32.53	-7.86
Reptile DB	*Saurodactylus brosseti*	32.53	-7.86
Reptile DB	*Saurodactylus brosseti*	32.53	-7.86
Reptile DB	*Saurodactylus brosseti*	32.53	-7.86
Reptile DB	*Saurodactylus brosseti*	32.66	-7.79
Reptile DB	*Saurodactylus brosseti*	32.66	-7.79
Reptile DB	*Saurodactylus brosseti*	32.66	-7.79
Reptile DB	*Saurodactylus brosseti*	31.62	-5.56
Reptile DB	*Saurodactylus brosseti*	32.14	-6.40
Reptile DB	*Saurodactylus brosseti*	31.75	-8.74
Reptile DB	*Saurodactylus brosseti*	29.09	-9.89
Reptile DB	*Saurodactylus brosseti*	29.09	-9.89
Reptile DB	*Saurodactylus brosseti*	30.42	-9.58
Reptile DB	*Saurodactylus brosseti*	31.68	-8.85
Reptile DB	*Saurodactylus brosseti*	32.13	-6.32
Reptile DB	*Saurodactylus brosseti*	31.10	-8.94
Reptile DB	*Saurodactylus brosseti*	31.10	-8.94
Reptile DB	*Saurodactylus brosseti*	31.89	-7.94
Reptile DB	*Saurodactylus brosseti*	30.81	-7.58
Reptile DB	*Saurodactylus brosseti*	30.81	-7.58
Reptile DB	*Saurodactylus brosseti*	31.33	-9.38
Reptile DB	*Saurodactylus brosseti*	31.28	-9.79

Reptile DB	*Saurodactylus brosseti*	31.26	-9.16	
Reptile DB	*Saurodactylus brosseti*	31.26	-9.16	
Reptile DB	*Saurodactylus brosseti*	31.51	-8.16	
Published in Rato and Harris 2008	*Saurodactylus fasciatus*	34.77	-5.52	EU014299/EU014343/-/-/-
Published in Rato and Harris 2008	*Saurodactylus fasciatus*	32.60	-7.81	EU014296/EU014340/-/-/-
Reptile DB	*Saurodactylus fasciatus*	34.15	-4.83	
Reptile DB	*Saurodactylus mauritanicus*	34.90	-3.59	

Appendix 1. – Information of the samples used for phylogenetic analyses.