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Integrative taxonomic study on selected reptiles from the Atlas Mountains, North Africa

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Resumo alargado

Quantificar a biodiversidade que nos rodeia é uma tarefa tão importante quanto complexa. Esta requer a integração de vários ramos da biologia, desde a evolução até à ecologia e conservação de espécies ameaçadas. A definição de espécie continua a ser objeto de grande controvérsia – basearmo-nos apenas em características morfológicas, levará à não identificação de "espécies crípticas", porém, se a separação de espécies tiver por base apenas diferenças ao nível molecular, pode levar a uma sobrestimação das mesmas. Na tentativa de contornar este problema, a ideia de complementar análises moleculares com observações morfológicas e modelagem ecológica começa a ser aceite numa abordagem designada por "taxonomia integrativa".

Os sistemas montanhosos são ricos em biodiversidade e endemismos, muitas vezes com pouco fluxo genético devido ao seu isolamento por habitats intermediários cujas características diferem significativamente e podem ser inadequadas para espécies de alta altitude. Essa especialização por parte dos organismos que habitam as montanhas pode ser comparada ao que ocorre nas ilhas, onde os indivíduos também se adaptam a condições muito específicas. Por estas razões, quaisquer flutuações ambientais, como as alterações climáticas nestes nichos ecológicos, terão um grande impacto nestas espécies, que são particularmente sensíveis, comprometendo a sua persistência no ecossistema.

O Norte de África, apesar de ser uma região extremamente rica não só em biodiversidade, mas também em história geológica, é uma região pouco estudada quando comparada com o Sul da Europa, apesar de ambos fazerem parte do hotspot de biodiversidade mediterrânica. A área de estudo deste trabalho é a Cordilheira do Atlas e regiões circundantes em Marrocos, onde (entre muitos outros organismos) ocorrem dois géneros endémicos de lagartos, *Quedenfeldtia* e *Atlantolacerta*, pertencentes às famílias Sphaerodactylidae e Lacertidae, respetivamente. *Atlantolacerta* é atualmente considerada monoespecífico, enquanto que, *Quedenfeldtia* compreende duas espécies. No entanto, as três espécies demonstram uma extensa diversidade genética, podendo por isso, ser consideradas complexos de espécies crípticas.

No manuscrito I, apresento um estudo filogeográfico em duas espécies crípticas endémicas de Marrocos: *Quedenfeldtia moerens* (Chabanaud, 1916) e *Quedenfeldtia trachyblepharus* (Boettger, 1874). Durante o trabalho de campo, tivemos a oportunidade de amostrar novas populações para ambas as espécies e de registar dados morfológicos que nos permitiram fazer um estudo integrativo e combinar as filogenias (marcadores mitocondriais) e redes de haplótipos (marcadores nucleares) com uma análise estatística das medições corporais e do número de escamas. Assim, foi-nos possível investigar a existência de diferenças morfológicas entre as populações de lagartos, que são de difícil perceção – variação fenotípica "críptica" – e relacioná-las com o modo como as linhagens se encontram organizadas ao nível molecular. Para além disso, foi ainda aplicado um estudo de modelagem da distribuição das espécies, para prever possíveis regiões que possam ser habitadas pelas espécies acima descritas, no sentido de planear futuro trabalho de campo para incluir novas populações. Este estudo permitiu incluir dados de todas as populações previamente amostradas e das novas populações amostradas entre 2019 e 2022.

A análise molecular com marcadores mitocondriais (12S rRNA e ND4 + tRNAs) e nucleares (MC1R, PDC, RAG2 e C-MOS) reconstruiu as linhagens identificadas em estudos anteriores realizados por Barata et al. (2012) e Harris et al. (2017) para ambas as espécies. *Quedenfeldtia moerens* compreende duas linhagens distintas: uma restrita da região Norte e outra do Sul do Atlas; o trabalho de campo realizado durante a Primavera permitiu amostrar uma população de *Quedenfeldtia trachyblepharus* de Jebel

Awlime, que se veio a confirmar relevante, no sentido em que, novos organismos sequenciados suportaram uma linhagem identificada por Harris, (2017) com apenas um indivíduo. Surpreendentemente, as populações de *Quedenfeldtia trachyblepharus* de M'goun e Jebel Sirwa pertencem à mesma linhagem, apesar de estarem espacialmente afastadas. Este resultado, indica que é importante amostrar entre as duas localidades para perceber se, de facto, a linhagem abrange indivíduos de toda a extensão de habitat.

A análise morfológica (PERMANOVA) dos tamanhos corporais, medidos durante o trabalho de campo para ambas as espécies, revelou que dentro de cada espécie existem diferenças significativas entre populações e entre sexos, separadamente. Para investigar o efeito das populações (fêmeas + machos) em cada uma das variáveis, foram aplicados testes estatísticos (ANOVA ou teste Kruskal-Wallis H) para ambas as espécies. ANOVA revelou existirem diferenças significativas no tamanho do membro posterior e no comprimento entre membros, entre as populações de *Quedenfeldtia moerens* e na largura da cabeça e tamanho do membro superior entre algumas populações de *Quedenfeldtia trachyblepharus.* O teste Kruskal-Wallis H mostrou haver variações significativas no comprimento da boca à cloaca entre as populações de Norte e Sul de *Quedenfeldtia moerens* e também, entre populações de *Quedenfeldtia trachyblepharus,* assim como, no comprimento entre membros e do membro posterior. O número de escamas para *Quedenfeldtia moerens* foi também analisado entre as duas populações de Norte e Sul. Foram testadas quatro variáveis: escamas supra e infralabiais, entre os olhos e pre-cloacais. A variação encontrada no número de escamas pre-cloacais entre as duas populações foi, surpreendentemente, significativa (*p*-value: 2.888 e-15). Este resultado é importante, uma vez que, o número de escamas se tem revelado conservado entre répteis.

A modelagem do nicho ecológico, baseada apenas em variáveis ambientais climáticas, para *Quedenfeldtia moerens* de Sul (QMS) e *Quedenfeldtia trachyblepharus* (QT) demonstrou que os habitats onde é mais provável haver ocorrências destas espécies, são áreas perto do litoral e Médio Atlas, para QMS, enquanto que, para os QT se restringem ao Médio Atlas. Para além disso, foi também possível determinar que a isotermalidade, a temperatura máxima do mês mais quente e o coeficiente de variação da precipitação sazonal (QMS) e a precipitação dos três meses mais quentes (QT) são as variáveis ambientais que mais influenciam a distribuição dos organismos. De um modo geral, estes modelos confirmaram as expectativas e são importantes para próximos trabalhos no campo, pois, indicam a possibilidade de ocorrência das espécies em áreas adjacentes às atualmente amostradas.

No manuscrito II, é apresentado um breve estudo com uma espécie endémica das montanhas do Atlas, *Atlantolacerta andreanskyi* (Werner, 1929). No decorrer do trabalho de campo, foram amostrados cinco novos indivíduos, de duas localidades novas e de outras previamente amostradas. A atualização da filogenia com estes novos indivíduos foi importante para compreender de que forma organismos de novas localidades se relacionam com os restantes: se cada localidade corresponde a uma nova linhagem ou se, por outro lado, localidades distintas e separadas entre si por habitat não suportável para esta espécie, pertencem à mesma linhagem. Os resultados foram consistentes com as expectativas, mostrando que, indivíduos de novas localidades podem pertencer a novas linhagens, mas que, também é possível, que mesmo numa extensão maior de habitat, diferentes organismos pertençam à mesma linhagem. Adicionalmente, foi feita uma comparação simples da morfologia de alguns dos espécimes amostrados no presente ano. Estes resultados remetem-nos para outras questões a ser estudadas no futuro, nomeadamente, a necessidade de realizar um estudo morfológico mais extenso, uma vez que, os indivíduos apresentam diferenças notáveis, nomeadamente, ao nível dos padrões de coloração.

Os estudos elaborados ao longo deste trabalho permitem realizar uma revisão taxonómica para o género *Quedenfeldtia* e, também, organizar informação e planear próximos estudos em *Atlantolacerta andreanskyi*. Com isto, visamos aumentar o conhecimento da fauna herpetológica do Norte de África e da biodiversidade em geral. Quantificar a diversidade de espécies existentes no planeta é importante, ainda mais, no contexto de alterações climáticas que vivemos.

Palavras-chave: Taxonomia integrativa; *Quedenfeldtia*; *Atlantolacerta andreanskyi;* morfologia; ecologia.

Abstract

Quantifying the biodiversity requires integration of various branches of biology, from evolution to the ecology and conservation of endangered species. The definition of species continues to be the subject of great controversy – using only morphological characters means that "cryptic species" will be overlooked, but an overemphasis on molecular distinction may lead to an overestimation of species numbers. In an attempt to address this, the idea of complementing molecular analyses with morphological observations and ecological modelling is beginning to be accepted in an "integrative taxonomy" approach.

Mountain systems are rich in biodiversity and endemism, often with little gene flowdue to their isolation by intermediate habitats whose characteristics differ significantly such that they may be unsuitable for montane species. North Africa, despite being an extremely rich region not only in biodiversity, but also in geological history, is a poorly studied region when compared to Southern Europe, despite both being part of the Mediterranean biodiversity hotspot.

Therefore, the main objective of the present work was to contribute to an increase in the knowledge of these two genera within North Africa. Fieldwork in North Africa was carried out to increase understanding of the distribution of these montane forms. An emphasis was made regarding *Quedenfeldtia*, and in the field morphological assessments were made, while in the laboratory additional DNA sequence data was collected and combined with previously published works. Environmental variables were compared to known distributions to better assess the potential factors which limit the species ranges. For *Atlantolacerta*, new localities were reported, and a new genetic lineage was identified highlighting the need for further fieldwork to fully understand the number of potential species within this complex.

Regarding *Quedenfeldtia*, the situation in one species *Quedenfeldtia moerens* is now relatively clear: there are distinct forms in the northern and southern parts of the range, which are highly distinct with mitochondrial DNA markers, and which show strong differences also with the 4 nuclear DNA markers employed. While these genetically distinct lineages are morphologically very similar, some diagnostic characters can be identified so that these should be considered distinct species with non-overlapping ranges. The situation in the other species, *Quedenfeldtia trachyblepharus*, remains more complex. While 4 lineages can be identified using mitochondrial markers, these are less distinct with the nuclear markers, and diagnostic characters were not evident from the morphological analyses. The range of one of these lineages was greatly extended through the additional fieldwork carried out during this thesis. While it seems possible that 4 species could be recognized within the *Quedenfeldtia trachyblepharus* complex, more fieldwork is still needed to better delimit the ranges, and possibly new molecular approaches using for example next generation sequencing methods may be needed to confirm how distinct the known lineages are to decide what taxonomic changes are needed.

Keywords: Integrative taxonomy; *Quedenfeldtia*; *Atlantolacerta andreanskyi;* morphology; ecology.

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List of abbreviations

ACM4 - Acetylcholinergic receptor M4
ArcGIS – Aeronautical Reconnaissance Coverage Geographic Information System
A.s.l – Above sea level
AUC – Area under the ROC Curve
BI – Bayesian inference
C-MOS – MOS proto-oncogene
DNA – Deoxyribonucleic acid
EDTA – Ethylenediaminetetraacetic acid
ENM – Ecological niche modelling
ENV – Environmental variables
FLL – Forelimb length
HH – Head height
HL – Head length
HLL – Hind-limb length
HW – Head width
ILL – Inter-limb length
MANOVA – Multivariate analysis of variance
MC1R – Melanocortin 1 receptor
ML – Maximum likelihood
Mt – Mount
MtDNA – Mitochondrial deoxyribonucleic acid
ND4 – NADH dehydrogenase unit 4
NE – Northeast
PCR – Polymerase chain reaction
PDC – Phosducin
PNC – Phylogenetic niche conservatism
RAG1 – Recombination activating gene 1
RAG2 – Recombination activating gene 2
ROC – Receiver operating characteristic curve
rRNA – Ribosomal ribonucleic acid
SBEY – Scales between the eyes
SUBLAB – Sub-labial scales until the limit of the mouth opening
SVL – Snout-vent length
SW – Southwest
TBE – Tris/borate/EDTA
tRNA – Transfer ribonucleic acid
UPLAB – Upper labial scales on the right side until the limit of the mouth opening
QGIS – Quantum geographic information system
12S rRNA – 12S ribosomal ribonucleic acid

CHAPTER I - Introduction

1.1. Quantifying biodiversity

In the XXI century, we may have the idea that our thoughts and our questions are new and creative. However, we can go back approximately twenty-five centuries and find that many philosophers already reflected on "our" main themes. Biology is no exception: Aristotle (384 – 322 BC) was the first intellectual interested in observing plants and animals separately, examining and characterizing them. At that time, the classification procedure consisted merely of separation based on the main visible differences in features and fundamental functions that resulted in two distinct Kingdoms: Plantae and Animalia (Shatalkin, 2005). That rudimentary practice of classification, that remained until the rise of molecular biology, opened the doors for what we now know as systematics (Zagris, 2022). Aristotle had many disciples that followed his principles, and more recently, many well-known biologists such as Carolus Linnaeus (1707-1778) and Charles Darwin (1809-1882), improved the way of characterization, classification, and the establishment of relationships between the organisms.

In the past, the interest in quantifying organisms was encouraged by the curiosity in understanding the basis of Nature. However, now we are facing a severe problem that requires our attention: the loss of biodiversity. Most of the causes of this loss are directly or indirectly human-related, including climatic changes, pollution, fires, introduction of exotic species and habitat destruction (Begon et al. 2006). How can we measure the loss of biodiversity without quantifying it precisely? More than ever, this is an extremely important task to be done and with the growing human impact on our planet, it will become even more necessary to implement conservation strategies of endangered species, and to prevent that other species become compromised (Thomson et al. 2018).

1.2. Integrated taxonomy

Taxonomy is an ancient discipline that has provided the universal characterization and classification system of biodiversity for centuries and continues effectively to accommodate new knowledge (Thomson et al. 2018). On which features taxonomy should be based remains in discussion, because after all these centuries we have much more information available. With the new technologies there are scientists that believe we are now entering a new era for taxonomy, named "cybertaxonomy", which aims to perform the classification of species and the publication of new data for everyone, everywhere in digital platforms (Schram, 2004).

In the meantime, taxonomy requires a quantifiable unit: a species. What is a species? The species concept has also been a challenge for biologists from the most variable fields since the first scientist tried to describe it. Historically, species were primarily identified based on their external morphological differences. With the advent of molecular biology and the use of these techniques to improve the classification method used before, some scientists suggested that we were facing a new problem – "taxonomic inflation" - which was leading to the separation of species that might not be divided considering other aspects of their biology. Some biologists suggested that, to avoid the undue separation of species, we should consider several fields in biology in one methodology – integrative taxonomy – which includes morphological, molecular, and ecological data in an attempt to get better supported results. This was another epicentre of controversy, far from being universally accepted, but it has been a focus of attention from the scientific community, and nowadays it is becoming better accepted.

1.3. Phylogeography

With the birth of molecular biology in the 1980's, a new discipline - phylogeography - was introduced (Avise, 1987). Typically, this field combines inseparable perspectives - geology and biology – to explain the evolutionary history (spatial and temporal dynamics) of a group of organisms, thereby incorporating aspects of population genetics, phylogenies, paleontology, earth history, geoclimatic analysis and ecology (Avise, 2004).

Why was molecular biology so crucial for the rise of phylogeography? One of the subdisciplines within phylogeography - intraspecific phylogeography - focusses on how physical and biological processes have influenced the spatial distributions of genetic lineages among closely related taxa or within the same species. To perform these studies, scientists began to use mitochondrial DNA (mtDNA) that are maternally inherited and evolve rapidly in nucleotide sequence in most animal taxa (Avise, 2004). Nuclear DNA haplotype networks can provide information about gene flow and introgression between lineages and corroborate, or not, our results from the mitochondrial data, which can be biased by various aspects such as mitochondrial introgression, or selective sweeps (Barata et al. 2012b; Harris et al. 2017).

1.4. Brief history of the species concept

Species are the basic unit used in taxonomy, systematics, evolution, and conservation (Padial et al. 2010; Wilkins, 2010). John Ray (1627-1705), a naturalist mostly interested in plants and their pharmacological properties, was the first biologist who tried to define species, writing in *Historia Plantarum* (his biggest work): ‘‘No matter what variations occur in the individuals or the species, if they spring from the seed of the same plant, they are accidental variations and not such as distinguish a species permanently; one species never springs from the seed of another nor vice versa”.

Approximately 50 years later, Linnaeus (1707-1778) identified a range of new species based on their features in *Species Plantarum* (Linnaeus, 1753). As a botanist, most of the species were plants and the characteristics relevant for the classification were mainly the floral structure and sexual features.

De Candolle (1778–1841), in his book *Théorie Élémentaire de la Botanique* (Candolle, 1813), developed further the species concept, by defining species as ‘‘…a collection of all the individuals which resemble each other more than they resemble anything else, which can by natural fecundation produce fertile individuals, and which reproduce themselves by generation, in such a manner that we may from analogy suppose them all to have sprung from one single individual”.

Later, Charles Darwin (1809 - 1882), even before the era of genetics, proposed in his work that the species are the fundamental unit of evolution, which can evolve rapidly in a favourable environment or become stable and unchanged over long time periods (Darwin, 1859). These new insights into the species concept were crucial for our current knowledge.

In the era of molecular biology and genetics, scientists have been proposing several definitions for what a species is (e.g., morphological, ecological, or pluralistic species concepts) (Mayr et al. 2003). One of the most widely accepted definitions is the biological species concept, proposed by Mayr in 1942 (De Queiroz, 2005). Mayr was a zoologist who described biological species as ‘‘groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups”. Mayr explained that following this idea, different groups of related organisms, even growing in the same area, will not interbreed with each other. Distinct organisms at the biological species level, occupying the same ecological niche, are not capable of reproducing with each other or generating fertile offspring (Mayr et al. 2003). However, this theory remains open to criticism: primarily, it cannot be applied to asexual organisms, and secondly, it does not apply to allopatric (geographically isolated) species (Stace, 1991).

1.5 Study area

1.5.1 Mountain systems

Mountains have attracted the attention of biologists and geologists for both evolutionary and ecological studies (Emerson, 2003). This interest lies in the fact that mountains are surprisingly biodiverse regions, frequently, with rich aggregations of small-ranged species that form centres of endemism, due to the isolation of species complexes, similar to what happens in islands (Emerson, 2003). Mountains are influenced by geological and climatic dynamics over deep time, hosting a substantial proportion of the world's species (Rahbek et al. 2019).

Mountainous regions are topographically complex and can emerge through the interaction of tectonic and volcanic dynamics (Antonelli et al. 2018). The uplift of a mountain promotes the heterogeneity of surface areas, creating new habitats where species can adapt and evolve (Fjeldså et al. 2012). For that reason, they are often called "hotbeds of speciation" (Rahbek et al. 2019). These systems are intrinsically unstable undergoing considerable changes in response to tectonic, erosional, and climatic processes over geologically short time scales (Rahbek et al. 2019). Overall, the emergence of the massif and associated climatic conditions create distinct and variable transient mountain environments (Fjeldså et al. 2012). These can act as promoters of division and subsequent isolation of species ranges, evolutionary adaptation to changing conditions, and consequently, population differentiation (Darwin, 1859).

Frequently, mountains provide habitat for many endemic species, which extend from plant taxa to reptiles and other vertebrates (Carranza et al. 2004). For the non-avian reptiles which are non-flying animals, there are a few distinct ways in which they can become endemic to a high-altitude region, that include: a) the migration of species inhabiting lowland regions during colder periods to higher altitudes after climatic amelioration; b) species that simply got adapted to the mountain environment as the montane was lifting up itself; c) competition between different taxa in the surrounding lowlands that force the displacement of a species to another environment – higher altitudes (Barata et al. 2012b; Carranza et al. 2004; Harris et al. 2017).

1.5.2 Maghreb region

Maghreb (from Arabic *al-Maghrib*, "the west") is a region in North-west Africa. In the narrower sense, this area includes the territory of Algeria, Morocco, and Tunisia; in a broader sense, it also includes Libya, Western Sahara, and Mauritania (Miralles et al. 2020).

Typically, there are distinct bioclimatic zones: humid and semi-arid areas in the north, and large, extremely dry areas in the south (McFarland & Majolo, 2013), with the high peaks of the Atlas Mountains being much cooler than the rest of North Africa. These mountains separate coastal, humid areas from the southern, dry part. They also retain much of the humidity that comes in from the Atlantic Ocean (McFarland & Majolo, 2013). The mountainous areas of Atlas and Rif, characterized by a suitable climate with high precipitation, are considerate especially rich in biodiversity (Walas & Taib, 2022).

1.5.3 High Atlas Mountains

The High Atlas of Morocco (Figure 1.1 and 1.2), representing the southernmost element of the Perimediterranean Alpine belts, is a typical example of intracontinental belt (Fekkak et al. 2018). It was formed within the North African plate during convergence of the African and European plates during the Cenozoic (Alessandro et al. 2012).

The High Atlas represents the highest mountain belt of Morocco, with peaks of over 4000 m a.s.l. (Mt. Toubkal, 4165 m), crossing the country along the SW-NE direction from the Atlantic Ocean

to Algeria, with a length of about 2000 km (800 km in Morocco) and a width ranging between about 50 km to 100 km, framed between the Meseta domains (Morocco Meseta and Oran Meseta) to the north, and the northern boundary of the West African Craton (Anti Atlas belt) to the south (Alessandro et al. 2012; Avella et al. 2020; Barata et al. 2012).

Figure 1.1 – High Atlas Mountains.

The position of the Atlas Mountains, one of various mountain belts within the Mediterranean basin, is exceptional for being close not only to the Mediterranean Sea but also to the Atlantic Ocean and to the Sahara Desert, which makes this area extremely interesting to evolutionary studies. Despite this, compared to other mountainous systems such; as the Western Balkans, Iberia and Pyrenees, the Atlas Mountains is a large area that remains relatively unstudied (Giraudi & Giaccio, 2017; Hughes & Woodward, 2008). Moreover, because this region suffered extensive glaciations during the Pleistocene (Hughes et al. 2020), it consisted in a refuge of plants and animals during the glacial periods which had an important contribution to the colonization of northern areas during interglacial stages (Hughes & Woodward, 2008). For this reason, montane-specialist species inhabiting the Atlas Mountains have an old evolutionary history to be unravelled.

Figure 1.2 – Oukaimeden in the High Atlas Mountains showing a glaciated valley (centre of image).

1.6 Ecological niche modelling

Species distribution modelling has an old tradition in applied ecology and with the advances in technology it is now possible to understand the current species distribution and predict it for the future in the face of climate change (Yackulic et al. 2013). Ecological niche modelling – hereafter referred as ENM – is particularly useful for cryptic species. For "hidden" lineages within species, morphological differences may be undetected, overlapping, or not fixed; reproductive barriers may be incomplete in recent divergences; and lineage sorting of rapidly evolving molecular loci may be incomplete (Chippindale et al. 2000; Raxworthy et al. 2007). This may lead to indecision and disagreement in identifying the ideal criteria to identify such species. With the analysis of the ecological niche of the study species, it is possible to demonstrate the geographical isolation between populations or lineages, by mapping the spatial distribution of probability or favourability of the species' occurrence, based on environmental variables (Raxworthy et al. 2007). In this way, it may become clear that distinct genetic lineages also occupy distinct niches, which would be further evidence that they are indeed separate entities.

1.7 Study group

1.7.1 "Cryptic" species

In recent decades, scientists have widely identified examples where the speciation processes occurred without obvious morphological features diverging, due to the molecular techniques now applied to study variation between species or populations (Bickford et al. 2007; Fišer et al. 2018). This is the main reason why "cryptic" species is becoming a common concept: we might know that groups of organisms are splitting and generated distinct lineages, but it is not evident since the overall morphological aspect does not change enough to be easily perceived (Egea et al. 2016). Sometimes "cryptic" species can also be named as sibling species, but "cryptic" refers to something hidden, and it is exactly this – new lineages are hidden through overall morphological similarities and may not necessarily be "siblings" or sister taxa (Barata et al. 2012b). Studies with an integrative taxonomy approach have the key tools to unravel and quantify "cryptic" species, due to the inclusion of data from multiple sources.

Which are the processes beyond the emergence of "cryptic" diversity? One of the postulates is that morphological features evolve and change only due to stochastic processes, after divergence, being a gradually process (Adams et al. 2009; Egea et al. 2016). Since the external features of the organisms do not appear to be distinct, species divergence must be recent (Zúñiga-Reinoso & Benítez, 2015). However, this theory seems to fail in explain the existence of older "cryptic" species. The morphological stasis hypothesis proposes that there are biological constraints implicit in morphological divergence in descendant species (Bickford et al. 2007). Typically, the correlation between morphology and ecological niche links this hypothesis with "phylogenetic niche conservatism" (PNC) (Fišer et al. 2018). The biological mechanisms inherent to PNC encompass stabilizing selection, gene flow swamping local adaptation, pleiotropic effects constraining adaptation and lack of genetic variation (Adams et al. 2009). Lastly, the third hypothesis is related with morphology convergence: similar morphological features have the capacity to evolve among distantly related species when submitted to similar selective pressures (Ingram & Mahler, 2013). According to (Fišer et al. 2018), this is quite a common observation, since 26% of the amphipod species analysed contained "cryptic" species that are not sister groups.

The discovery of newly identified "cryptic" species provides unique opportunities to study evolutionary mechanisms and to answer many questions that remains poorly explored in this topic: "What evolutionary and ecological processes lead to genetic divergence and reproductive isolation in the absence of morphological differentiation?"; "Is cryptic speciation more common in allopatry,

parapatry, or sympatry?", and "What kinds of genetic evidence are necessary for demonstrating and formally describing cryptic species?" (Bickford et al. 2007).

1.7.2 Reptiles

Class Reptilia (phylum Chordata) are a heterogenous group of organisms that continue to be grouped together, despite phylogenetic analysis demonstrating that they are not monophyletic, since the monophyletic group that includes reptiles, also includes birds (Wollenberg et al. 2019). This issue began with the classical system of classification proposed by Carolus Linnaeus, where the external morphological similarities – phenetics – were the basis of classification, and in which birds were separated from the reptiles.

Reptiles include various distinct groups: tuatara (1 species), squamates (lizards and snakes; ~10,000 species), turtles (~350 species), and crocodilians (27 species) (Uetz, 2022)). The main shared characters of reptiles are that they are all covered by scales, and not being able to regulate their own body temperature.

The order Squamata includes snakes, lizards, and geckos (Shine, 2006), and includes the species which are the main focus of this thesis.

1.7.3 *Quedenfeldtia* Boettger, 1883

The genus *Quedenfeldtia*, Boettger, 1883 (Squamata, Sphaerodactylidae) was described with two species: *Quedenfeldtia moerens* (Chabanaud, 1916) and *Quedenfeldtia trachyblepharus* (Boettger, 1874). However, some authors in the early and middle 20th century did not agree and considered only *Quedenfeldtia trachyblepharus* as a single species within the genus (Loveridge, 1947). After that, morphological differences were perceived (Bons, 1959), which highlighted for the existence of two distinct species, regarding those different phenotypes. A few decades later, in his extensive reassessment, Arnold (1990) confirmed the two original species (Figure 1.3) based on external features, such as body measurements and pholidosis. Both species exhibit sexual dimorphism and are distinguishable on a morphological and ecological level (Arnold, 1990; Barata et al. 2012b). Variation can be perceived in body measurements, pholidotic characters (scales-related features, e.g.: number and shape) and colour patterns (Arnold, 1990; Barata et al. 2012b; Harris et al. 2017). They inhabit different altitude ranges, along the slopes of the massifs and typically are found in slits of big and smooth dark metamorphic rocks. *Quedenfeldtia moerens* is also found inhabiting lower areas. Typically, it is found from sea level (s. l.) to 3000 m above sea level (a. s. l.). *Quedenfeldtia trachyblepharus*, on the other hand, inhabit higher altitudes, from 1400 m a.s.l. to 4000 m a.s.l. (Harris et al. 2017; Barata et al. 2012b).

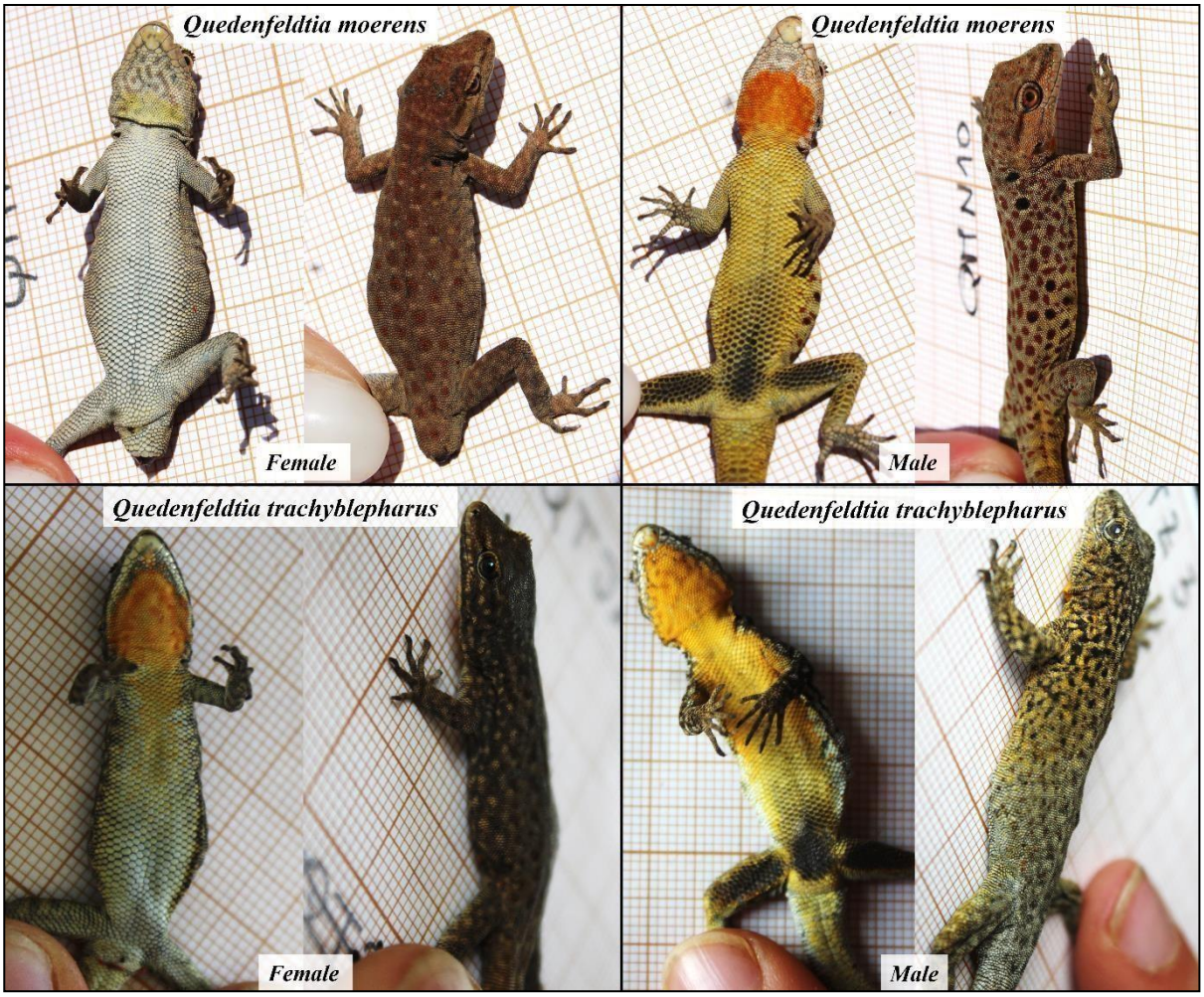

Figure 1.3 – Female and male specimens of *Quedenfeldtia moerens* and *Quedenfeldtia trachyblepharus.*

The first study analysing molecular divergences between each species was carried by Barata et al. (2012b) combining morphological and ecological data in some lineages. The phylogenies were estimated based on two mitochondrial markers (12S rRNA and ND4 + tRNAs) and four nuclear markers (MC1R, PDC, RAG1, and ACM4). The two main groups recovered were consistent with the two current species (*p*-distance = 13%). Within *Quedenfeldtia moerens* two further lineages were separated (*p*-distance = 8%), corresponding to geographical distant groups: one from the North and one from the South with slightly differences between central and southern individuals. *Quedenfeldtia trachyblepharus* was also separated into two distinct lineages (*p*-distance = 8.7%): one grouping Jebel Sirwah population and near localities, and one including Oukaimeden and Jebel Toubkal (Barata et al. 2012b). Overall, the phylogeny based on nuclear DNA was mostly congruent (*p*-distance between each species = 1.5%). However, ACM4 and RAG1 did not recovered the two lineages within *Quedenfeldtia moerens* and *Quedenfeldtia trachyblepharus*, respectively. Within *Quedenfeldtia trachyblepharus* the population of Jebel Toubkal was grouped with Jebel Sirwah instead of Oukaimeden. Nuclear divergence between North and South *Quedenfeldtia moerens*, and Oukaimeden and Jebel Sirwah populations for *Quedenfeldtia trachyblepharus* were 0.8% and 0.5%, respectively.

A more recently study by Harris et al. (2017), continued the work on this species complex with previously unknown localities and identification of new lineages based on molecular markers used by Barata et al. (2012b) (12S, ND4 + tRNAs, and MC1R). Topology resulting from Bayesian Inference reconstruct the main lineages within *Quedenfeldtia moerens*, described by Barata et al. (2012b) as

Northern and Southern lineages. For *Quedenfeldtia trachyblepharus*, two additional lineages were identified: one comprises only one individual from the far South and the other one includes all the newly sequenced individuals from the North, an unsampled region in Barata et al. (2012b).

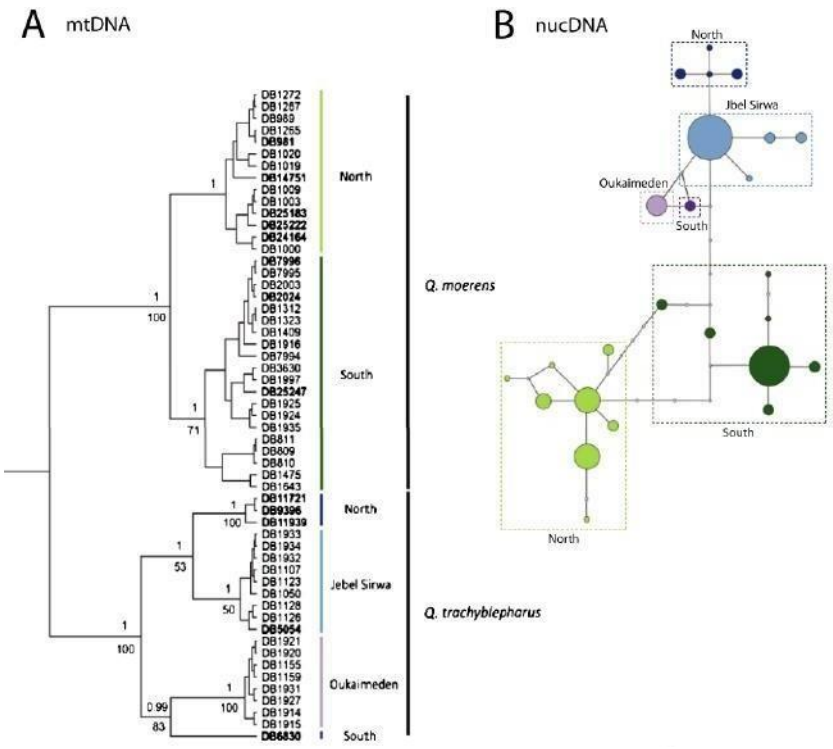

Figure 1.4 - (A) Bayesian tree based on mitochondrial DNA (mtDNA) sequences (12S and ND4 + tRNA) of *Quedenfeldtia*. (B) Parsimony Network based on nuclear DNA sequences (MC1R). Haplotypes are coloured according to the mitochondrial lineages (Adapted from Harris et al. 2017).

These new lineages appeared to be highly divergent from the previously known ones: Northern lineage was 8% (ND4 + tRNAs, *p*-distance) divergent from Jebel Sirwah lineage and far South lineage was 9% (ND4 + tRNAs, *p*-distance) divergent from Oukaimeden lineage. The parsimony network based on MC1R revealed high genetic distances between groups (*p*-distance = 1%). These divergences are higher than previously proposed ones for other "cryptic" species of geckos (Rocha et al. 2011).

An early morphological assessment based on seven linear measurements (SVL, HH, HL, HW, ILL, HLL, FLL) and five pholidotic traits (UPLAB, SUBLAB, SNEY, PRECL, and LAM) confirmed the existence of differences between species, population, and sex. Overall, the variation in size was mostly explain by sex, while the variation found in shape were explained by species and populations factors (Barata et al. 2012b). Typically, males revealed wider heads, and females, larger inter-limb lengths. *Quedenfeldtia moerens* showed longer posterior and anterior members and head, while *Quedenfeldtia trachyblepharus* seemed to have wider heads and longer trunks. Intraspecific variation was also identified (Barata et al. 2012b). For pholidosis, populations revealed variation for all traits except for UPLAB (SNEY and PRECL, *p*-value = 0.001; SUBLAB and LAM, *p*-value = 0.00). Individuals from Agoudal and Tafraoute populations, corresponding to Northern and Southern lineages of *Quedenfeldtia moerens* were also well differentiated (Barata et al. 2012b).

Ecological niche modelling prediction was also done by Barata et al. (2012b) showing that both species can live in sympatry, however, it has not been confirmed in the field (Harris et al. 2017). The two lineages of *Quedenfeldtia moerens* have been found isolated, approximately by 250 km of distance,

which indicates that they might had evolved and adapt to distinct environments (Harris et al. 2017).

Despite all the work carried by herpetologists in the field during the last decade, it is lacking a study of all the sampled populations (previously analysed and new ones), since after the morphological assessment in 2012, more lineages have been identified and new populations were sampled between 2019 and 2022.

1.7.4 *Atlantolacerta andreanskyi,* (Werner, 1929)

Lacertid lizard (Figure 1.4), endemic to the High Atlas Mountains, distributed across 440 km (straight line) from the western to the central areas of the massif (Figure 1.5) (Bons & Geniez, 1996). This species complex inhabits altitudes from 2400 m a.s.l. to 3800 m a.s.l. under small rocks and in small spiky bushes, often near to watercourses or snowmelt. The different populations of *A. andreanskyi* are separated, in a way similar to island endemic species, due to the surrounding environment simply not being suitable to inhabit (i.e., areas under 2400 m a.s.l.) (Barata et al. 2012a).

A past study on this species complex revealed the occurrence of seven highly divergent lineages, separated approximately by 10 km (Barata et al. 2012a), corresponding to the populations from J. Awlime, J. Sirwah, Tizin n' Tichka, J. Azourki, Outabati, J. Ayache, and Oukaimeden and nearby Toubkal that were grouped together. Populations presented a very high level of genetic divergence between them (5.5 – 16.5% in the ND4 + tRNAs and 2.5 – 6.6% in the 12S). However, the morphology of the different lineages appeared to be very similar. Other studies were done on this species, for example S'Khifa et al. (2020), with behavioural tests and assessment of environmental variables that further demonstrated the importance in studying high mountains ranges of "cryptic" species facing the actual climatic change. Unlike has previously been found on *Quedenfeldtia*, each different *Atlantolacerta andreanskyi* populations seems to be distinct lineages (except Toubkal and Oukaimeden). Therefore, it is not clear to which lineage will belong samples from any new localities. Regarding this, further sampling and additional molecular assessment are essential to understand if the new populations will belong to a previously described lineage or, contrarily, will continuing to indicate new lineages.

Figure 1.5 - Specimen of *Atlantolacerta andreanskyi.*

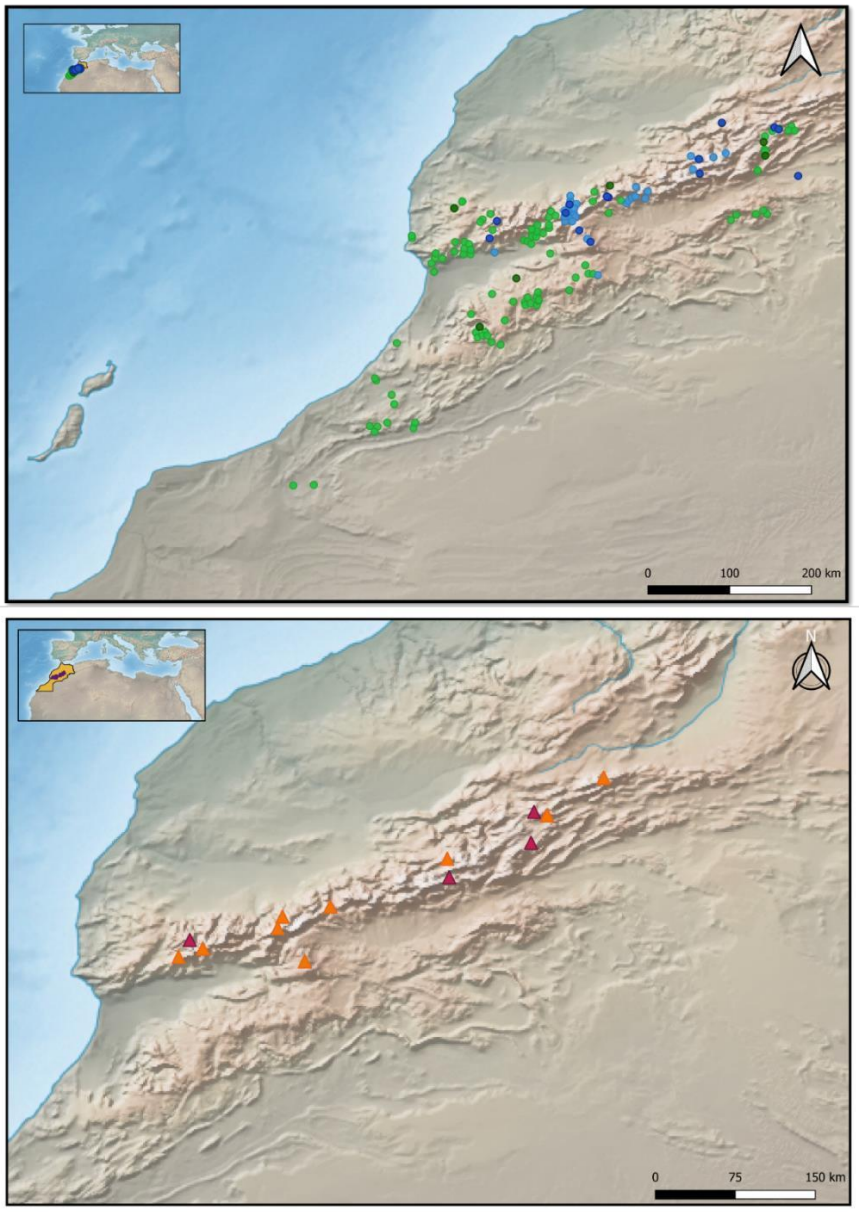

Figure 1.6 - Map distribution of A) *Quedenfeldtia spp*.: *Quedenfeldtia moerens* from Bons & Geniez, 1996; Barata et al. 2012; and Harris et al. 2017 (light green dots); *Quedenfeldtia trachyblepharus* from Bons & Geniez, 1996; Barata et al. 2012; and Harris et al. 2017 (light blue dots); Distribution of new samples from *Quedenfeldtia moerens* used in the present study (dark green dots) and from *Quedenfeldtia trachyblepharus* (dark blue dots); B) *Atlantolacerta andreanskyi* from Bons & Geniez, 1996, Barata et al. 2012, and Avella et al. 2020 (orange triangles) and new samples (pink triangles).

1.8 Objectives

The present work focuses primarily on *Quedenfeldtia* (Boettger, 1883), that are presented in Manuscript I. In this study we aim to apply a multiperspective approach, including molecular, morphological, and ecological data, to revise the taxonomy of *Quedenfeldtia* with previously known and unsampled localities. Firstly, we intent to confirm how the lineages within each species are phylogenetic related and to assess if parsimony networks based on nuclear DNA reveal introgression between them or if the same pattern occurs with both mitDNA and nucDNA. Some questions that remain to solve will be discussed in this thesis: is the divergence between *Quedenfeldtia moerens* Northern and Southern lineages higher enough to consider the existence of a new "cryptic" species? Do the newly sequenced individuals of *Quedenfeldtia trachyblepharus* belong to previously identified lineages? Which ones?

Secondly, we planned to perform a morphological analysis in an attempt to investigate significant differences in body measurements and pholidotic traits within each species: is there phenotypic variation that support the genetic divergence between *Quedenfeldtia moerens* North and South lineages? A less extensive analysis with body lengths will be applied to *Quedenfeldtia trachyblepharus* to include all the recently sampled localities.

The expected results are the confirmation of existence of two highly separated lineages within *Quedenfeldtia moerens*, with significant divergence, regarding genetic and morphological differences, and habitat isolation; in parallel, we aim to achieve a clearer understanding of *Quedenfeldtia trachyblepharus* molecular and morphological divergence, between the different populations, in particular, with the new sampled localities. However, this species is more challenging to study since they have been found in a narrower range of localities.

The ecological niche modelling would be valuable to predict for *Quedenfeldtia moerens* Northern and Southern lineages, however, there is no occurrences available for the Northern one. Regarding this, ENM will be estimated for *Quedenfeldtia moerens* from the South and for *Quedenfeldtia trachyblepharus* to assess for environmental variables that might shape their distribution and local adaptation in a context of climatic change and to search for other suitable areas where these species might occur for further fieldwork.

In Manuscript II, the focus of study is *Atlantolacerta andreanskyi*, another species that we had the opportunity to sample in Morocco and to include in this work. The main objective of this section is to understand if newly sequenced individuals from unsampled localities correspond to new lineages (as it has been found) or belong to previously identified ones. The expected result is that new individuals might belong to known lineages as well as distinct ones and it would be valuable to know to plan future projects on this species complex.

Overall, this thesis will contribute for the increasing in knowledge of high-altitude endemic lizards from Morocco, addressing questions that remained from Barata et al. (2012a, b) and Harris et al. (2017). Mountain living organisms are adapted to restricted conditions and for that reason, are considered more sensitive to environmental changes. In the present context, with the global warming, those species become even more vulnerable. Regarding this, it is important to contribute for the quantification of biodiversity, particularly in North Africa, a region rich in endemic species and poorly unravelled.

Below, I give a detailed overview of the study area, species of interest, and additional information about some of the methods applied, that will be detailed described in the manuscripts.

CHAPTER II - METHODS

2.1 Fieldwork

An expedition to the Atlas Mountains, in Morocco was conducted from the 9th of May to the 26th of May 2022. Eight populations of *Quedenfeldtia* were sampled: one population of *Quedenfeldtia moerens* in the North, and two populations of *Quedenfeldtia moerens* in the South (one of which in a new locality); five populations of *Quedenfeldtia trachyblepharus* (Jebel Awlime, Oukaimeden, Jebel Sirwa, Jebel Azourki and from the North – the last two are new localities). Additionally, one new locality, M'goun, which was previously sampled are included in this work. For *Atlantolacerta andreanskyi* four new localities were collected, two of which were quite near previously sampled localities (Jebel Awlime, Toumliline, Outabati and M'goun).

The capture method employed differs between the two genera, due to the type of rock where they preferentially inhabit: to sample *Quedenfeldtia*, which are found in large metamorphic rocks (Figure 2.2) (personal observation), often in regions with climatic conditions that make it difficult to see them unless they are active (e.g., cold, wind), the most efficient method is to use a noose (Figure 2.1) with a floss/fishing knot at the end that holds them across the anterior portion of the head. The individuals are then carefully removed and kept in conditions that cause them the least possible stress prior to obtaining the remaining necessary data (e.g., tip tail, measurements, photographs, records).

Figure 2.1 - Sampling method to capture *Quedenfeldtia.*

On the other hand, *Atlantolacerta andreanskyi* typically found under small rocks on the ground or active around the base of thorny bushes (Figure 2.2) (personal observation). For this reason, the most efficient method to sampling them is lifting and picking the lizard up by hand, meaning the lizards can be caught either when active or inactive.

Figure 2.2 - (A) Large metamorphic rocks where we typically can find *Quedenfeldtia*. (B) Small and dispersed rocks where *Atlantolacerta andreanskyi* can be found.

All the collected individuals were photographed, and the posterior portion of the tail was removed: whenever it was possible, because some of the tails were already damaged, a small piece of tail was twisted by hand so that it breaks by segment (Figure 2.3), where there is a weaker tissue composition, instead of breaking randomly to prevent damage. This can minimize the haemorrhage caused, since this technique has a greater vasoconstrict effect, and simulates the natural autotomy process (Barr et al. 2019; Jacyniak et al. 2017; McLean & Vickaryous, 2011). Tail samples were stored in 1.5ml tubes containing 96% ethanol. After all the data collection (tail tissue, measurements, and photographs), all the specimens were released unharmed at the same capture sites.

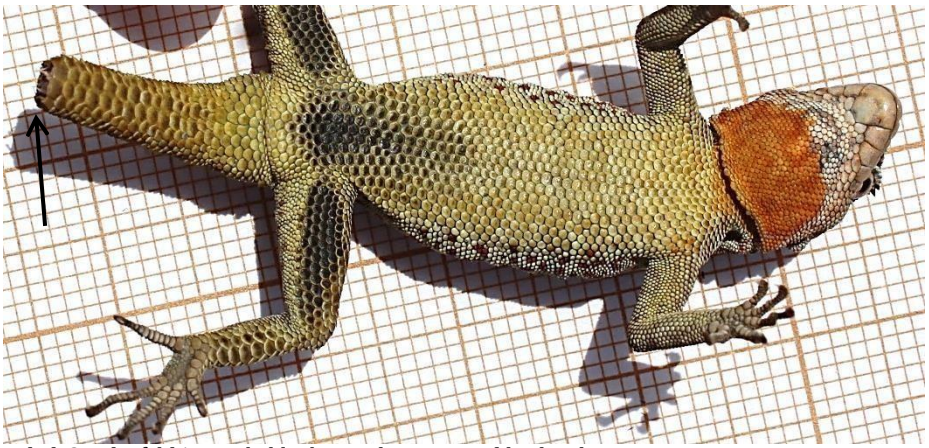

Figure 2.3 - Sampled *Quedenfeldtia trachyblepharus* showing a tail broken by segment.

2.2 Molecular experiments

2.2.1 DNA Extraction, amplification, and sequencing

To perform DNA extraction (Figure 2.4A and 2.4B), the chosen samples were submitted to a high-salt protocol (Sambrook et al. 1989). Primarily, a very small piece of tail (approximately 1mm) was cut and crushed with the aid of tweezers and a scalpel. The procedure was carried out with a lamp, in a near-sterile environment. The first step consists of placing the pieces of tissue in a 1.5 mL eppendorf tube with a solution of 600 µL lysis buffer (0.5M tris; 0.1M EDTA; 2% SDS; pH 8.0) and 10 µL proteinase K (25 mg/mL) and left overnight at 56 ºC to activate the proteinase K, for the digesting of proteins. Briefly, the second and third steps comprise cycles of centrifugation with ammonium acetate (7M; pH 8.0; at 4ºC), ice-cold isopropanol, and ice-cold ethanol (70%) to precipitate the proteins that result from the digestion and the DNA, and to wash off the DNA impurities, respectively. Finally, the samples were hydrated with TBE buffer (Tris-borate-EDTA) from 3h to overnight, in a seesaw.

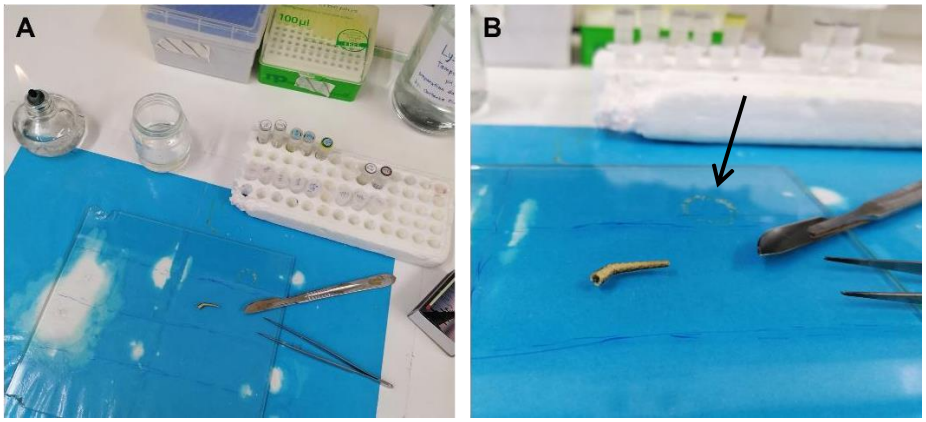

Figure 2.4 - (A) DNA extraction bench with the material used: glass (base for DNA extraction), lamp, beaker with 70% ethanol, tweezers, scalpel, and tubes. (B) Pieces of tissue already smashed (arrow) and a piece of another tail to cut approximately 1 mm of tissue with the scalpel.

To confirm the DNA extraction, electrophoresis in agarose gel was performed (Figure 2.5A and 2.5B). PCR for two mitochondrial markers (12S and ND4) and four nuclear markers (MC1R, PDC, RAG2 and C-MOS) were performed for *Quedenfeldtia*, and two mitochondrial markers (12S and ND4) for *Atlantolacerta andreanskyi*.

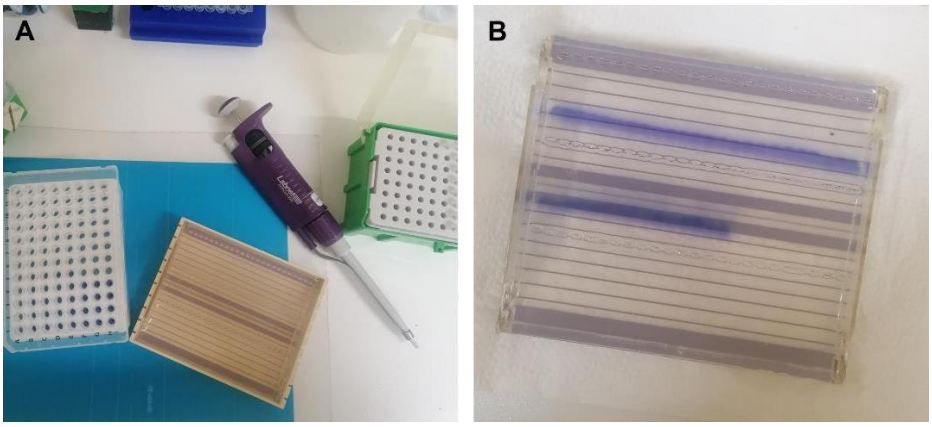

Figure 2.5 – Electrophoresis agarose gel before (A) and after (B) being ran at 300 V in 0.5x TBE.

The mitochondrial and nuclear markers were chosen regarding past studies in *Quedenfeldtia* and *Atlantolacerta* (Barata et al. 2012a; Barata et al. 2012b; Harris et al. 2017; Rato et al. 2010).

All the PCR were performed using published primers. Sequences of used markers and primers are available in Table 2.1. The PCR product was evaluated using agarose gel electrophoresis. This procedure consisted in running 2 μL of PCR product from each sample, on a 2.0 % (w/v) agarose gel — previously stained with GelRed (Biotarget) — at 300 V in 0.5x TBE. A standardised ladder, a negative, and positive control were also run. Positive PCRs were sent to GENEWIZ, Germany, for Sanger sequencing.

Table 2.1 – Molecular markers and primers used for their amplification (PCR).

Marker	Primers	Sequence (5' - 3')	Authors
ND4	ND4 Leu	CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC CAT TAC TTT TAC TTG GAA TTT GCA CCA	Arévalo et al. (1994)
12S	12Sa 12Sb	CTG GGA TTA GAT ACC CCA TAT GAG GGT GAC GGG GCG GTG TGT	Kocher et al. (1989)
MC1R	MC1RF MC1RR	GGC NGC CAT YGT CAA GAA CCG GAA CC CTC CGR AAG GCR TAG ATG ATG GGG TCC AC	Pinho et al. (2010)
PDC	PHOF2 PHOF1	AGATGAGCATGCAGGAGTATGA TCCACATCCACAGCAAAAAACTCCT	Bauer et al. (2007)
C-MOS	G73 G74	GCGGTAAAGCAGGTGAAGAAA TGAGCATCCAAAGTCTCCAATC	Saint et al. (1998)
RAG2 1st-PCR	31FN Lung460R	TTYGGICARAARGGGGITGGCC GCATYGRGCATGGACCARTGICC	Modified from: Venkatesh et al. (2001) and Hoegg et al. (2004)
RAG2 2nd-PCR	Lung35F Lung320R	GGCCCAAGAGRTCYTGTCCXACTGG AYCACCCATATYRCTACCAAACC	

2.2.2 Genetic data treatment

The sequences obtained for all the mitochondrial and nuclear markers were aligned separately in BioEdit (v. 7.2.5) or using Muscle (v. 5). For *Quedenfeldtia*, lengths of the alignments were 400 base pairs (bp) for 12S, 691 bp for ND4 + tRNAs (ND4: 633 bp; tRNA: 58 bp), 564 bp for MC1R, 358 bp for PDC, 713 bp for RAG2, and 282 bp for C-MOS. For *Atlantolacerta andreanskyi*, lengths of the alignments were 327 base pairs (bp) for 12S, 741 bp for ND4 + tRNA (ND4: 603 bp; tRNAs: 138 bp). *Saurodactylus spp.* and *Podarcis tiliguerta* were used as outgroups for *Quedenfeldtia* and *Atlantolacerta*, respectively. Further analyses are detailed in the manuscripts.

2.3 Mapping - Geographic Information System (GIS)

ArcGIS online (v. 10.5.1) and QGIS (v. 3.20.0) were used to produce all the maps included in this thesis.

2.4 Morphological characterization of *Quedenfeldtia*

Morphological measurements took place during the expedition to Morocco, in 2022 (Figure 2.6 and 2.7). In total, seven linear measurements and two pholidotic features (Table 2.2) described by Arnold (1990) were used to perform statistical tests. All the body lengths were measured with a digital calliper to minimize the error and were recorded by the same individual (A.C.P.). Additionally, photographs of individuals were taken with a camera by Diana Vasconcelos and Joaquim Faria for further analysis and counting of pholidotic traits.

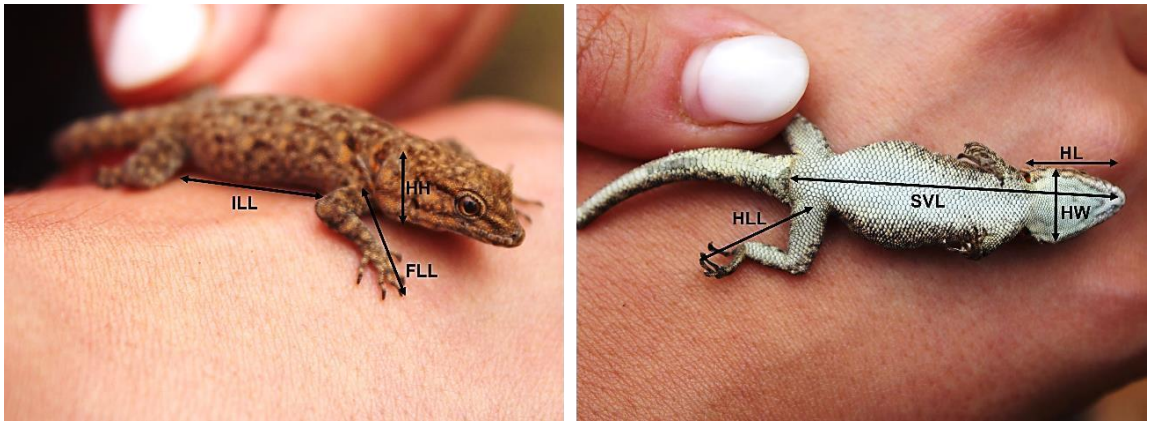

Figure 2.6 - Morphological lengths measured during the fieldwork. All the measurements were done with stretched limbs (arrows are merely representative of the start and end points of the lengths).

Figure 2.7 - Measurement of body lengths using a digital caliper.

Table 2.2 - Morphological features included in the present study.

Body measurements	**Pholidotic features**
SVL - snout-vent length, from the tip of the snout until the cloaca.	SUBLAB- number of sub-labial scales until the limit of the mouth opening counted on the right side of the head.
HL - head length, from the tip of the snout to the posterior ear cavity.	UPLAB- number of upper labial scales on the right side until the limit of the mouth opening counted on the right side of the head.
HW - total head width at its widest part at the level of the temporal region.	SBEY – number of linear scales between the eyes, counted from the top (dorsal pictures).
HH - head height from occipital to jaws.	PRECL – number of linear pre-cloacal scales.
ILL - inter-limb length from the posterior edge of forelimb insertion to the anterior edge of hindlimb insertion.	
HLL - hind-limb length from the longest toe to the base of the hindlimb.	
FLL - forelimb length from the longest toe to the base of the forelimb.	

CHAPTER III - Manuscript I

This manuscript is still in preparation, and it will include more analyses for both species, particularly, in the morphology. There is no prevision about the journal where it would be submitted.

Integrative taxonomy study suggests the possibility of a new "cryptic" species within *Quedenfeldtia,* high-altitude geckos from Morocco

Abstract

Quedenfeldtia is an endemic genus of high-altitude geckos from Morocco with two currently recognized species. Previous molecular assessments of this species using mitochondrial and nuclear DNA markers identified two distinct lineages within *Quedenfeldtia moerens* (Chabanaud, 1916) and four lineages within *Quedenfeldtia trachyblepharus* (Boettger, 1874). A morphological assessment of five populations (Barata et al. 2012b) demonstrated lower phenotypic variation within *Quedenfeldtia moerens* compared to *Quedenfeldtia trachyblepharus*, possibly related to their distribution ranges. However, not all lineages were assessed in this study, we extended the genetic analysis, and further assessed morphological variation across all known lineages. Phylogenetic reconstruction based on mitochondrial markers (12S rRNA and ND4) and parsimony networks based on nuclear markers (MC1R, PDC, RAG2, and C-MOS) corroborate the previously described lineages and clearly separate *Quedenfeldtia moerens* in two distinct lineages: (North and South). Regarding morphological variation, SVL, ILL, and HLL lengths and the number of pre-cloacal scales are significantly different between *Quedenfeldtia moerens* from the Northern and the Southern lineage. These combined results suggest the possibility of defining a new "cryptic" species within *Quedenfeldtia*. Within *Quedenfeldtia trachyblepharus*, the four previously described linages were recovered. Morphological analysis, comparing body lengths, revealed significant differences in SVL, HLL, ILL, HW, and FLL between populations. However, this species is more complex regarding the range of habitat where they have been found. Ecological niche modelling was used to predict the most suitable niches for *Quedenfeldtia moerens* "South" and *Quedenfeldtia trachyblepharus*, supporting their habitat range differences, and identifying potential suitable localities based on the selected environmental variables used in the ENMs prediction.

Keywords: *Quedenfeldtia*; Atlas Mountains; phylogeny; "cryptic" phenotypic variation; ecological niche modelling

Introduction

The genus *Quedenfeldtia*, Sphaerodactylidae, comprises two species of diurnal geckos: *Quedenfeldtia moerens* (Chabanaud, 1916) and *Quedenfeldtia trachyblepharus* (Boettger, 1874). Both species are endemic to Morocco (Arnold, 1990), inhabiting different altitude ranges along the slopes of the Atlas Massif. *Quedenfeldtia moerens* is known to occur from the sea level to 3000 m a.s.l, while *Quedenfeldtia trachyblepharus* is more restrict to the highlands, from 1200 m a.s.l. to 4000 m a.s.l. (Barata et al. 2012b). Typically, they can be found on metamorphic rocky faces, frequently in dry places, but also near watercourses (Schleich et al. 1996). In the last decade, two relevant studies were carried out on these species. A molecular assessment on 18 populations using two mitochondrial and four nuclear markers uncovered notable divergences within each species (Barata et al. 2012b). Morphological analyses were performed in five populations. The overall results were linked to the distribution range of each species: typically, *Quedenfeldtia moerens* have higher phenotypic variation, presumably linked to the wider range of habitats where they can be found, while *Quedenfeldtia trachyblepharus* demonstrated the opposite, as they occur across a smaller area (Barata et al. 2012b). In general, geckos present high genetic variation with more conservative morphology (Gamble et al. 2008; Rato & Harris, 2008). In order to clarify the knowledge within *Quedenfeldtia*, an ecological niche modelling was predicted,

showing the importance of integrated taxonomy studies and the demand for further sampling and for the searching of contact zones between the two species (Barata et al. 2012b). After the initial assessment of genetic diversity of Barata et al. (2012b), additional fieldwork was performed, and a new molecular assessment was conducted with two mitochondrial and one nuclear markers, with the overall confirmation of previously identified lineages and the report of two additional distinct lineages within *Quedenfeldtia trachyblepharus* (Harris et al. 2017). This means that some lineages had not been assessed morphological, and demonstrates how additional fieldwork can recover new, previously unreported diversity. The aim of this study therefore was to perform a new integrated taxonomy study, to combine all the known lineages in a molecular, morphological, and ecological analyses, particularly, including previously unsampled localities.

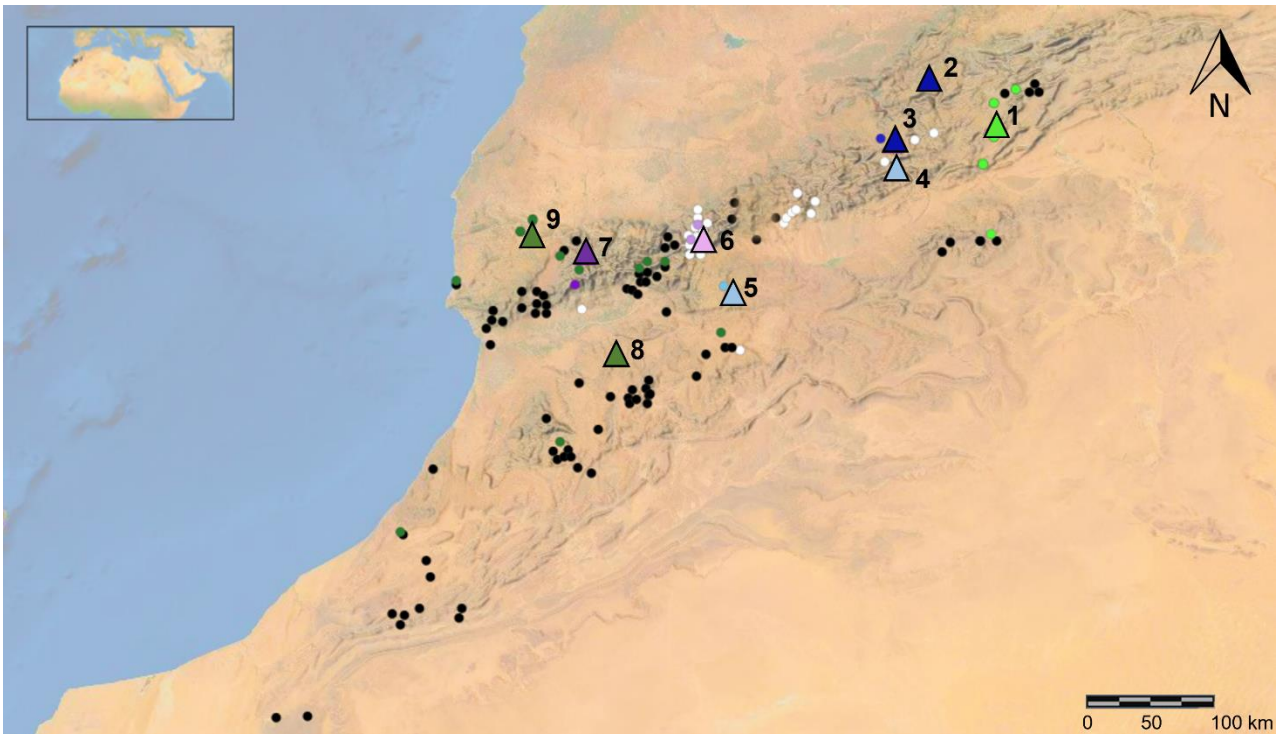

Figure 3.1 - Map distribution of *Quedenfeldtia moerens* (black dots) and *Quedenfeldtia trachyblepharus* (white dots) based on Bons and Geniez (1996), Barata et al. (2012) and Harris et al. (2017). Localities of the newly sampled individuals, analysed in the present study, are represented with numbers (1-9), and coloured according to BI resulting main lineages. 1 – *Quedenfeldtia moerens* from the North (Agoudal) 2 and 3 – *Quedenfeldtia trachyblepharus* from the North (Jebel Azourki); 4 – *Quedenfeldtia trachyblepharus* from M'goun; 5 - *Quedenfeldtia trachyblepharus* from Jebel Sirwah; 6 - *Quedenfeldtia trachyblepharus* from Oukaimeden; 7 - *Quedenfeldtia trachyblepharus* from Jebel Awlime, and 8, 9 - *Quedenfeldtia moerens* from the South.

Methods

Fieldwork

All geckos were captured under permit from the *Haut Commissariat aux Eaux and Forests of Morocco*. Fieldwork was carried out in the Spring of 2019 and 2022 (Figure 3.1). Specimens were captured with a noose and tip tail samples were collected and preserved in tubes filled with ethanol 96%. All individuals were then released in the place of capture. In total, 212 individuals were analysed for seven linear measurements (121 males (M) and 91 females (F)) from eight different localities: north region (Figure 3.1: (1)) for *Q. moerens* (17 M, 11 F), south region (Figure 3.1: (9)) for *Q. moerens* (18 M, 13 F), new locality in the south (Figure 3.1: (8)) for *Q. moerens* (15 M, 10 F); far north (Figure 3.1: (2)), (6 M, 16 F), Jebel Azourki (Figure 3.1: (3)), (15 M, 10 F), Jebel Sirwa (Figure 3.1: (5)), (20 M, 13 F), Oukaimeden (Figure 3.1: (6)), (16 M, 9 F), Jebel Awlime (Figure 3.1: (7)), (14 M, 9 F), for *Quedenfeldtia trachyblepharus*. Four pholidotic features described by Arnold, (1990) were used to

perform statistical tests on *Quedenfeldtia moerens*. All the body lengths were measured with a digital calliper to minimize the error and were recorded by the same individual (A.C.P.). Additionally, photographs of individuals were taken for further pholidosis analysis.

Extraction, amplification, and sequencing

Total genomic DNA was extracted from tip tail samples, using standard high-salt protocols (Sambrook, 1989). Following DNA extraction, two portions of mitochondrial DNA and four nuclear markers were amplified via polymerase chain reaction (PCR): 12S rRNA (12S), partial NADH dehydrogenase 4 (ND4) and flanking tRNA (tRNA-His), MC1R, PDC, C-MOS, and RAG2, using previously published primers from Kocher et al. (1989) and Arévalo et al. (1994), Pinho et al. (2010), Bauer et al. (2007), Saint et al. (1998), Venkatesh et al. (2001) and Hoegg et al. (2004) respectively. The PCR thermocycler conditions used started with 95 °C for 10 min followed by thirty-five cycles of 30 s at 95 °C, 30 s at 50 ºC (12S and MC1R) or 52 ºC (ND4 and PDC), and 30 s at 72 °C with a final extension at 72 °C for 10 min. For C-MOS, the PCR thermocycler conditions started with 95 °C for 10 min followed by seven cycles of 30 s at 95 °C, 30 s at 54 ºC and 30 s at 72 °C, and 33 cycles of 30 s at 95 °C, 30 s at 51 ºC, and 30 s at 72 °C, with a final extension at 72 °C for 10 min. The amplification of RAG2 fragment was carried out using a 2-step nested PCR. The thermocycler conditions used were 95 °C for 10 min followed by thirty-five cycles of 30 s at 95 °C, 30 s at 55 ºC (first PCR) and 57 ºC (second PCR with 1µl of the product from the first PCR), and 30 s at 72 °C with a final extension at 72 °C for 10 min. PCR success was assessed through electrophoresis, by running 2µl of the product on an agarose gel stained with GelRed (Biotarget). All samples showing PCR product of the intended length were sent to GENEWIZ (Germany) for purification and standard Sanger sequencing.

Sequence Analysis

Sequences were edited and aligned using Muscle (v. 5.0) and Geneious (v. 4.8.5). Available sequences from Barata et al. (2012), Harris et al. (2017) and Lyra et al. (2017) were included in the alignment. The ND4 fragment was translated into amino acids in order to assess the reading frame and confirm that these corresponded to the expected protein. Sequences of *Saurodactylus mauritanicus, Saurodactylus fasciatus, Saurodactylus brosseti* were included for outgroup purposes. Two methods of phylogenetic inference were employed: Maximum Likelihood (ML) and Bayesian Inference (BI) (Ronquist et al. 2012). Best-fit nucleotide models and best-fit partitions schemes for both analyses were selected using Partition Finder (v. 1.1.1) (Lanfear et al. 2012). ML analysis was performed with MEGAX (v. 10.1.8) using the model GTR + G and random addition replicates, while nodal support was assessed by bootstrapping with 5000 replicates. The BI analysis was carried out using MrBayes (v. 3.2.6) (Ronquist et al. 2012) and GTR + G + I (12S), GTR + G (ND4 1^{st} and 2^{nd} codon position), HKY (ND4 3^{rd} codon position), and HKY + G (tRNAs) models were used. Two independent runs of $2x10^6$ generations were performed, with a sampling frequency of 100 and 25% of the trees were discarded as burnin.

Haplotype networks

Haplotype networks for nuclear DNA markers were performed on Concatenator (v. 1.1.0). Gene genealogies were estimated using TCS (v. 1.23) and visualised with tcsBU (Múrias Dos Santos et al. 2016), and coloured according to the main lineages recovered from Bayesian Inference.

Statistical analysis

Statistical analyses were performed in R (v. 4.1.2). All measurements were checked for normality (Shapiro-Wilks test) and homoscedasticity (Levene's test) assumptions, in order to apply the most appropriate statistical test for the different variables and groups. Regarding the body measurements of both species, to exclude the effect of the variation in size and shape, we performed an isometric correction (Somers, 1986) to the total variation analysed. For *Quedenfeldtia moerens*, the populations from the South were considered as one population. Permutational multivariate analysis of variance (PERMANOVA), based on 1000 permutations of the Euclidean distance matrix between group means was used to examine the difference in population communities' composition among body measurements. Principal component analysis (PCA) was performed on the linear measurements. $P < 0.05$ was considered statistically significant. Univariate one-way ANOVA or Kruskal-Wallis H tests were performed on individual groups to explore the effects of each population and measurement variable independently.

Ecological niche modelling analysis

ENM prediction was computed in R (v. 4.1.2). Species occurrences were downloaded from Global Biodiversity Information Facility (GBIF). Presence-only coordinates were converted to spatial points for mapping. Locality data from previously published studies (Barata et al. 2012b; Bons & Geniez, 1996; Harris et al. 2017) and the new sampled localities were also converted in spatial points. An area of 300 and 380 km of buffer from all localities was chosen to develop ecological niche models on *Quedenfeldtia moerens* from the South, and *Quedenfeldtia trachyblepharus*. Bioclimatic variables (Table 3.1) were downloaded from WorldClim and the spatial correlation among them was tested in RStudio ($r < 0.7$). Selected variables were chosen regarding their contribution to the spatial species distribution. Generalized linear models (GLM) were predicted for probability and favourability of the species distribution (Sillero et al. 2021). Within the selected variables, the species occurrences were visualised regarding the significant ones in a spatial environment.

Table 3.1 - Bioclimatic variables from WorldClim using in the present study.

Bioclimatic variables	Meaning
BIO1	Annual Mean Temperature
BIO2	Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3	Isothermality (BIO2/BIO7) (×100)
BIO4	Temperature Seasonality (standard deviation ×100)
BIO5	Max Temperature of Warmest Month
BIO6	Min Temperature of Coldest Month
BIO7	Temperature Annual Range (BIO5-BIO6)
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 (Coefficient of Variation)
BIO16	Precipitation of Wettest Quarter
BIO17	Precipitation of Driest Quarter
BIO18	Precipitation of Warmest Quarter
BIO19	Precipitation of Coldest Quarter

Results

Molecular data

Topology resulting from Bayesian Inference (Figure 3.2) recovered the major lineages identified by Barata et al. (2012b) and Harris et al. (2017). Within *Quedenfeldtia moerens*, the two previously described northern and southern lineages were retrieved, with a notable divergence of 5.77% ± 0.61% (12S rRNA + ND4 and flanking tRNAs). The southern lineage includes a new sampled locality with 3 newly sequenced individuals. For *Quedenfeldtia trachyblepharus*, the four previously described lineages by Harris et al. (2017) were also recovered. The northern lineage includes a new population (Jebel Azourki), which extends the range of this lineage into the Middle Atlas region (Figure 3.1). The far southern lineage, recovered by a single individual in Harris et al. (2017) is grouped with nearby individuals from Jebel Awlime. The two previously described lineages which includes the populations of Oukaimeden and Jebel Sirwah, were also confirmed, however the "Jebel Sirwah" lineage included individuals from the new locality of M'goun.

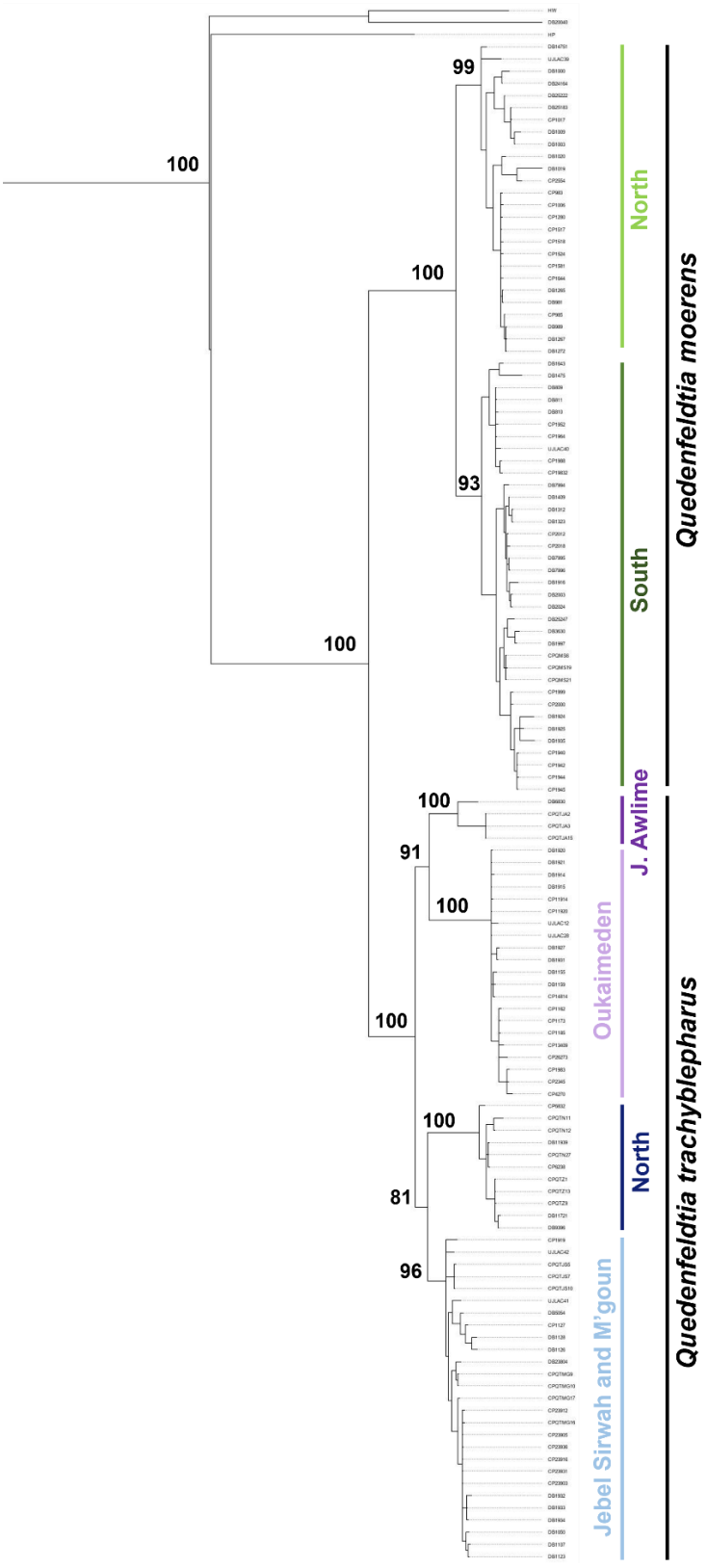

Figure 3.2 - Bayesian tree based on mitochondrial DNA (mtDNA) sequences (12S and ND4 + tRNAs) of *Quedenfeldtia*. Bayesian posterior probabilities (>0.8) are shown above branches. The main lineages within the species are represented by distinct colours according to Harris et al. (2017). New samples used are named with codes beginning with "CP". Codes beginning "DB" are from Barata et al. (2012b), in other cases GenBank numbers are indicated.

Parsimony networks, overall, demonstrated that at the nuclear level, the main lineages recovered from Bayesian Inference are separated. MC1R and RAG2 (Figure 3.3A and 3.3C) clearly splits both the lineages within *Quedenfeldtia moerens*, while C-MOS and PDC (Figure 3.3B and 3.3D) demonstrates sharing of haplotypes in the two main lineages. On the other hand, C-MOS and RAG2 undoubtedly separate the 4 main lineages within *Quedenfeldtia trachyblepharus*.

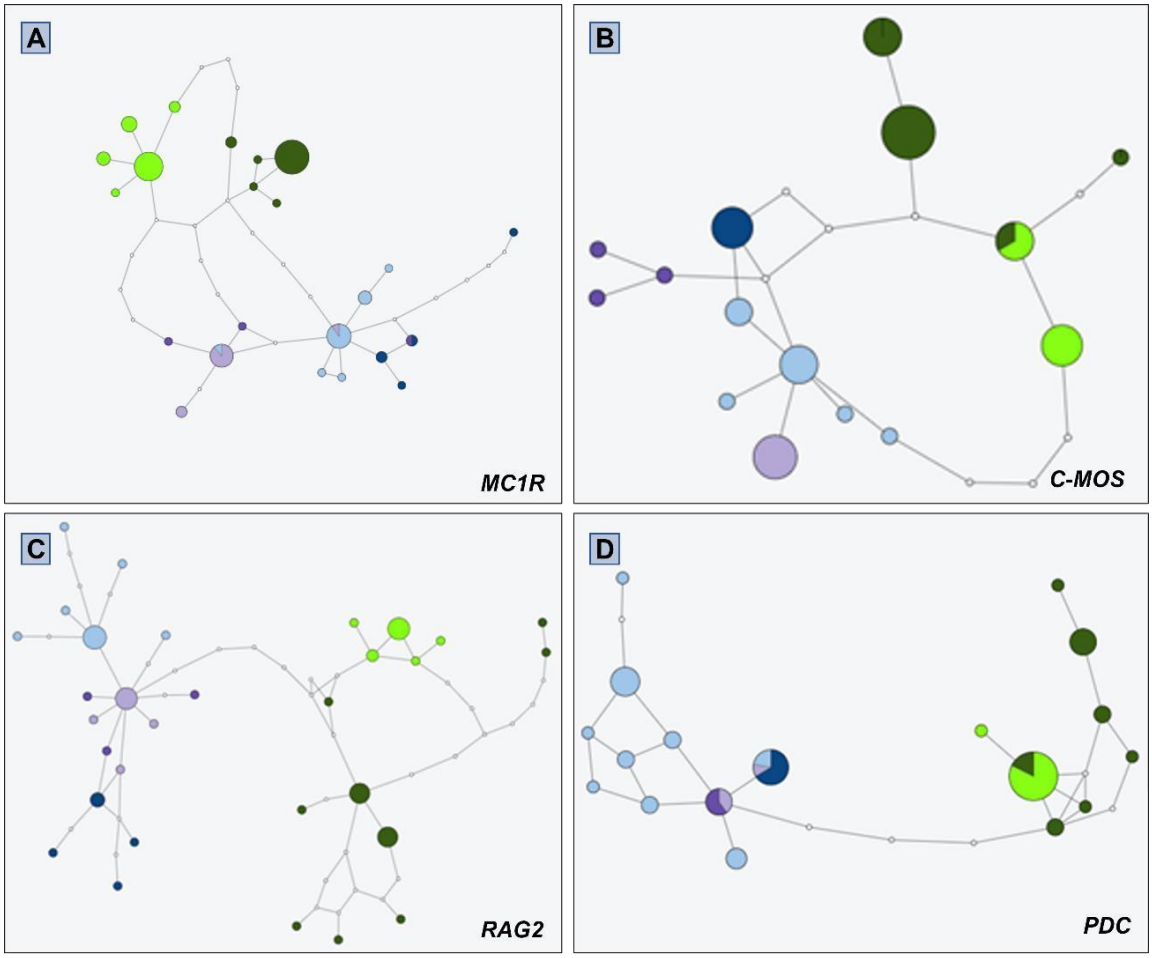

Figure 3.3 - Parsimony Network based on nuclear DNA sequences: A) MC1R; B) C-MOS; C) RAG2 D) PDC. Haplotypes are represented by circle with size proportional to their frequency.

Morphology

Linear measurements

PERMANOVA results demonstrated differences between Species, Population, and Sex in size and shape ($p<0.05$), however, no significant differences were detected for the interaction between population and sex. Details of PERMANOVA are described in Table 3.2.

Table 3.2 - PERMANOVA results for *Quedenfeldtia moerens* and *Quedenfeldtia trachyblepharus*. Df: degrees of freedom; SS: sum of squares; R2: variance explained; F: F value by permutation; P(perm): *p*-values based on 1000 permutations. POP: populations; SEX: sex. ($p < 0.05$).

Quedenfeldtia moerens					
Source	**Df**	**SS**	**R2**	**F**	***p*-value**
POP	1	56.35	0.09700	9.1398	**0.001***
SEX	1	24.58	0.04231	3.9866	**0.003***
POP x SEX	1	6.80	0.01170	1.1028	0.349
Residuals	80	493.27	0.84899		
Totals	83	581.00	1		
Quedenfeldtia trachyblepharus					
Source	**Df**	**SS**	**R2**	**F**	***p*-value**
POP	4	125.2	0.1408	5.2773	**0.001***
SEX	1	34.69	0.03902	5.8490	**0.001***
POP x SEX	4	29.26	0.03291	1.2333	0.219
Residuals	118	699.85	0.78724		
Total	127	889.00	1		

Principal component analysis with all the iso-corrected linear measurements allowed us to observe the individual variation and to group it by population in order to have a clear understanding of the phenotypic variation within each group, for both species (Figures 3.4 and 3.5).

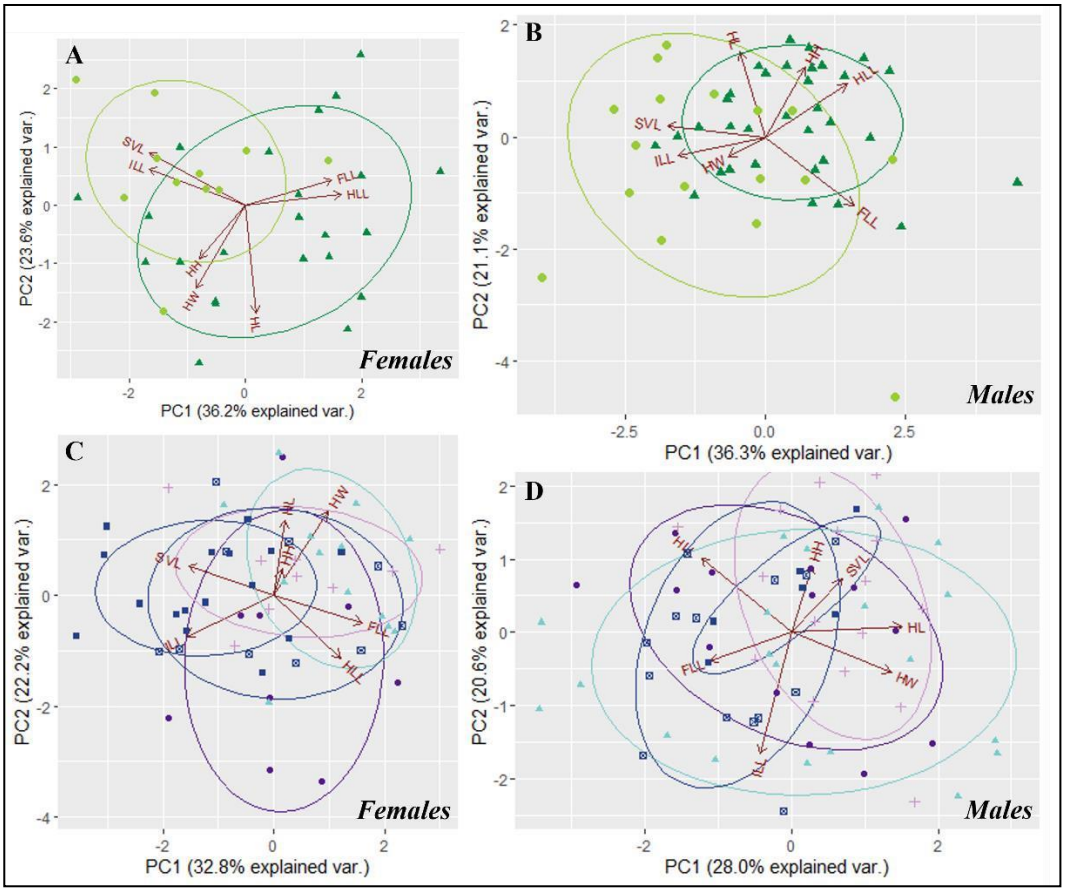

Figure 3.4 – PCA of iso-corrected linear measurements for *Quedenfeldtia moerens* females (A) and males (B), and *Quedenfeldtia trachyblepharus* females (C) and males (D). *Quedenfeldtia moerens* north are represented by light green dots and *Quedenfeldtia moerens* south are represented by dark green triangles. For *Quedenfeldtia trachyblepharus*, dark blue with squares (QTN), dark blue with squares and cross (QTZ), light blue and triangles (QTJS), dark purple and dots (QTJA), and light purple and crosses (QTO).

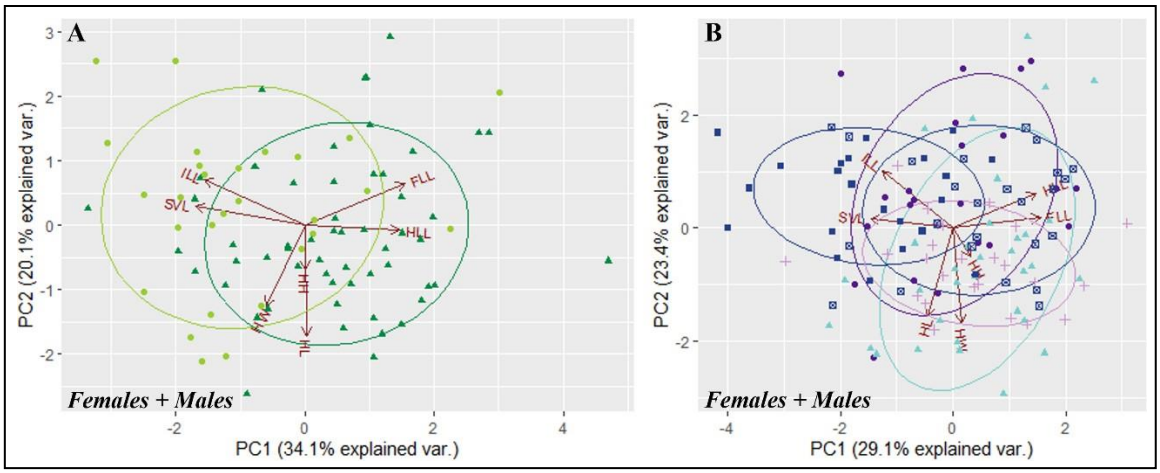

Figure 3.5 – PCA of iso-corrected linear measurements for *Quedenfeldtia moerens* (A) and *Quedenfeldtia trachyblepharus* (B) with all the individuals (females + males). *Q. moerens* north are represented by light green dots and *Quedenfeldtia moerens* south are represented by dark green triangles. For *Quedenfeldtia trachyblepharus*, dark blue with squares (QTN), dark blue with squares and cross (QTZ), light blue and triangles (QTJS), dark purple and dots (QTJA), and light purple and crosses (QTO).

Given the results obtained with the PERMANOVA analysis, further tests were performed at the individual level for *Quedenfeldtia moerens* and *Quedenfeldtia trachyblepharus*, considering the populations (males + females), through one-way ANOVA or Kruskal-Wallis H tests, to compare the effects of the populations on the measurement variables.

Table 3.3 - Output of one-way ANOVA analysis for the measurements HL, HW, HH, ILL, HL, for *Quedenfeldtia moerens*. ($p < 0.05$).

One-way ANOVA						
	Source of Variation	**SS**	**Df**	**MS**	**F**	***p*-value**
HL	Between Groups	1.166493	1	1.166493	2.238547	0.138446
	Within Groups	42.72967	82	0.521094		
	Total	43.89617	83			
HW	**Source of Variation**	**SS**	**Df**	**MS**	**F**	***p*-value**
	Between Groups	0.206653	1	0.206653	0.762285	0.384516
	Within Groups	29.82066	110	0.271097		
	Total	30.02731	111			
HH	**Source of Variation**	**SS**	**Df**	**MS**	**F**	***p*-value**
	Between Groups	0.356102	1	0.356102	1.735715	0.191352
	Within Groups	16.82322	82	0.205161		
	Total	17.17933	83			
ILL	**Source of Variation**	**SS**	**Df**	**MS**	**F**	***p*-value**
	Between Groups	9.370181	1	9.370181	6.904655	**0.010259***
	Within Groups	111.2807	82	1.357082		
	Total	120.6509	83			
HLL	**Source of Variation**	**SS**	**Df**	**MS**	**F**	***p*-value**
	Between Groups	45.66439	1	45.66439	40.53203	**1.05E-08***
	Within Groups	92.38322	82	1.126625		
	Total	138.0476	83			

Table 3.4 - Kruskal-Wallis H test for the measurements SVL and FLL, for *Quedenfeldtia moerens*. (p <0.05).

Kruskal-Wallis

	H Test Statistic	***p*-value**
SVL	17.990006	**2.22E-05***
FLL	1.275	0.258831

Significant differences were observed for SVL, ILL and HLL, between the north (QMN) and south populations (QMS) (p < 0.05, Table 3.3 and 3.4).

Results of one-way ANOVA analysis for the measurements HW and FLL for *Quedenfeldtia trachyblepharus* revealed that variation within this species is significant (*p*-value: 6.245e-06*(HW); *p*-value: 0.00190*(FLL)). In order to assess which populations contributed for these results, Tukey post-hoc test was applied for both variables (Tables 3.5 and 3.6).

Table 3.5 – Output of Tukey test comparison between populations of *Quedenfeldtia trachyblepharus* for HW length. (p < 0.05).

Group 1	**Group 2**	**Differences of means**	***p* - value**	**95% confidence interval**	
				Lower	**Upper**
QTJA	QTJS	0.8322	**0.0001***	0.3261	1.3383
QTJA	QTN	0.1895	0.879	-0.3661	0.7452
QTJA	QTO	0.8583	**0.0002***	0.3199	1.3966
QTJA	QTZ	0.4024	0.24	-0.136	0.9407
QTJS	QTN	-0.6427	**0.0064***	-1.1555	-0.1298
QTJS	QTO	0.0261	0.9999	-0.468	0.5201
QTJS	QTZ	-0.4298	0.1199	-0.9239	0.0642
QTN	QTO	0.6688	**0.008***	0.1241	1.2135
QTN	QTZ	0.2129	0.8155	-0.3318	0.7576
QTO	QTZ	-0.4559	0.1236	-0.9829	0.0711

For the head width length (HW), Jebel Awlime population showed significant differences compared to Jebel Sirwah and Oukaimeden populations. *Quedenfeldtia trachyblepharus* from the North were also distinct from Jebel Sirwah and Oukaimeden populations.

Table 3.6 – Output of Tukey test comparison between populations of *Quedenfeldtia trachyblepharus* for FLL length. ($p < 0.05$).

Group 1	Group 2	Differences of means	*p* - value	95% confidence interval	
				Lower	Upper
QTJA	QTJS	0.5339	0.3636	-0.2757	1.3435
QTJA	QTN	-0.6545	0.2539	-1.5433	0.2343
QTJA	QTO	-0.0824	0.9989	-0.9436	0.7787
QTJA	QTZ	0.3286	0.8282	-0.5325	1.1898
QTJS	QTN	-1.1884	**0.001***	-2.0087	-0.368
QTJS	QTO	-0.6163	0.2023	-1.4066	0.174
QTJS	QTZ	-0.2052	0.9518	-0.9955	0.585
QTN	QTO	0.5721	0.368	-0.2992	1.4433
QTN	QTZ	0.9831	**0.0186***	0.1119	1.8544
QTO	QTZ	0.4111	0.6605	-0.4319	1.2541

For the forelimb length (FLL), *Quedenfeldtia trachyblepharus* from the North revealed to be significant different from Jebel Sirwah population and also from Jebel Azourki population, which belongs to the same phylogenetic lineage. SVL, ILL, and HLL also showed significant differences between populations (Table 3.7).

Table 3.7 - Kruskal-Wallis H test for the measurements SVL, HL, HH, ILL, and HLL for *Quedenfeldtia trachyblepharus*. ($p < 0.05$).

Kruskal-Wallis		
	H Test Statistic	***p*-value**
SVL	31.674873	**2.23e-06***
HL	10.060572	0.03942
HH	10.785959	0.02908
ILL	14.975889	**0.00475***
HLL	16.167929	**0.00280***

Pholidosis

For *Quedenfeldtia moerens*, Kruskal-Wallis H tests for UPLAB and SUBLAB revealed no significant differences between both populations and sexes (Table 3.8). However, significant differences were detected for PRECL measurements ($p < 0.05$, Table 3.8), which through the post-hoc Dunn test, could

be attributed to both populations as a whole, as well as the interactions between populations and sex (Table 3.9).

Table 3.8 - Kruskal-Wallis H test for the measurements UPLAB, SUBLAB, and PRECL, for *Quedenfeldtia moerens*. ($p < 0.05$).

Kruskal-Wallis		
	H Test Statistic	P-value
UPLAB	5.781	0.328
SUBLAB	6.780	0.238
PRECL	77.433	**2.888 e-15***

Table 3.9: Kruskal-Wallis H test Post-Hoc Dunn's test for measurement PRECL for *Quedenfeldtia moerens*. F: females; M: males. ($p < 0.05$).

Groups	***p*-value**
QMN x QMS	**7.184101e-10***
QMN-F x QMN-M	0.207944
QMS-F x QMS-M	0.31337
QMN-M x QMS-M	**0.000014***
QMN-M x QMS-F	**0.000421***

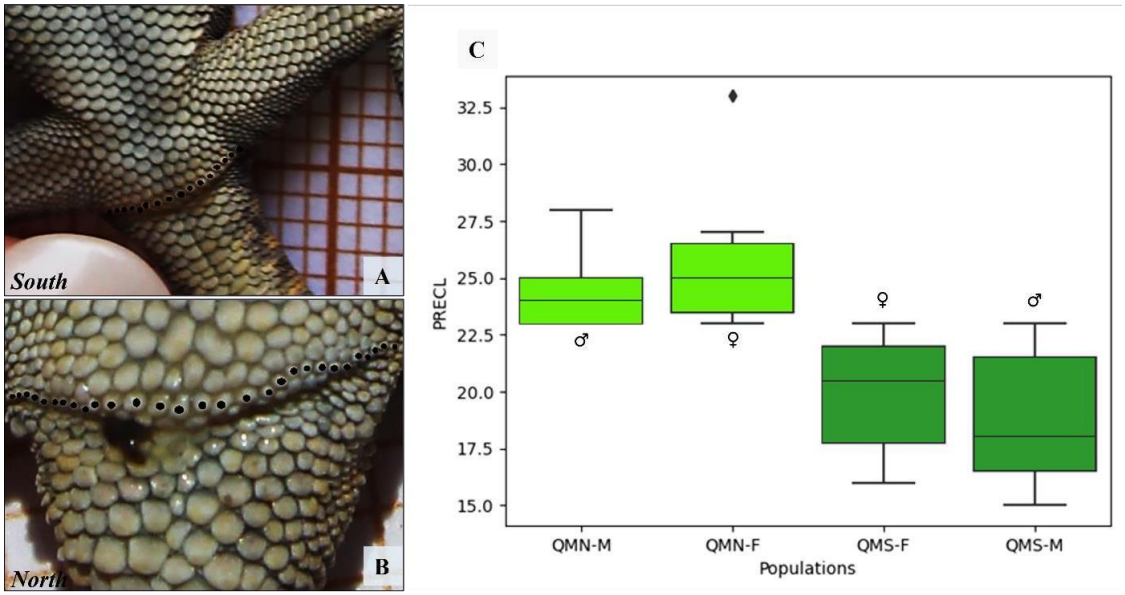

Figure 3.6 – Pre-cloacal scale counts in (A) *Quedenfeldtia moerens* from the Southern and (B) Northern lineages. (C) Boxplot depicting the results observed through the Kruskal-Wallis H test with post-hoc Dunn's test, for the PRECL variable. Light green represents *Quedenfeldtia moerens* North population (QMN – males and QMN – females) and *Quedenfeldtia moerens* South population (QMS – females and QMS – males).

Regarding the SBEY measurements, a two-way ANOVA analysis was performed, however no significant differences were detected between populations, sexes, or their interaction (Table 3.10).

Table 3.10 - Two-way ANOVA analysis for the measurement SBEY of *Quedenfeldtia moerens*. ($p < 0.05$).

Source	**Df**	**SS**	**MS**	**F**	***p*-value**
POP	1	9.545455	9.545455	1.907501	0.173263
SEX	1	1.517395	1.517395	0.303226	0.584271
POP:SEX	1	2.270038	2.270038	0.453629	0.503657
Residual	51.0	255.212567	5.004168		

Ecological niche modelling

The contribution of the environmental variables for *Quedenfeldtia moerens* "South" and *Quedenfeldtia trachyblepharus* revealed to be different. Isothermality, (BIO 3; *p*-value: 0.000374), Maximum Temperature of Warmest Month (BIO 5; *p*-value: 4.37 x 10e-15), and Precipitation Seasonality (Coefficient of Variation), (BIO 15; *p*-value: 0.000596) were the significant variables shaping the distribution of *Quedenfeldtia moerens* (South), while for *Quedenfeldtia trachyblepharus*, environmental variables seemed to not affect significantly their distribution (Precipitation of Warmest Quarter - BIO 18; *p*-value: 0.237).

Computed GLM favourability demonstrated the correlation between the selected environmental variables (Figure 3.8) and the species occurrences. GLM test were performed for each ENMs indicating good modelling performance (Figures 3.10 and 3.11) (AUC = 0.808 (QMS); AUC = 0.999 (QT); TSS > 0.5 for both ENMs). The distribution of *Quedenfeldtia moerens* occurs proximate to the coast, and extends to the Middle Atlas, while the *Quedenfeldtia trachyblepharus* are more restricted to the Middle Atlas (Figure 3.9). Overall, our results suggest that, for the selected environmental variables, there are other suitable areas close to our sampled localities, where species might occur and/or adapt, which can be valuable for further fieldwork.

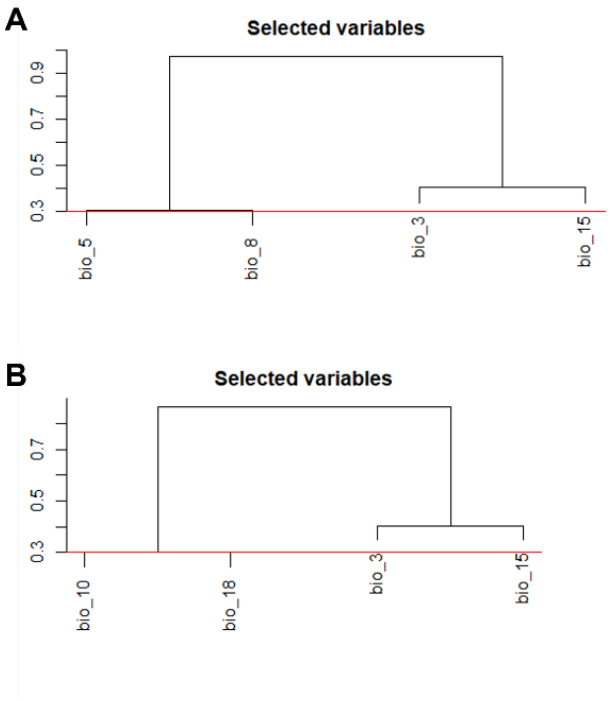

Figure 3.7 – Selected environmental variables used to predict ENMs in the present study, regarding their contribution to the species distribution for (A) *Quedenfeldtia moerens* (South) with BIO_3, BIO_5, and BIO_15 being significant, and (B) *Quedenfeldtia trachyblepharus*.

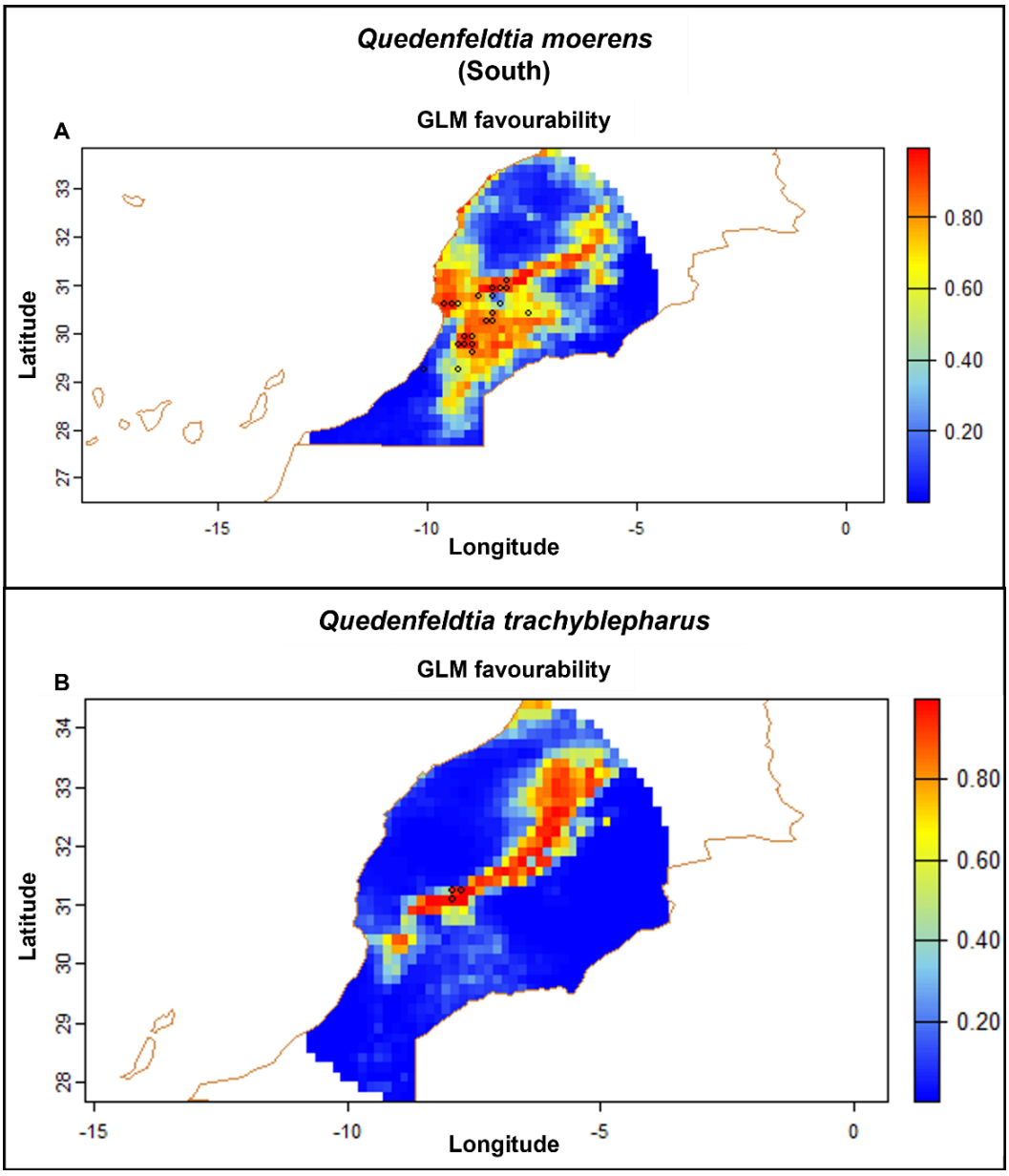

Figure 3.8 – ENMs prediction for *Quedenfeldtia moerens* South (A) and *Quedenfeldtia trachyblepharus* (B) endemic from Morocco (country delimitation is represented in orange) with GLM favourability correlating species occurrences with selected environmental variables. Species occurrences are spatial represented by black dots.

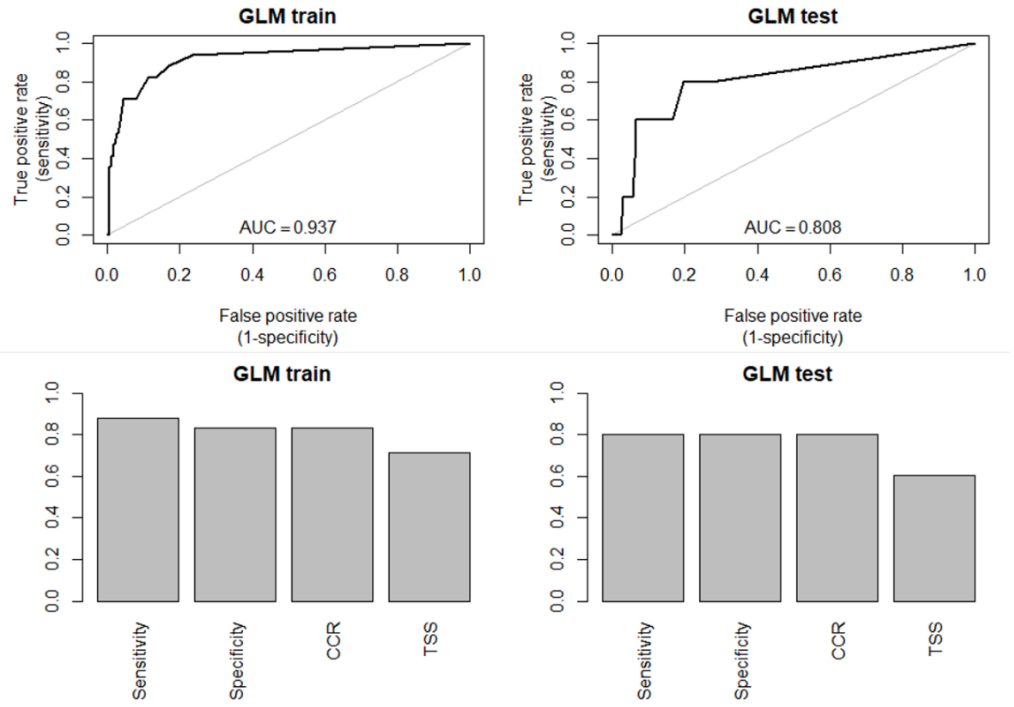

Figure 3.9 – Generalized Linear Model test accuracy for ENM performed for the *Quedenfeldtia moerens* from the South. Area under the ROC curve (AUC = 0.808); Sensitivity and specificity for GLM train and test; True skill statistic (TSS > 0.5) for GLM train and test.

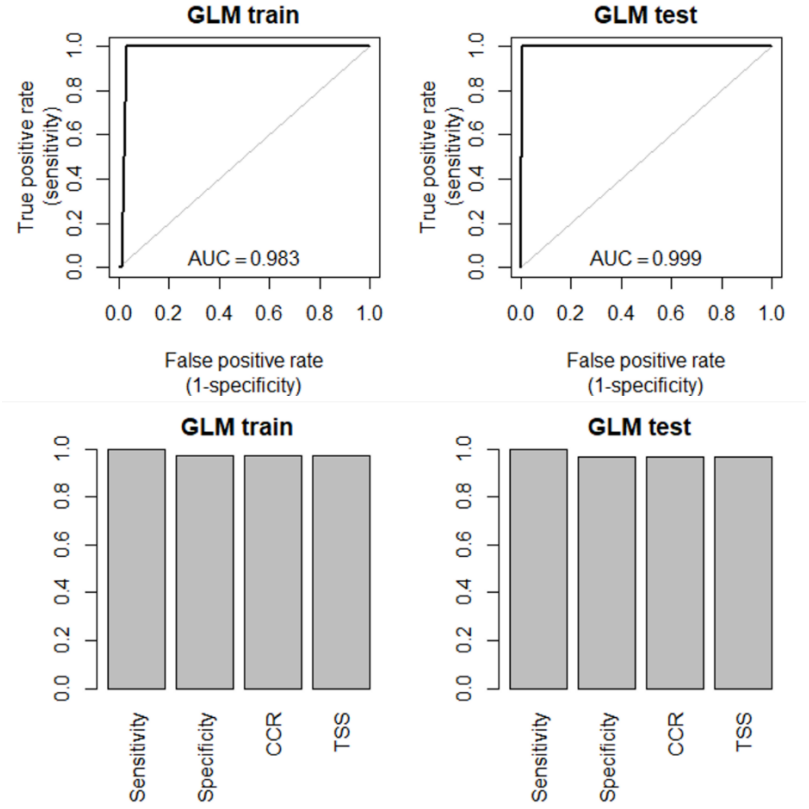

Figure 3.10 – Generalized Linear Model test accuracy for ENM performed for the *Quedenfeldtia trachyblepharus* from the South. Area under the ROC curve (AUC = 0.999); Sensitivity and specificity for GLM train and test; True skill statistic (TSS > 0.5) for GLM train and test.

Discussion

The last study carried out by Harris et al. (2017) recovered the main lineages within each species from Barata et al. (2012b), confirming a clear separation between *Quedenfeldtia moerens* Northern and Southern lineages and showed the emergence of a new group within *Quedenfeldtia trachyblepharus*. However, further work was required to assemble molecular data with a morphological study with all the known lineages and to assess the environment where these species inhabit to understand if the separation within *Quedenfeldtia moerens* with molecular data is supplemented for phenotypic variation and differences in the most suitable habitat for both groups. In this study, newly sampled individuals from previously known and new localities were crucial to assess if new localities would have the same pattern as the previously known ones and to have a clear understanding of *Quedenfeldtia trachyblepharus* populations which are more complex to organize since they have a narrower range of habitat. The level of variation between *Quedenfeldtia moerens* North and South of 5.77% ± 0.61% (12S rRNA + ND4 and flanking tRNAs) was higher than variation between other groups of high-altitude geckos (Hang Do et al. 2020), indicating a possibility of a new "cryptic" species within *Quedenfeldtia*. Parsimony networks based on nuclear markers also demonstrated the same pattern, although PDC and C-MOS showed a few shared haplotypes, which are common to occur even across populations that diverged hundreds of generations ago (Gusev et al. 2012).

The variation in linear measurements on both species revealed to be significant between populations (*p*-value: 0.001 for QM and QT) and sexes (*p*-value: 0.003 for QM and *p*-value: 0.001 for QT). The variables that contributed much for this variation where the snout-vent length, inter-limb length, and hind-limb length. Within *Quedenfeldtia moerens*, pholidosis analysis allowed to identify significant differences in the pre-cloacal scales (*p*-value: 2.888e-15 on Kruskal-Wallis test). PRECL largely differed between North and South populations (*p*-value: 7.184101e-10), in particular between males from those populations (QMN – males and QMS – males) (*p*-value: 0.000014), and between males from the Northern population (QMN – males) and females from the Southern population (QMS – females) (*p*-value: 0.000421). The variation found for PRECL is an important finding, since the number of scales is normally a conserved feature in reptiles (Tulli et al. 2016), as we could confirm with other pholidotic characters: UPLAB, SUBLAB, and SBEY. Previously studies on Squamata suggest that the number of scales can be driven by climate, in particular, by precipitation and temperature (Wegener et al. 2014). However, dorsal scales would have a higher response to selection than ventral ones (i.e., PRECL) since they are directly exposed to climate conditions (Tulli et al. 2016). Overall, our results suggest that the divergence within *Quedenfeldtia moerens* are not only based on genetic differences, but also in conserved morphological phenotypes. Furthermore, although this study did not include colour pattern assessment, it is important to mention the existence of distinct morphs on both ventral and dorsal plan in both sexes. The assessment of morphological variation within *Quedenfeldtia trachyblepharus* allowed us to search for differences between the five known populations. Head width and forelimb lengths revealed to be the most variable measurements, although not consensual in terms of populations. *Quedenfeldtia trachyblepharus* is a challenging group and more work is needed to clarify differences between lineages.

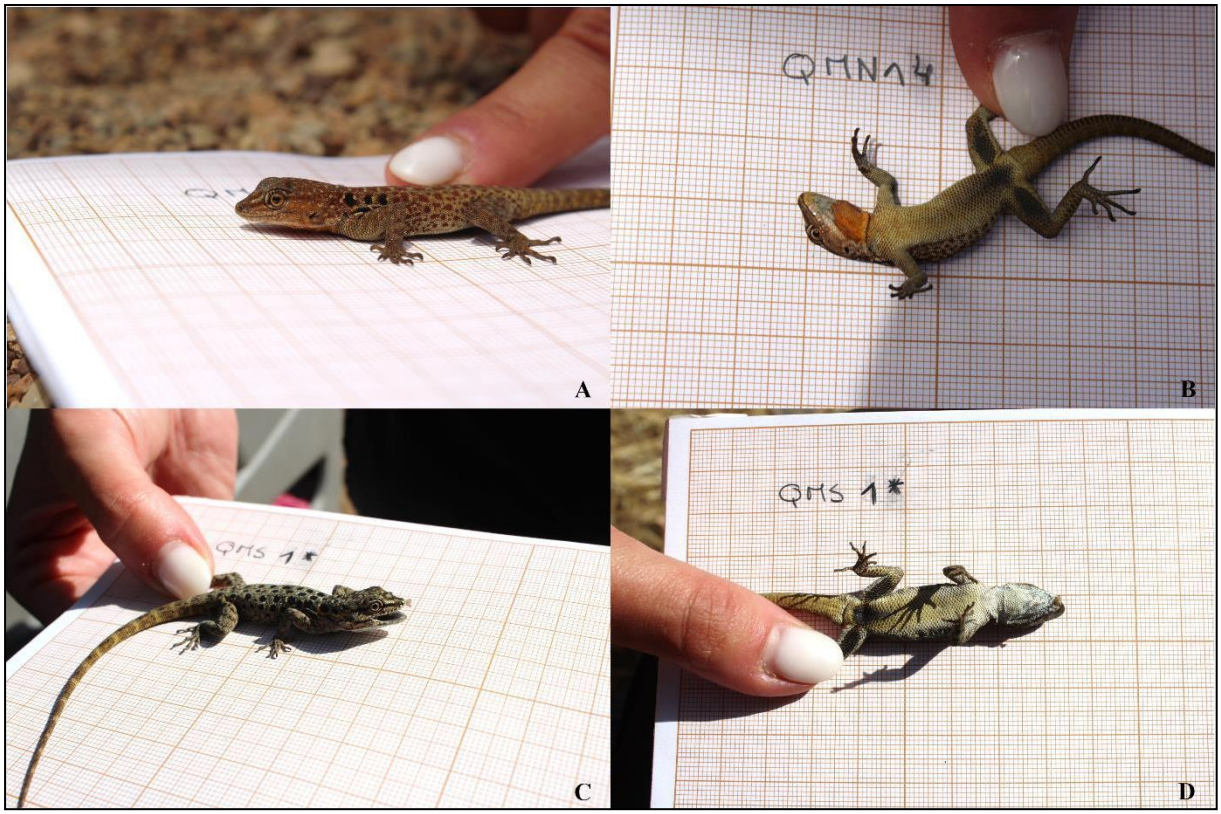

Figure 3.11 – *Quedenfeldtia moerens* male from the Northern population (A and B) and from the Southern population (C and D).

Although it would be valuable to predict the ENM for *Quedenfeldtia moerens* from the North in order to have a comparison between the two distinct lineages, there are not enough available occurrences to provide robustness for the modelling. The most suitable niche for *Quedenfeldtia moerens* South is more proximate to the coast, and Middle Atlas, where their occurrences have been identified, however in high-altitude areas. For future studies, it will be crucial to include altitude data and combine it with the environmental climatic variables, as these species inhabit in high-altitude habitats. The ENM for *Quedenfeldtia moerens* South leads us to expect a very different suitable ecological niche for *Quedenfeldtia moerens* North and therefore, to support their clearly isolation into two distant habitats. However, it is still valuable to sample between this range. In the past, herpetologists identified the occurrence of two *Quedenfeldtia moerens* in far south, near Guelmin. Those regions are much closer to the desert. Therefore, it is difficult to observe and capture geckos. However, since *Quedenfeldtia moerens* can inhabit lower ranges of altitude and the coastal area seems to be suitable for their occurrence, these results might indicate that more effort can be done in an attempt to sample more individuals in that region.

Conclusion

Overall, our results confirmed the expectations of previously studies carried by Barata et al. (2012b) and Harris et al. (2017) regarding genetic lineages in both species. Our findings suggest that *Quedenfeldtia moerens* might have a "cryptic" species hidden through morphological similarities (in particular, on scales, since the colour patterns reveal more perceptible variation). However, in our analysis we describe a significant variable trait – pre-cloacal scales. In the last decade, several studies have been correlating the variation found in number and shape of scales in reptiles with the climatic changes (Calsbeek et al. 2006; Oufiero et al. 2011; Tulli et al. 2016; Wegener et al. 2014). Our results, also suggests the importance on climatic environmental variables in the shape of species distribution. Combining all data,

the variation between *Quedenfeldtia moerens* lineages appears to be present at both genetic and morphological levels. For *Quedenfeldtia trachyblepharus*, the additional fieldwork carried out in the present year allowed to have a clearer understanding on the four known lineages with additional sampled populations.

CHAPTER IV - Manuscript II

This manuscript will include parsimony networks based on MC1R and C-MOS nuclear markers, and another previously unknown locality. We intent to submitted it to the “Journal of Herpetology”.

New localities and lineages of the Atlas dwarf lizard *Atlantolacerta andreanskyi* identified using mitochondrial DNA markers

Abstract

Atlantolacerta andreanskyi (Werner, 1929) is an endemic lizard from the High Atlas Mountains region of Morocco. A previous molecular assessment of this species using mitochondrial and nuclear DNA markers uncovered extensive genetic diversity with seven lineages indicative of a species complex. A morphological assessment of six of these lineages did not establish simple diagnostic features, and proposed these should be considered as cryptic species, while highlighting the need for greater sampling across the range. In this study, we sampled 5 individuals from 4 previously unsampled localities and carried out genetic analyses to compare these populations to the known variation. Phylogenetic reconstruction based on mitochondrial markers (12S rRNA and ND4) corroborates the previously described lineages and identified a new one. Interestingly, the two samples that account for this newly identified lineage have been collected from distinct localities – M'goun and Toumliline– that form a sister taxon to the population of Jebel Azourki.

Keywords: *Atlantolacerta andreanskyi*, phylogeny, morphology, evolutionary history

Introduction

Atlantolacerta andreanskyi (Werner, 1929), is a lacertid lizard endemic to the High Atlas Mountains in Morocco, distributed across 440 km (straight line) from the western to the central areas of the massif (Bons & Geniez, 1996; Mármol et al. 2019). It can be found in areas from 2400 m a.s.l. to 3800 m a.s.l., often under small rocks near watercourses and around the base of cushion-like thorny plants that offer a buffered microclimate (Bons & Geniez, 1996). The species presents a patchy spatial distribution, with populations separated by regions of unsuitable, lower elevation habitats.

Despite extensive herpetological surveys across the Atlas Mountains in recent years (eg Avella et al. 2019; Harris et al. 2010), there have been few additional populations reported, with minimal differences between the recent field guide and ones from much earlier (eg distribution reported from Mármol et al. 2019), compared to Bons & Geniez, 1996). A molecular assessment of 8 populations widely distributed across the range using two mitochondrial and five nuclear markers (Barata et al. 2012a) revealed extreme genetic diversity among seven of the eight populations analysed, demonstrating divergence levels indicating that *A. andreanskyi* should be considered a species complex.

A later assessment of phenotypic variability of this species, employing linear measurements, pholidotic and coloration characters in six of the previously analysed populations of *A. andreanskyi* (Barata et al. 2015), indicated that despite the high genetic divergence previously detected, morphological variation among populations was low. However, with almost every population studied representing a unique lineage, distribution of these cryptic forms remains essentially unknown.

The present study is the result of multiple additional fieldtrips to the region, and the collection of samples from additional localities to compare to the known genetic lineages. The aim was to increase the knowledge of the distribution of this species complex, and to include new samples within a phylogenetic framework.

Figure 4.1 – A) Distribution map of *Atlantolacerta andreanskyi*. Colored dots are based on the distribution map populations of Barata et al. (2012a). White dots surrounded by a black line represent the distribution points of Bons & Geniez, (1996). The yellow triangle in Jebel Awlime represents a specimen sampled in Jebel Toubkal (Avella et al. 2019). The localities with n ew sampled individuals are identified with numbers (1-4): Outabati (1), Toumliline (2), M'goun (3), and Jebel Awlime (4). B, C) Typical habitat of *Atlantolacerta andreanskyi* in Jebel Awlime and in Toumliline, respectively. Photos taken by Diana Vasconcelos and D. James Harris.

Methods

Fieldwork

All lizards were captured under permit from the *Haut Commissariat aux Eaux and Forests of Morocco*. Fieldwork was carried out in the Spring of 2019 and 2022, and resulted in the identification of *A. andreanskyi* from 4 new localities (Figure 4.1): 1) near the previously sampled population of Outabati (32°12'29.2"N 5°27'25.2"W; 2659 m a.s.l.), 2) near Toumliline, a new locality over 30km from any previously reported populations (31°54'46.1"N 5°29'08.2"W; 2609 m a.s.l.), 3) near M'goun, the second highest peak of the Atlas Mountains (31°34'52.9"N 6°16'11.8"W; 2712 m a.s.l.), south of the sampled populations of Jebel Azourki, and 4) north of the sampled populations in the furthest South, at Jebel Awlime (30°58'50.9"N 8°45'18.7"W; 2541 m a.s.l.). Specimens were captured by hand and photographed for later comparison of external phenotypic differences between lineages. Tip tail samples were collected and preserved in tubes filled with ethanol 96%. All individuals were then released in the place of capture.

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from tip tail samples, using standard high-salt protocols (Sambrook et al. 1989). In total five new individuals were included, two from the population near Jebel Awlime, and one from each of the remaining locations. Following DNA extraction, two portions of mitochondrial DNA were amplified via polymerase chain reaction (PCR): 12S rRNA (12S) and partial NADH dehydrogenase 4 (ND4) and flanking tRNA (tRNA-His), using previously published primers from Kocher et al. (1989) and Arévalo et al. (1994) respectively. The PCR thermocycler conditions used started with 95 °C for 10 min followed by thirty-five cycles of 30 s at 95 °C, 30 s at 50 ºC (12S) or 52 ºC (ND4), and 30 s at 72 °C with a final extension at 72 °C for 10 min. PCR success was assessed through electrophoresis, by running 2µl of the product on an agarose gel stained with GelRed (Biotarget). All samples showing PCR product of the intended length were sent to GENEWIZ (Germany) for purification and standard Sanger sequencing.

Sequence analysis

Sequences were edited and aligned using Muscle (v. 5.0) in Geneious (v. 4.8.5). Available sequences from Barata et al. (2012a) were included in the alignment. The ND4 fragment was translated into amino acids in order to assess the reading frame and confirm that these corresponded to the expected protein. Sequences of *Podarcis tiliguerta* were included for outgroup purposes. Two methods of phylogenetic inference were employed: Maximum Likelihood (ML) and Bayesian Inference (BI). Best-fit nucleotide models and best-fit partitions schemes for both analyses were selected using Partition Finder (v. 1.1.1) (Lanfear, Calcott, Ho, & Guindon, 2012). ML analysis was performed with MEGA (v. 10.1.8) using the model HKY + G + I and random addition replicates, while nodal support was assessed by bootstrapping with 5000 replicates. The BI analysis was carried out using MrBayes (v. 3.2.6) (Ronquist et al. 2012) applying SYM + G model for 12S, HKY + G + I, HKY + I, SYM + G models for ND4, and HKY + G + I model for tRNAs. Two independent runs of $3x10^6$ generations were performed, with a sampling frequency of 1000 and 25% of the trees were discarded as burnin.

Results

Both Bayesian Inference and Maximum Likelihood analysis produced almost identical topologies, differing slightly in the shallow nodes within populations (Figure 4.2). As expected, the major lineages identified by Barata et al. (2012a) were again recovered. The two newly sequenced individuals from locality (4) north of Jebel Awlime were strongly supported as a clade along with samples from Barata et al. (2012a) from nearby. Likewise, the individual from locality (1), near Outabati, was similarly most closely related to individuals from Barata et al. (2012a) from nearby, although divergence was notable (3.00 ± 0.62% (SE) with the ND4 marker, 0.33 ± 0.30% (SE) with the 12S rRNA marker). Unexpectedly, the individuals from M'goun (3) and Toumliline (4), despite being geographically separated by over 60 km formed a well-supported clade, distinct from the sister taxa population of Jebel Azourki (8.15 ± 0.95% (SE) with the ND4 marker, 3.34 ± 0.98% (SE) with the 12S rRNA marker). The overall estimate of relationships between lineages is concordant with Barata et al. (2012a): the northern Outabati and Jebel Ayache populations are sister taxa with strong support, and with weaker support that the sister taxon to these is the population from Jebel Awlime in the south; populations from Jebel Sirwah are sister taxa to those from Oukaimeden and Jebel Toubkal; and populations from the central region of Tizi n' Tichka and Jebel Azourki form a clade along with the newly identified lineage from M'goun and Toumliline.

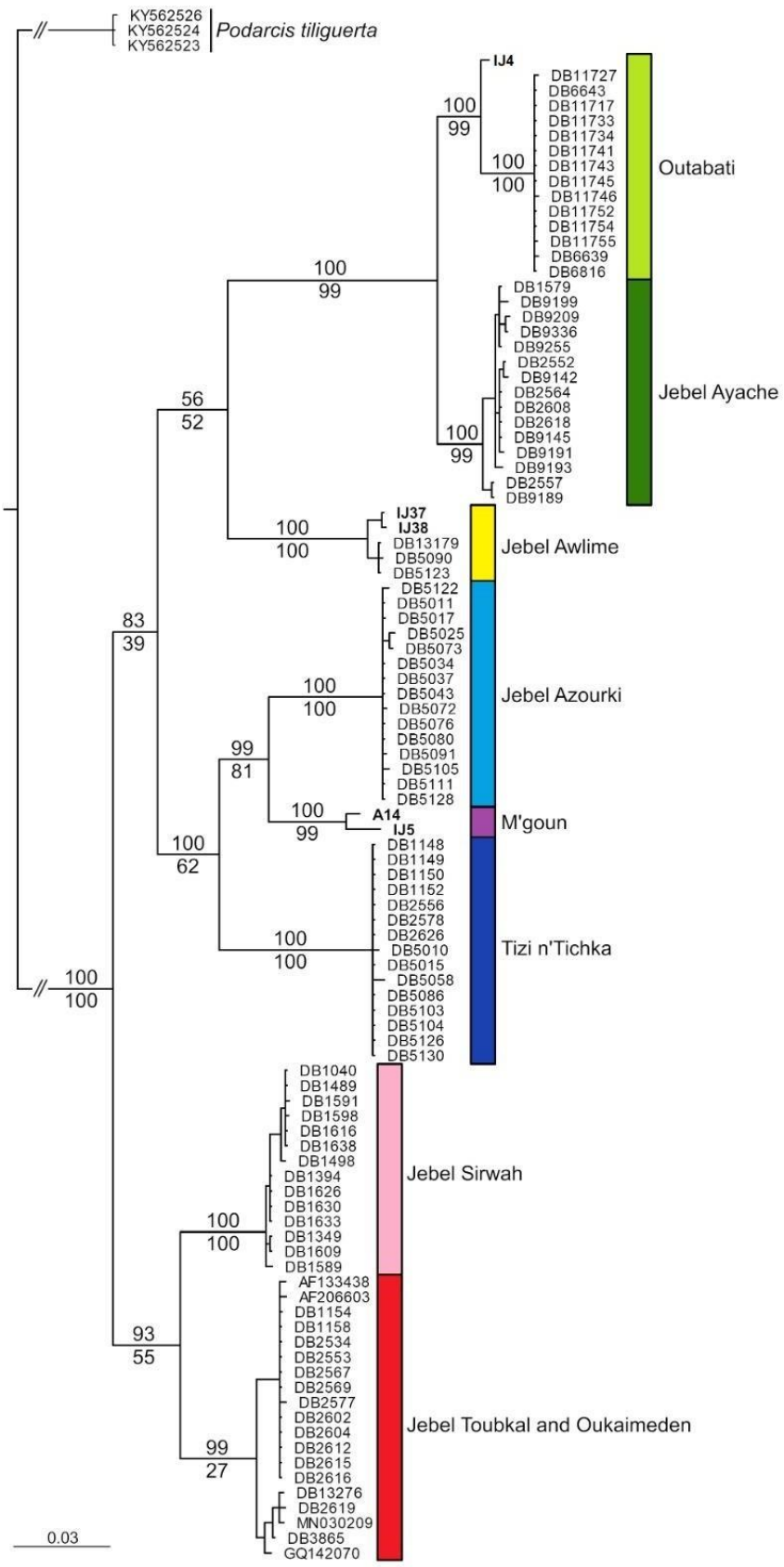

Figure 4.2 - Bayesian tree based on mitochondrial DNA (mtDNA) sequences (12S and ND4 + tRNAs) of *Atlantolacerta andreanskyi*. Bayesian posterior probabilities (>0.9) are shown above branches; bootstrap values for Maximum Likelihood are shown below branches. The main lineages within the species are represented by distinct colours. New samples used are named as IJ4 (1 - Outabati), IJ5 and A14 (2, 3 – Toumliline and M'goun), and IJ37 and IJ38 (4 – Jebel Awlime). Codes beginning "DB" are from Barata et al. (2012a), in other cases GenBank numbers are indicated.

Discussion

The earlier molecular study of Barata et al. (2012a) clearly indicated that *A. andreanskyi* could be considered a potential species complex, with important conservation implications since six lineages were identified using both mitochondrial and nuclear markers. However, these authors highlighted that the mountainous habitat was difficult to sample, and that the potential for additional cryptic forms to exist was high. In this study, by including four additional localities, we have demonstrated that this is indeed the case, with a divergent lineage occurring in M'goun and Toumliline. The level of divergence, approximately 8% with the ND4 marker, is similar or higher than variation between mountain species of the lacertid lizard genus *Iberolacerta* in the Pyrenees (Garcia-Porta et al. 2019), indicating another potential cryptic species within the *A. andreanskyi* complex.

Although Barata et al. (2015) reported some morphological differences between lineages, they also found that these could only be determined by examining multiple individuals and characters – simple diagnostic characters that could be used to classify specimens in the field were not identified. Colour pattern variation tended to match phylogenetic relationships, with the populations from Azourki and Tizi n' Tichka having a tendency towards more dark spots on the ventral head region, and a more intense ventral spotted pattern in general (Barata et al. 2015). The male specimen from Toumliline shows a similarly heavily spotted ventral pattern (Figure 4.3), although with a single specimen observed, this clearly needs further assessment to see if this pattern variation is maintained within the new lineage.

Figure 4.3 – Male specimens of *Atlantolacerta andreanskyi* (Werner, 1929) sampled in Toumliline (A-B) and Jebel Awlime (C-D). Photos taken by Diana Vasconcelos.

In the far south of the range of *A. andreanskyi*, additional populations have recently been identified both in this study (locality 4) and in the Tichka plateau (Avella et al. 2019). Genetically these seem to belong to the same lineage as those first identified from Jebel Awlime by Barata et al. (2012a). This population has never been included in a morphological assessment, but based on the few specimens

observed show a greatly reduced pigmentation pattern on the ventral (Figure 4.3), similar to specimens in the occidental lineage (Oukaimeden and Jebel Sirwah) of Barata et al. (2015). The specimen collected from about 10 km northeast of the known Outabati population, while clearly related to these, was also notably genetically distinct, highlighting just how much variation remains unknown. The Outabati population shows a trend towards an absence of dorso-lateral lines in males (Barata et al. 2015), however, again with a single specimen observed (Figure 4.3), it is not possible to assess if this feature is maintained in this new, nearby population.

Conclusion

Overall, our additional fieldwork within the range of *A. andreanskyi* confirms the expectation of Barata et al. (2012a) that additional lineages occur, but also that single lineages can occur across larger areas. The identification of the population at Toumliline shows that the range of the *A. andreanskyi* species complex is greater than previously considered, and combined with the considerable diversity already known highlights how important continuing surveys across the region are. Complete morphological assessments of unsampled lineages are needed to better determine morphological variation, even if this is slight, so that an integrated taxonomic revision can be performed. A recent assessment noted that ecophysiological conservativeness of *A. andreanskyi* demonstrates its vulnerability to climate change (S'khifa et al. 2022), while also highlighting that low elevation populations are the most vulnerable. Detailed assessments of the range of lineages, including altitudinal ranges, will also therefore be essential to develop appropriate conservation management plan for this cryptic species complex.

CHAPTER V - General Discussion

The overview of this thesis was to better understand the evolutionary histories of high-altitude reptiles from North Africa and to assess genotypic and phenotypic variation within each study group. Despite all the work that has been carried out by herpetologists in the field, North Africa is a region with high biodiversity still requiring additional studies. Furthermore, sampling high-altitude organisms is a challenge since these regions are difficult to access. The High Atlas Mountains have been the focus of study for some biologists that are interested in unravelling the biodiversity patterns of reptiles from this region (Barata et al. 2012a, b; Carranza et al. 2004; Harris et al. 2017). During the Spring of the present year, fieldwork was carefully planned and performed between the 9th to the 26th of May. Two groups of reptiles were sampled with the intent that they would be the focus of this thesis: *Quedenfeldtia* and *Atlantolacerta andreanskyi*.

For *Quedenfeldtia,* past studies carried out by Harris et al., (2017) and Barata et al., (2012b) a few years ago demonstrated the needed of a new approach on these species with an assessment of not only molecular data but combining genetics with morphological analyses and ecological niche modelling with all the known lineages. One of the intents was to assess the separation between *Quedenfeldtia moerens* Northern and Southern lineages, regarding past studies and their habitat distance, and also to sample two new localities for *Quedenfeldtia trachyblepharus*: Jebel Azourki and Jebel Awlime, regarding past questions that remained to be answered. Additionally, one more locality (M'goun) was included in the molecular and ecological analysis.

For *Atlantolacerta andreanskyi,* our intent was to increase the sampled areas to assess if they were as divergent as the already known ones and also to collect more specimens from the region around Jebel Awlime, for which only a few specimens were included in a previous study (Barata et al. 2012a), and to determine how widespread single lineages actually may be in a local area. Additionally, one more locality (M'goun) was sampled and analyzed.

5.1 Detecting "cryptic" phenotypic variation

During the last decades, the levels of biodiversity – in terms of accepted species numbers - have increased in this study area, and this is partially due to the greater application of molecular approaches. Sometimes, the genetic divergence resulting from these studies is not initially perceived in terms of morphology, at the first sight. While this might be due to truly "cryptic" species, in which morphological variation really is minimal, it is also often clear that morphological differences do occur but are either extremely difficult to identify in the field or require expert knowledge to distinguish. These aspects can only be separated if a detailed "integrative" taxonomy is performed, as was attempted during this thesis for *Quedenfeldtia*.

5.2 Final remarks

For *Quedenfeldtia*, phylogenies and haplotype networks similar to those from past studies were recovered, but by including additional localities, and by assessing morphological variation across all known genetic lineages, we were able to draw additional conclusions. In particular, *Quedenfeldtia moerens* North and South lineages are clearly separated, and can be distinguished in the field – both for males and females – by counting the numbers of precloacal scales Thus, these two groups are actually not completely "cryptic", but another example where morphologically diagnostic characters can be found if enough populations are assessed. Genetic divergence combined with morphological variation and their isolation into two distinct and markedly separated niches, indicate that these should be considered as two distinct species. However, the situation within *Q. trachyblepharus* remains more complicated, as morphological variation was lower, and there was more haplotype sharing with the nuclear markers, despite the overall high levels of mtDNA variation between lineages. Furthermore,

with so few known populations of each lineage, separate environmental niche modelling for each group could not be performed

For *A. andreanskyi*, our results confirmed the hypothesis proposed by Barata et al. (2012a), that there are additional lineages still to be identified within this species, with a new divergent lineage found in two localities. On the other hand, the additional sampling also showed that it is possible to find the same lineage across a greater range.

5.3 Future perspectives

This work allowed some previous questions to be answered, but it also highlighted certain areas in which additional research into the biodiversity of these high-altitude geckos and lizards from North Africa is still needed.

For the *Quedenfeldtia*, it is important to search for possible contact zones between *Q. trachyblepharus* and *Q. moerens*. Also, regarding the unexpected lineage in which individuals sampled in M'goun belong, more localities between Jebel Sirwah and M'goun should be sampled, to confirm that within that range, these geckos belong to the same lineage. Another interesting task that could be done is to confirm (or not) the existence of *Q. moerens* in the far southern historic records (near the desert). This might be difficult to achieve, since confirming the non-occurrence of a species is always harder than demonstrating that it does occur, but it would be important to study how they are related to the other lineages, and their morphological characteristics, inhabiting such a distinct environment. This might also make a difference for modelling approaches, as it is not clear what habitat can be suitable for Q. moerens in this region. Finally, a particularity of these geckos is the capacity to emit sounds. Thus, behavioral ecology could be included in integrated taxonomy studies through the analysis of their vocals in each population.

For the *Atlantolacerta andreanskyi*, it is necessary to focus on the Toubkal locality, to study what patterns will be observed if an intensive sampling in the area was done, as we only have one sampled individual from there. It would be valuable to assess for the possible existence of unsampled regions to study if they have the same pattern that have been found in the known localities. Finally, a morphological analysis for all the known lineages – including the new one identified in this thesis – are essential, to complement the earlier work (Barata et al. 2015), and to determine if diagnostic characters can be found, as was the case of *Q. moerens* in this thesis. Further, an ecological approach should be essential regarding a recent study (S'Khifa et al., 2022) on ecophysiology in these lizards. In this way future conservation plans for this unique biodiversity can be better developed and enacted.

Finally, other projection for future studies in these species complexes is to combine Next Generation Sequencing with morphology, as it has been revolutionizing the fields of phylogenetic and population genomics with the capacity to detect slightly divergences between lineages (Rittmeyer & Austin, 2014).

All the photographs in this thesis were taken by A. Carolina Pereira, D. James Harris, Diana Vasconcelos, and Joaquim Faria.

CHAPTER VI - REFERENCES

6.1 Chapter I

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Quedenfeldtia moerens from the North, Morocco (31°48'06.1"N 5°28'00.1"W), 11th May 2022.