# Integrating geospatial methods into evolutionary biology and conservation: case studies on selected Western Palearctic herpetofauna

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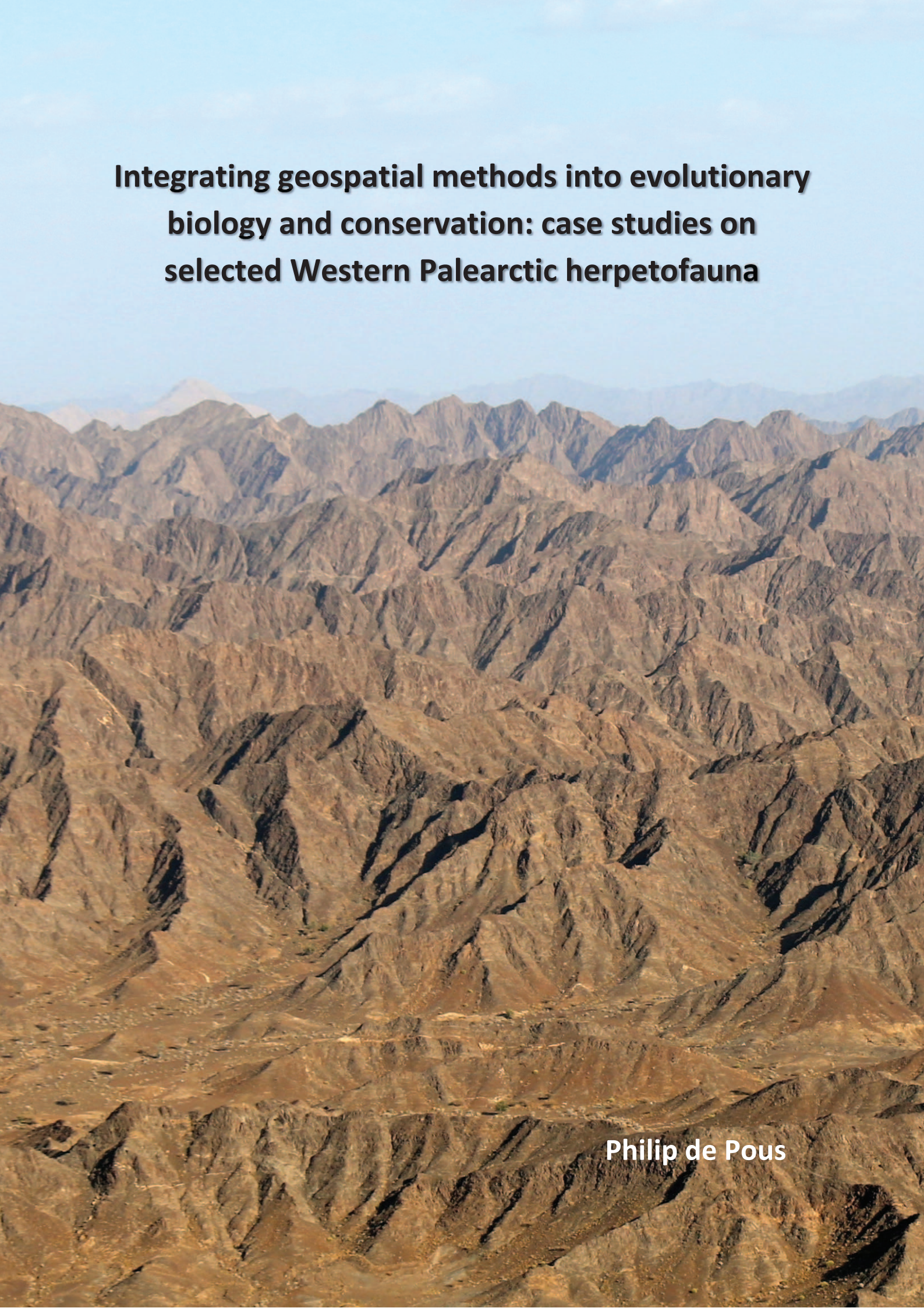

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# Integrating geospatial methods into evolutionary biology and conservation: case studies on selected Western Palearctic herpetofauna

Dissertation by

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to obtain the degree of Doctor at the University of Lleida
Gestió multifactorial de superficies forestals

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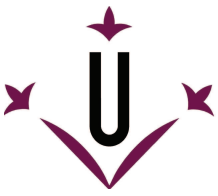

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d'Enginyeria Agrària

Then take me disappearin' through the smoke rings of my mind
Down the foggy ruins of time, far past the frozen leaves
The haunted, frightened trees, out to the windy beach
Far from the twisted reach of crazy sorrow
Yes, to dance beneath the diamond sky with one hand waving free
Silhouetted by the sea, circled by the circus sands
With all memory and fate driven deep beneath the waves
Let me forget about today until tomorrow

-Bob Dylan-

Wie schoonheid vrij wil maken
moet eerst z'n naakte waarheid grondig reinigen
van aangekleefd humaan

-Hans Verhagen-

**To Trui, Niels, Steffi and Anke**

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I am most indebted to Delfi Sanuy and Salvador Carranza who allowed me to pursue my dream of becoming a herpetologist under their excellent supervision.

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During my PhD I have collaborated with many colleagues from around Europe and Africa. The many people listed as co-authors and mentioned in the acknowledgements of the individual chapters illustrate the importance of collaboration during my PhD.

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I especially want to thank Wouter Beukema who helped me to rediscover my passion when I was somewhere halfway between the gutter and the stars… Wouter has been my friend and colleague during the last years and together we have completed many nice projects and experienced some incredible adventures.

I am indebted to Simon Rietveld for his enthusiasm in nature and for telling me influential stories about fishing and herpetology when I was a young boy.

My biggest supporters have always been my parents and sister. They have continuously supported me during my long struggle to find my passion. My love Anke van Kempen has been my main supporter and help during this PhD and helped me tackle many problems along the way. It would have been impossible to finish this thesis without her constant support and love. To express my sincere gratitude, I dedicate this dissertation to my parents, sister and wife.

# Abstract

Many evolutionary processes are influenced by spatio-temporal environmental variation, including speciation, genetic divergence among populations, and evolutionary change in physiology, morphology and behaviour. However, despite the extensive environmental data available from Geographic Information Systems (GIS) most evolutionary biologists and conservationists have not taken advantage of this data until relative recently. Especially species distribution models (SDMs) have been used in conjunction with genetic data and methods to address questions related to estimating ancestral distributions and niche divergence/conservatism of sister taxa (including identification and delineation of cryptic species). Furthermore, many studies have used SDMs along with past climate data to identify refugia and assess the role of stability on phylogeographic structure.

The general objective of this thesis was to integrate and expand the use of geospatial methods in evolutionary biology (biogeography, phylogeography and systematics) and conservation research. This general objective was accomplished through three specific objectives spread over six chapters, which compromised both methodological developments and their application in a series of case studies on Western Palearctic herpetofauna: The specific objectives aimed to: (1) study the phylogenetics, biogeography and phylogeography of the Western Mediterranean *Alytes maurus*, *Natrix natrix*, *Discoglossus* and the Arabian *Bunopus spatalurus*; (2) review the systematics, biogeography and natural history of Moroccan amphibians using an integration of both traditional (e.g. morphology, bioacoustic) and contemporary methods (phylogenetics, species distribution modelling); (3) develop and use a novel framework that combines SDM, high-resolution gridded climate data, dispersal modelling and data on both genetic diversity and evolutionary history to explore the effects of climate change on the potential distribution and genetic variation of the endemic Pyrenean newt *Calotriton asper* over the period 2020-2080

The enormous development of both conceptual and methodological approaches has revolutionised evolutionary and conservation research but there is still potential for major improvements related to reducing uncertainties. This thesis explored and successfully used a number of promising new geospatial methods in combination with more traditional molecular analyses. Such integrative approaches will ultimately allow us to better consider and examine the range of potential histories underlying both inter and intraspecific divergence patterns. Finally, as shown is this thesis, the combination of geospatial methods such as SDMs and dispersal models together with phylogeographic (or population genetic data) has major implications for conservation biology and should be further explored in during the next years.

# Resum

Molts processos evolutius són influenciats per la variació ambiental espai-temporal, incloent l'especiació, la divergència genètica entre les poblacions i el canvi evolutiu en la fisiologia, morfologia i comportament. No obstant això, malgrat la gran quantitat de dades ambientals disponibles dels Sistemes d'Informació Geogràfica (SIG), la majoria de biòlegs evolutius i experts en conservació no han aprofitat l'enorme potencial d'aquestes dades fins fa relativament poc temps. Especialment els models de distribució d'espècies (SDM) s'han utilitzat en conjunció amb les dades genètiques i mètodes per abordar qüestions relacionades amb l'estimació de distribucions ancestrals i divergència / conservació del nínxol ecològic de taxons germans (incloent la identificació i delimitació d'espècies críptiques). A més, molts estudis han utilitzat SDM juntament amb les dades climàtiques del passat per identificar refugis i avaluar el paper de l'estabilitat de l'estructura filogeogràfica.

L'objectiu general d'aquesta tesi era integrar i ampliar l'ús de mètodes geoespaials en la biologia evolutiva (biogeografia, filogeografia i Sistemàtica) i en la conservació.

Aquest objectiu general es va dur a terme a través de tres objectius específics que es distribueixen en sis capítols, que incloïen els diferents desenvolupaments metodològics i la seva aplicació en una sèrie d'estudis de casos sobre l'herpetofauna del Paleàrtic Occidental: Els objectius específics s'han centrat en: (1) l'estudi de la filogènia, biogeografia i filogeografia dels següents grups de la Mediterrània Occidental: *Alytes maurus*, *Natrix natrix*, *Discoglossus* i dels *Bunopus spatalurus* de la península Aràbiga; (2) revisar la sistemàtica, la biogeografia i la història natural dels amfibis marroquins integrant mètodes tradicionals (per exemple, morfologia, bioacústica) i mètodes contemporanis (filogènia, modelat de distribució d'espècies); (3) desenvolupar i utilitzar un marc innovador que combina SDM, dades climàtiques d'alta resolució, modelatge de la dispersió i dades sobre la diversitat genètica i la història evolutiva per tal d'explorar els efectes del canvi climàtic al llarg del període 2020-2080 en la distribució potencial i la variació genètica del tritó pirinenc, *Calotriton asper*, un endemisme pirinenc.

El recent desenvolupament dels marcs conceptuals i metodològics ha revolucionat la investigació en biologia evolutiva i conservació, però encara hi ha potencial per a millores importants relacionades amb la reducció d'incerteses. En aquesta tesi s'ha explorat i utilitzat amb èxit una sèrie de nous mètodes geoespaials prometedors en combinació amb les anàlisis més tradicionals moleculars. Aquests plantejaments integradors ens han permès i ens permetran examinar i entendre millor com es generen els patrons de divergència inter e intra-específics. Finalment, com es mostra en aquesta tesi, la combinació de mètodes geoespaials com SDM i models de dispersió juntament amb dades filogeogràfiques o de genètica de poblacions té importants implicacions per a la biologia de la conservació i, per tant, el seu potencial hauria de ser explorat en profunditat en els propers anys.

# Resumen

Muchos procesos evolutivos son influenciados por la variación ambiental espacio-temporal, incluyendo la especiación, la divergencia genética entre las poblaciones y el cambio evolutivo en la fisiología, morfología y comportamiento. Sin embargo, a pesar de la gran cantidad de datos ambientales disponibles de los Sistemas de Información Geográfica (SIG), la mayoría de biólogos evolutivos y expertos en conservación no han aprovechado el enorme potencial de estos datos hasta hace relativamente poco tiempo. Especialmente los modelos de distribución de especies (SDM) se han utilizado en conjunción con los datos genéticos y métodos para abordar cuestiones relacionadas con la estimación de distribuciones ancestrales y divergencia / conservación del nicho ecológico de taxones hermanos (incluyendo la identificación y delimitación de especies crípticas). Además, muchos estudios han utilizado SDM junto con los datos climáticos del pasado para identificar refugios y evaluar el papel de la estabilidad de la estructura filogeográfica.

El objetivo general de esta tesis era integrar y ampliar el uso de métodos geoespaciales en la biología evolutiva (biogeografía, filogeografía y Sistemática) y en la conservación.

Este objetivo general se llevó a cabo a través de tres objetivos específicos organizado en seis capítulos, que incluyen los diferentes desarrollos metodológicos y su aplicación en una serie de estudios sobre la herpetofauna del Paleártico Occidental: Los objetivos específicos se han centrado en: (1) el estudio de la filogenia, biogeografía y filogeografía de los siguientes grupos del Mediterráneo Occidental: *Alytes maurus*, *Natrix natrix*, *Discoglossus* y de *Bunopus spatalurus* de la península Arábica; (2) revisar la sistemática, la biogeografía y la historia natural de los anfibios marroquíes integrando métodos tradicionales (por ejemplo, morfología, bioacústica) y métodos contemporáneos (filogenia, modelado de distribución de especies); (3) desarrollar y utilizar un marco innovador combinando SDM, datos climáticos de alta resolución, modelado de la dispersión y datos sobre la diversidad genética y la historia evolutiva para explorar los efectos del cambio climático a lo largo del período 2020- 2080 en la distribución potencial y la variación genética del tritón pirenaico, *Calotriton asper*, un endemismo pirenaico.

El reciente desarrollo de un marco conceptual y metodológico robusto ha revolucionado la investigación en biología evolutiva y conservación, pero todavía hay potencial para mejoras importantes relacionadas con la reducción de incertidumbres. En esta tesis se ha explorado y utilizado con éxito una serie de nuevos métodos geoespaciales prometedores en combinación con los análisis más tradicionales moleculares. Estos planteamientos integradores nos han permitido y nos permitirán examinar y entender mejor cómo se generan los patrones de divergencia inter e intra-específicos.

Finalmente, como se muestra en esta tesis, la combinación de métodos geoespaciales como SDM y modelos de dispersión junto con datos filogeográficos o de genética de poblaciones tiene importantes implicaciones para la biología de la conservación y, por lo tanto, su potencial debería ser explorado en profundidad en los próximos años.

# The thesis includes the following papers

**Chapter 1: Phylogeography of the endemic *Alytes maurus* (Amphibia; Alytidae) from Morocco, inferred using mtDNA sequences and ecological niche modeling**

Authors: Philip de Pous, Margarita Metallinou, David Donaire, Salvador Carranza and Delfi Sanuy

Journal: Herpetological Journal 23: 153-160

Year: 2013

IF 2014: 1.338

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**Chapter 2: New insights on phylogeography and distribution of Painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula**

Authors: Miguel Vences, Philip de Pous, Violaine Nicolas, Jesús Díaz-Rodríguez, David Donaire, Karen Hugemann, J. Susanne Hauswaldt, Felix Amat, Juan A.M. Barnestein, Sergé Bogaerts, Abdellah Bouazza, Salvador Carranza, Pedro Galán, Juan Pablo González de la Vega, Ulrich Joger, Aziza Lansari, El Hassan El Mouden, Annemarie Ohler, Delfi Sanuy, Tahar Slimani and Miguel Tejedo

Journal: Amphibia-Reptilia 35: 305-320

Year: 2014

DOI: 10.1163/15685381-00002954

IF 2013: 1.138

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**Chapter 3: Molecular phylogeny of grass snakes (*Natrix*) with emphasis on the biogeography of northern African (*N. natrix*) populations**

Authors: Philip de Pous, Margarita Metallinou, Delfi Sanuy and Salvador Carranza

Journal: Submitted to Journal of Zoological Systematics and Evolutionary Research

Year: 2015

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**Chapter 4: Review of the systematics, distribution, biogeography and natural history of Moroccan amphibians**

Authors: Wouter Beukema, Philip de Pous, David Donaire-Barroso, Sergé Bogaerts, Joan Garcia-Porta, Daniel Escoriza, Oscar J. Arribas, El Hassan El Mouden and Salvador Carranza

Journal: Zootaxa 3661: 001-060

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**Chapter 5: Taxonomy and biogeography of *Bunopus spatalurus* (Reptilia; Gekkonidae) from the Arabian Peninsula**

Authors: Philip de Pous, Luis Machado, Margarita Metallinou, Jan Červenka, Lukáš Kratochvíl, Nefeli Paschou, Tomáš Mazuch, Jiří Šmíd, Marc Simó-Riudalbas, Delfi Sanuy and Salvador Carranza

Journal: Accepted in Journal of Zoological Systematics and Evolutionary Research

Year: 2015

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**Chapter 6: Range contraction and loss of genetic variation of the Pyrenean endemic newt *Calotriton asper* due to climate change**

Authors: Philip de Pous, Albert Montori, Fèlix Amat and Delfi Sanuy

Journal: Regional Environmental Change

Year: 2015

DOI: 10.1007/s10113-015-0804-3

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**Papers related to the dissertation and previously published by the author of this dissertation**:


Beukema W, de Pous P, Donaire D, Escoriza D, Bogaerts S, Toxopeus AG, de Bie CAJM, Carranza S (2010) Biogeography and contemporary climatic differentiation among Moroccan *Salamandra algira*. Biological Journal of the Linnean Society **101**: 626-641

de Pous P, Mora E, Metallinou M, Escoriza D, Comas M, Donaire D, Pleguezuelos JM, Carranza S (2011) Elusive but widespread? The potential distribution and genetic variation of *Hyalosaurus koellikeri* in the Maghreb. Amphibia-Reptilia **32**: 385-397

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Litvinchuk SN, Crottini A, Federici S, de Pous P, Donaire D, Andreone F, Kalezić ML, Džukić G, Veith M, Lada GA, Borkin LJ, Rosanov JM (2013) Phylogeographic patterns of genetic diversity in the common spadefoot toad, *Pelobates fuscus*, reveals evolutionary history, postglacial range expansion and secondary contact. Organisms Diversity and Evolution **13**: 433-451

Sillero N, Campos J, Corti C, Creemers R, Crochet P-A, Crnobrnja Isailović J, Denoël M, Ficetola GF, Kuzmin S, Lymberakis P, de Pous P, Rodríguez A, Sindaco R, Speybroeck J, Toxopeus B, Vieites DR, Vences M (2014) Updated distribution and biogeography of amphibians and reptiles of Europe. Amphibia-Reptilia **35**: 1-31

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# Introduction

Biogeography is the study of the geographical distribution of plants and animals at different taxonomic levels, past and present, the habitats in which they occur, and the ecological relationships involved. In other words, biogeography seeks to understand the spatio-temporal distribution of biodiversity. Phylogeography, coined by Avise et al. (1987), seeks to study the geographic distribution of genealogical lineages in order to determine the evolutionary history of species, subspecies and populations. In this sense, phylogeography can be considered as biogeography based on intraspecific gene geography. Since 1987 there have been major advances in phylogeography resulting in a vast literature fuelled by theoretical and methodological developments (Avise 2000). Coalescent theory and the continued development of statistically rigorous methods for inferring historic demographic processes and testing among alternative hypotheses of population differentiation have drastically changed and revolutionized the field (Knowles 2004, 2009; Hickerson et al. 2010; Nielsen and Beaumont 2009).

The extensive environmental data (e.g. vegetation, climate, geology) available from geographic information systems (GIS) and the development of predictive modelling approaches have revolutionized evolutionary, ecological and conservation research. Correlative approaches such as species distribution models (SDMs) – also known as "ecological niche models", "bioclimatic envelope models", or "habitat suitability models" - use associations between aspects of the environment (e.g. climate) and known occurrences of species across landscapes of interest to define sets of conditions under which species are likely to maintain viable populations (see Peterson et al. 2011; Araujo and Peterson 2012). There have been major theoretical and methodological advances since the early days of SDMs (Guisan and Zimmermann 2000) and the field now ranks among the most widely reviewed topics in the ecological literature. This is partly due to the wide applicability of SDMs to answer a variety of interesting questions. For example, SDMs can be applied to: discover new populations and species (e.g. Feria and Peterson 2002; Raxworthy et al. 2003; Bourg et al. 2005), conservation planning (e.g. Williams et al. 2005, Wilson et al. 2005, de Pous et al. 2011; Araujo et al. 2011), identifying potential sites for species' reintroductions (e.g. Adhikari et al. 2012), assessing the risk of invasive species (e.g. Broennimann et al. 2007; Peterson et al. 2008; Villemant et al. 2011), mapping the risk of disease transmission (e.g. Peterson et al. 2006, 2007a), studying niche evolution (e.g. Beukema et al. 2011), predicting effects of climate change on species' distributions (e.g. Thuiller et al. 2005; Araujo et al. 2006; Huntley et al. 2008, Lawler et al. 2009) and on phylogenetic diversity (Thuiller et al. 2011), and identifying refugia in phylogeography studies using past climate data (e.g. Waltari et al. 2007, Carnaval and Moritz 2008, Vega et al. 2010).

Since the development of the first SDM package in 1984, a large number of SDM algorithms have been developed. These algorithms can be classified to type of biological input data as follows: (1) Presence-only approaches (BIOCLIM, Busby 1991; HABITAT Walker and Cocks 1991), (2) Presence/absence approaches (generalised linear models GLM; generalized additive models GAM; Multivariate Adaptive Regression Splines MARS; classification and regression trees CART; boosted regression trees BRT; artificial neural networks ANN; support vector machines SVM, (3) Presence/background approaches (e.g. maximum entropy modelling, Phillips et al. 2006; Ecological Niche Factor Analysis ENFA, Hirzel et al. 2002), and (4) Presence/pseudoabsence approaches (GARP, Stockwell 1999) (see Peterson et al. 2011 for more information). There are several widely used software packages that have incorporated these algorithms. For example, the BIOMOD (Thuiller et al. 2009) and DISMO (Hijmans et al. 2012) R packages both include a large number of algorithms and provide options to create ensembles models. Other SDM software are openModeller (de Souza Muñoz et al. 2011), Maxent (Phillips et al. 2006), ModEco (Guo and Liu 2010) among others.

Among these predictive models, the presence/background algorithm Maxent (Phillips et al. 2006) is particularly popular and widely used with over 1500 applications published since 2006. This is mainly due to the relative easy use of the software and the fact that it has been shown that it produces high quality predictions that are often more successful when evaluated and compared with other predictive models (Hernandez et al. 2006; Giovanelli et al. 2010; Merow et al. 2013). Additionally, Maxent has a successful prediction power even when using low sample sizes (Pearson and Dawson 2003; Wisz et al. 2008) which is often the case for many species. The Maxent algorithm uses environmental parameters in combination with geographical coordinates in order to predict the distribution of the species of interest. Maximum entropy is achieved by the constraint that the expected value for each variable under the estimated distribution has to match its empirical average (the mean value of a random set of coordinates within the distribution (Phillips et al. 2006). In other words, this means that the model minimizes the relative entropy between two probability densities (one from the presence data and one from the landscape) defined in covariate space (Elith et al. 2011). In recent years, there have been many studies on SDM using Maxent, resulting in a much better understanding of what the model does, why inputs and settings matter and how we can make better predictions of species' distributions (Peterson et al. 2007b; Phillips and Dudík 2008; Warren et al. 2010, 2011; Anderson and Gonzalez 2011; Kramer-Schadt et al. 2013; Merow et al. 2013; Syfert et al. 2013; Yackulic et al. 2013; Elith et al. 2011; Brown 2014; Fourcade et al. 2014; Muscarella et al. 2014; Radosavljevic and Anderson 2014). The overall performance of this algorithm combined with the ever increasing understanding and methodological developments have resulted that Maxent is now the most widely used software for SDM.

Despite their usefulness, all correlative SDM approaches are subject to a wide range of problems arising from sampling bias, the choice and resolution of predictor variables, the quality of species distribution data, spatial autocorrelation, thresholds and the choice of the extent of the study region (Franklin 2009; Peterson et al. 2011). Nevertheless, promising model improvement methods are continuously being developed. For example, there are now useful tools and methods that facilitate the production of SDMs by reducing sampling bias (e.g. spatial autocorrelation) and evaluating (or tuning) optimal model settings (Warren et al. 2010; Brown 2014; Muscarella et al. 2014; Aiello-Lammens et al. 2015).

In parallel with the rise of SDMs there has been an enormous development of other geospatial methods that can be applied in evolutionary biology, ecology and conservation research. For example, Chan et al. (2011) reviewed most spatially explicit methods of use to biogeographers and provided several interesting case studies. The study of niche evolution has received considerable attention (and debate) during the last years (reviewed in e.g. Wiens et al. 2005, 2010; Peterson 2011). Niche conservatism, or the tendency of species to retain ancestral ecological characteristics, carries implications for a range of evolutionary and ecological phenomena such as the role of ecology in speciation (Wiens and Graham 2005) or the adaption of invasive species to new habitats (Schulte et al. 2012). A useful framework for measuring niche overlap has been developed by Broennimann et al. (2011) and is already widely applied to study niche evolution between species in evolutionary biology, systematics and biological invasions (e.g. Schulte et al. 2012; Wielstra et al. 2012; Ahmadzadeh et al. 2013; Koch et al. 2013). Another important yet understudied research topic has addressed the visualisation and spatial interpolation of intraspecific variation (reviewed in Thomassen et al. 2010a). The development of methods to interpolate genetic data to the landscape holds enormous promise to predict the spatial distribution of biodiversity (Rodríguez-Robles et al. 2010; Thomassen et al. 2010b; Vandergast et al. 2011; Tarroso et al. 2015). Finally, there have been important developments in incorporating dispersal constraints into projections of species distribution models (e.g. Engler et al. 2012). These methods have not yet been widely used despite their potential to illuminate problems in both evolutionary biology and conservation research (Espíndola et al. 2012; Engler et al. 2012).

Many evolutionary processes are influenced by spatio-temporal environmental variation, including speciation, genetic divergence among populations, and evolutionary change in physiology, morphology and behaviour. However, despite the extensive environmental data available from geographic information systems (GIS) most evolutionary biologists have not taken advantage of this data until relative recently (Kozak et al. 2007). Especially SDMs have been used in conjunction with genetic data and methods to address questions related to estimating ancestral distributions, niche divergence/conservatism of sister taxa (including identification and delineation of cryptic species) and

as proxies for a species dispersal potential (Graham et al. 2004; Knowles et al. 2007; Rissler et al. 2006; Stockman and Bond 2007). Furthermore, many studies have used SDMs along with past climate data (e.g. Last Glacial Maximum (LGM)) to identify refugia and assess the role of stability on phylogeographic structure (Waltari et al. 2007; Carnaval et al. 2009; Werneck et al. 2012). The integration of SDMs into phylogeography has received particular attention and is a common part of many phylogeography studies (e.g. reviewed in Alvarado-Serrano and Knowles 2014).

In recent years the application of SDMs has received much attention in conservation biology (Franklin 2010; Peterson et al. 2011). Species distribution models can potentially illuminate challenges relating to (1) estimating extinction risks for species, (2) identifying potential sites for reintroductions, (3) assessing the effects of climate change and land use, and (4) providing a base framework for conservation planning (Peterson et al. 2011). Reliable forecasts of climate change impacts on extinction risks are critical for effective conservation management responses and the use of SDMs to infer current and project future species distributions has been applied to a wide variety of taxa in many regions of the world. Under the assumption that species will track the geographic position of their niche, SDMs can be used to predict a range shift under climatic change (Peterson et al. 2011). Despite an ever increasing number of studies reporting climate change induced range shifts, few of these have incorporated species' specific dispersal constraints into their models despite the availability of suitable geospatial methods. Moreover, the impacts of climate change on genetic variation within populations and species have rarely been assessed while this is considered an important and promising research topic that requires further development (Pauls et al. 2013; Anderson 2013; Franklin 2013). Genetic variation at the intraspecific level is the most fundamental level of biodiversity and provides the basis for any evolutionary change. In order to fully understand the evolutionary consequences of climate change and its long term effects on biodiversity, it is necessary to assess the effects of climate change on intraspecific genetic diversity and evolutionary history (Moritz 2002; Pauls et al. 2013).

Several studies have already started to use the results of phylogeography studies in a conservation context and the use of geospatial methods has proven extremely useful. For example, Malaney and Cook (2013) used an integrative approach that combined molecular analyses and SDMs to highlight the importance of biogeographical history in establishing conservation priorities. Several other studies have shown the usefulness of integrating geospatial methods and phylogeography data to inform conservation planning (Vandergast et al. 2008), identify evolutionary hotspots (Wood et al. 2013) or to assess the impacts of climate change on intraspecific genetic diversity (e.g. D'Amen et al. 2013). The continued development of new methods to interpolate phylogeographic data onto the landscape (e.g. Tarroso et al. 2015) further strengthens this tie between evolutionary biology and conservation and we are now on the verge of a completely new integrative research field that applies

and combines methods from many disiplines including spatial ecology, evolutionary biology and conservation.

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# Objectives

The **general objective** of this thesis was to **integrate and expand** the use of geospatial methods in evolutionary biology (biogeography, phylogeography and systematics) and conservation research.

This general objective was accomplished through three **specific objectives**, which compromised both **methodological developments** and their **application** in a series of case studies on Western Palearctic herpetofauna.

**Specific objectives:**

**1)** To study the phylogenetics, biogeography and phylogeography of the Western Mediterranean *Alytes maurus*, *Natrix natrix*, *Discoglossus* and the Arabian *Bunopus spatalurus*

a) To develop phylogenies using up to date methods
b) To assess the role of past climate and dispersal barriers on intraspecific genetic variation using paleodistribution models
c) To identify contact zones between species, subspecies and lineages
d) To explore the application of a new phylogeographic interpolation method to infer lineage occurrence and contact zones
e) To make systematic changes that increase the taxonomic stability of the groups studied

**2)** To review the systematics, biogeography and natural history of Moroccan amphibians using an integration of both traditional (e.g. morphology, bioacoustic) and contemporary methods (phylogenetics, species distribution modelling)

**3)** To develop and use a novel framework that combines species distribution modelling, high-resolution gridded climate data, dispersal modelling and data on both genetic diversity and evolutionary history to explore the effects of climate change on the potential distribution and genetic variation of the endemic Pyrenean newt *Calotriton asper* over the period 2020-2080

# Chapter 1

## Phylogeography of the endemic *Alytes maurus* (Amphibia; Alytidae) from Morocco, inferred using mtDNA sequences and ecological niche modelling

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Authors: Philip de Pous, Margarita Metallinou, David Donaire, Salvador Carranza and Delfi Sanuy

We aimed at determining the effects of past climatic conditions on contemporary intraspecific genetic structuring of the endemic Moroccan midwife toad *Alytes maurus* using mitochondrial DNA (12S, 16S and cytochrome b) analysis and ecological niche modelling. Unexpectedly, our genetic analyses show that *A. maurus* presents a low level of variability in the mitochondrial genes with no clear geographical structuring. The low genetic variation in mtDNA can be explained by a much broader climatic suitability during the Last Glacial Maximum that allowed the connection among populations and subsequent homogenization as a consequence of gene flow.

**Author contribution**: First authorship reflects that I was the main contributor to the paper. I have developed the concept, written the first draft, contributed to geographical sampling design and conducted all the spatial analyses.

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# Integrating mtDNA analyses and ecological niche modelling to infer the evolutionary history of *Alytes maurus* (Amphibia; Alytidae) from Morocco

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We aimed at determining the effects of past climatic conditions on contemporary intraspecific genetic structuring of the endemic Moroccan midwife toad *Alytes maurus* using mitochondrial DNA (12S, 16S and cytochrome *b*) analysis and ecological niche modelling. Unexpectedly, our genetic analyses show that *A. maurus* presents a low level of variability in the mitochondrial genes with no clear geographical structuring. The low genetic variation in mtDNA can be explained by a much broader climatic suitability during the Last Glacial Maximum that allowed the connection among populations and subsequent homogenization as a consequence of gene flow.

*Key words:* biogeography, haplotype network, Köppen-Geiger, Maghreb, Maxent, midwife toad, Pleistocene glaciations

## INTRODUCTION

The Moroccan midwife toad (*Alytes maurus* Pasteur & Bons, 1962), endemic to northern Morocco, is the only African representative of the family Alytidae which includes only four other species: *Alytes obstetricans* Laurenti 1768 distributed across western Germany, the Netherlands, northern Switzerland, southern Belgium, Luxembourg, France and the northern half of the Iberian Peninsula; *Alytes muletensis* Sanchiz & Adrover 1979 restricted to few populations in the Serra da Tramuntana in Mallorca; *Alytes dickhilleni* Arntzen & García-París 1995 restricted to the mountainous massifs of southeastern Spain and *Alytes cisternasii* Boscá 1879 distributed across the central and southwestern Iberian Peninsula.

*Alytes maurus* was considered under the nomen *A. obstetricans* before it was described as a separate subspecies (*A. o. maurus*) by Pasteur & Bons (1962) based on divergent tadpole morphology when compared to European populations. Subsequently, the species received little scientific attention (but see e.g., Arntzen & Szymura, 1984; Libis, 1985) until the almost simultaneous publications of Bons & Geniez (1996), Schleich et al. (1996) and Salvador (1996) provided reviews of the species' geographic distribution and natural history. More recently, Donaire-Barroso & Bogaerts (2003) proposed to elevate the Moroccan midwife toad to the species level and, together with Donaire-Barroso et al. (2006), provided a substantial increase in ecological knowledge and geographic distribution. The specific status of *A. maurus* was later confirmed by osteological, mitochondrial DNA and nuclear DNA evidence (Fromhage et al., 2004; Martínez-Solano et al., 2004; Gonçalves et al., 2007). Phylogenetic analyses of the genus *Alytes* suggest that *A. maurus, A. dickhilleni* and *A. muletensis*, which together compose the subgenus *Baleaphryne*, form a clade, although weakly supported by mtDNA data (Fromhage et al., 2004; Martínez-Solano et al., 2004). The results also suggest that the three species split almost simultaneously, most probably as a result of the collapse of the Gibraltar land bridge at the end of the Messinian Salinity Crisis (MSC), in the late Pliocene around 5.3 mya, which led to diversification of the *Baleaphryne*, isolating *A. maurus* on the African continent, *A. dickhilleni* in southeast Iberia and *A. muletensis* in the Balearic Islands (Martínez-Solano et al., 2004).

At present, *A. maurus* is known only from about twenty fragmented localities (200–2,050 m a.s.l.) in the Rif and Middle Atlas Mountains in northern Morocco (see Fig. 1) and it has been assessed as Near Threatened (NT) according to the IUCN Red List of Threatened Species (Donaire-Barroso et al., 2009). The species is associated with humid sites in areas with montane karst, boulders and escarpments. Adult specimens live near permanent streams, pools and other waterbodies and generally inhabit fissures and cracks in rocks, as well as under stones on clay or humus-rich soils. The

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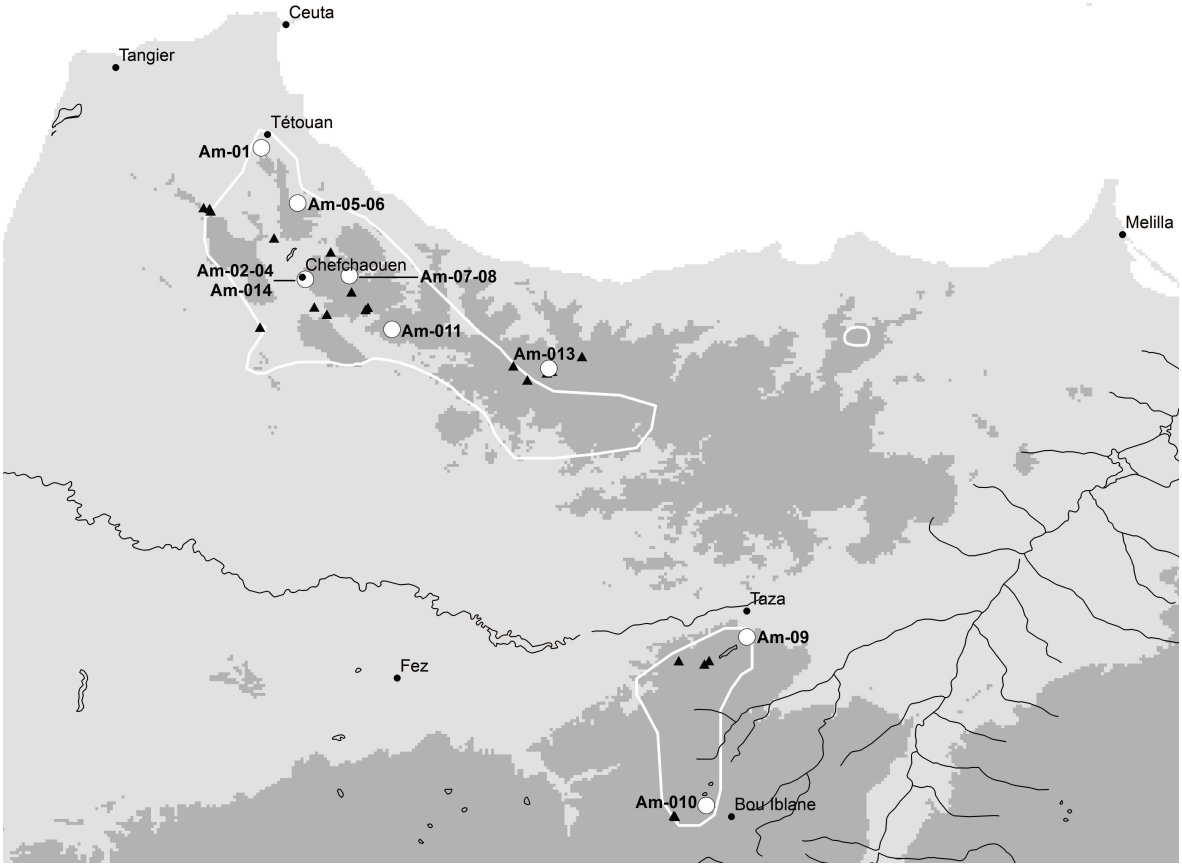


**Fig. 1.** The study area in northern Morocco showing the specimens included in the molecular analyses (Am-01–013, white dots), all known distribution records (black triangles) as well as the distribution range according to the IUCN Red List assessment (white lines). The main locality names and the Moulouya river basin are indicated. Areas above 800 m a.s.l. are indicated in darker grey colour.

typical surrounding vegetation may consist of oak and Atlas cedar forests, scrubs and orchards. Although the species can be locally common, future threats include the destruction and fragmentation of habitat and the domestic pollution of waterbodies (see Donaire-Barroso et al., 2009; Schleich et al., 1996). Furthermore, the species may also be threatened by climate change (PdP, unpublished data) and the recently detected chytrid fungus (*Batrachochytrium dendrobatidis*) in its vicinity (El Mouden et al., 2011).

In recent years, there has been a substantial increase in the knowledge of the evolutionary biology of the genus *Alytes*, both at the interspecific and intraspecific level. Fromhage et al. (2004) and Martínez-Solano et al. (2004) used mtDNA data to provide biogeographic scenarios for the evolutionary history of the genus, while the latter also used osteological data to propose a phylogenetic hypothesis. Gonçalves et al. (2007) used both mtDNA and nuclear DNA markers to infer phylogenetic relationships in *Alytes* and to assess the possible role of recent reticulation between deeply divergent lineages in the evolutionary history of the genus. More recently, Gonçalves et al. (2009) provided a detailed study on the intraspecific genetic variation of *A. cisternasii* and reported high population subdivision resulting from multiple refugia during Pleistocene glaciations. Finally, Pinho et al. (2010) and Agata et al. (2011) developed important new nuclear and microsatellite loci for *Alytes*. The species of the genus *Alytes* therefore constitute an interesting group for studying the effects of Pleistocene climatic oscillations. However, the intraspecific genetic variation of the Moroccan endemic *A. maurus* has never been investigated, while an interesting pattern of genetic differentiation between the Rif and Middle Atlas populations, as observed in *Salamandra algira* (e.g., Beukema et al., 2010), could be expected.

In this paper we use a modern framework of combining genetic data with ecological niche modelling (e.g., Waltari et al., 2007) to make inferences on the evolutionary history of *A. maurus* in Morocco. We specifically aim for the first time to assess the intraspecific genetic variation of *A. maurus* through mtDNA sequences, and to evaluate the potential distribution of the species in Morocco under present and past (Last Glacial Maximum) climatic conditions.

## MATERIALS AND METHODS

A total of 13 specimens of *A. maurus* covering most of its distribution range were included in the molecular analyses. Nucleotide sequences of three specimens were downloaded from GenBank. Specimen data and GenBank accession numbers of the gene fragments sequenced are listed in Table 1 and a map with all the localities of *A. maurus* included in the molecular study is shown in Fig. 1. Three mitochondrial gene fragments were sequenced: cytochrome *b* (cytb) (281 bp), 12S rRNA (12S) (298 bp) and 16S rRNA (16S) (495 bp).

**Table 1.** Codes, geographic localities (WGS 1984), mtDNA haplotypes and GenBank accession numbers in parentheses of all specimens of Moroccan *Alytes maurus* used in this study. The localities are indicated in Fig. 1.

| Map | Specimen Code | Locality | Latitude | Longitude | 12S | 16S | cytb |
|---|---|---|---|---|---|---|---|
| Am-01 | SPM000759 | South of Tetouan | 35,53889 | -5,38639 | H1 (AY333673) | H3 (KF145143) | H1 (KF145147) |
| Am-02 | SPM000309 | Chefchaouen | 35,16556 | -5,26138 | H1 (AY333673) | - | H1 (KF145147) |
| Am-03 | SPM000323 | Chefchaouen | 35,16556 | -5,26138 | H1 (AY333673) | H3 (KF145143) | H1 (KF145147) |
| Am-04 | SPM001757 | Chefchaouen | 35,16556 | -5,26138 | H1 (AY333673) | H3 (KF145143) | H1 (KF145147) |
| Am-05 | SPM002112 | North Jebel Kelti | 35,38214 | -5,28326 | H1 (AY333673) | H4 (KF145144) | H2 (KF145145) |
| Am-06 | SPM002145(34) | North Jebel Kelti | 35,38214 | -5,28326 | H1 (AY333673) | H3 (KF145143) | H1 (KF145147) |
| Am-07 | SPM004484 | Stream near Talassemtane | 35,17614 | -5,13647 | H1 (AY333673) | - | H3 (KF145146) |
| Am-08 | SPM004485 | Stream near Talassemtane | 35,17614 | -5,13647 | H1 (AY333673) | - | H3 (KF145146) |
| Am-09 | SPM004914 | Taza | 34,15018 | -4,00657 | H1 (AY333673) | - | H1 (KF145147) |
| Am-10 | SPM004915 | Bou-Iblane | 33,67165 | -4,12203 | H1 (AY333673) | - | H1 (KF145147) |
| Am-11 | SPM003892 | Northwest of Anasar | 35,02333 | -5,01530 | H1 (AY333673) | - | H3 (KF145146) |
| Am-12 | Aom01 | Rif Mountains | | | H1 (AY333673) | H1 (AY333711) | - |
| Am-13 | MNCN20917 | Ketama | 34,91263 | -4,56969 | - | H1 (AY442030) | H4 (AY442022) |
| Am-14 | MNCN40768 | Chefchaouen | 35,16556 | -5,26138 | - | H1 (AY442029) | H1 (AY442021) |

Primers used in both amplification and sequencing were: cb1 (5′-CCATCCAACATCTCAGCATGATGAAA-3′) and cb2 (5′-CCCTCAGAATGATATTTGTCCTCA-3′), both shortened at 5′ from original primers L14841 and H15149 (Kocher et al., 1989), respectively, for the cytb gene; 12Sa (5′-AAACTGGGATTAGATACCCCACTAT-3′) and 12Sb (5′-GAGGGTGACGGGCGGTGTGT-3′), both shortened at 5′ from original primers L1091 and H1478 (Kocher et al., 1989), respectively, for the 12S gene; and 16Sar-5′ and 16Sbr-3′ (Palumbi, 1996) for the 16S gene. PCR cycling conditions for all three gene fragments used were as follows: 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute, ending with 5 minutes of final extension at 72°C. Geneious v.5.3 (Drummond et al., 2010) was used for contig assembly, visualization of sequences and as a platform for exporting into different formats. Sequences were aligned using MAFFT v.6 (Katoh et al., 2002) available online, and applying default parameters (gap opening penalty=1.53, gap extension=0.0). A median-joining haplotype network for the separate 12S, 16S and cytb gene fragments was constructed using Fluxus Phylogenetic Network Analysis software v.4.6.0.0. (Bandelt et al., 1999) and is presented in Fig. 2.

A total of 19 BioClim variables were downloaded from the WorldClim database v.1.4 (http://www.worldclim.org/) to form the present and past (Last Glacial Maximum; LGM) climatic datasets (Hijmans et al., 2005) at a scale of 2.5 arc minutes (nearly 5 × 5 km). Two general atmospheric circulation models (GCM) were used to generate past climate scenarios for each period: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The two GCMs were assembled using ArcGIS v.10 (ESRI). Collinearity of the initial variables was measured with Pearson's correlation coefficient in ENMtools v.1.3 (Warren et al., 2010). A total of eight variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient) were retained. Selection of predictor variables was based on ecological understanding of the species (e.g., rainfall in the breeding season). The final set of environmental predictor variables used for ecological niche modelling (ENM) consisted of: Temperature Seasonality (BIO4), Max Temperature of Warmest Month (BIO5), Mean Temperature of Wettest Quarter (BIO8), Mean Temperature of Driest Quarter (BIO9), Annual Precipitation (BIO12), Precipitation Seasonality (BIO15) and Precipitation of Warmest Quarter (BIO18).

A total of 37 distribution records were assembled from both literature and fieldwork. The distribution records were filtered to remove duplicate records within unique grid cells in ENMtools v.1.3 (Warren et al., 2010). After filtering, a total of 26 distribution records were used for ENM (Fig. 3).

The ecological niche models were generated by the presence/background algorithm Maxent, v.3.3.3k (Phillips et al., 2006). Maxent was used with default settings (Convergence threshold=0.00001, maximum number of iterations=500 and $\boldsymbol{\beta}_j$=1) while partitioning the geographical records between training and test samples (default settings). Several studies have recently addressed the importance of selecting pseudo-absence or background locations in ENM (e.g., VanDerWal et al., 2009). We therefore followed the background approach of Webber et al. (2011) and Thompson et al. (2011) and downloaded Köppen–Geiger polygons from the CliMond database (Kriticos et al., 2011; www.climond.org). Subsequently, models were projected onto a larger area (Fig. 3).

The average of ten pseudo-replicated models with randomly selected test samples was used to produce ENMs, which were plotted in logistic format. The final models were reclassified in ArcGIS v.10 (ESRI) into binary presence-absence maps based on two different thresholds: (i) following the assumption that ten percent of the records were either wrongly identified or georeferenced (Raes et al., 2009), the average ten percentile threshold (TPT) was used, meaning, the 10%

of model outputs with the lowest predicted probabilities fall into the 'absence' region of the thresholded model, and 'presence' regions include the 90% of distribution records with the highest model values, and (ii) the average lowest presence thresholds (LPT=minimum training presence threshold of Maxent software), guaranteeing that all presences are predicted as suitable (Pearson et al., 2007).

All models were tested with receiver operating characteristics (ROC) curve plots and the area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model. A comparison of the environmental variables used for projection with those used for training the model were made using visual interpretation of multivariate similarity surface pictures and the most dissimilar variable (Elith et al., 2010).

## RESULTS AND DISCUSSION

Both 16S and cytb mitochondrial gene fragments have three variable positions, and haplotype diversity is estimated at 0.75 and 0.60, respectively. The results of the network analyses are shown in Fig. 2 and indicate that among the sampled individuals, depending on the marker, up to four haplotypes are found, all interconnected with single-mutation steps, while they lack clear geographic structure. In accordance with the low level of genetic variability detected in the 16S and cytb genes, all 12 sequences of the 12S gene fragment included in this study were identical and therefore a single haplotype was detected (Table 1).

As shown in Fig. 3, Maxent produced models of high predictive accuracy, according to the average test AUC for the present (0.947±0.040) and past (LGM) models (0.943±0.040). The present ENM (LPT threshold; Fig. 3B) of *A. maurus* reveals a relatively large potential distribution in both south and eastward directions, with suitable areas in the Middle Atlas region (including Ifrane National Park), the area between Agadir and Marrakech and more southwards, as well as in large parts of northeast Morocco (e.g., the Beni Snassen Massif and Debou) and in northwestern Algeria (e.g., Tlemcen region). The models under the TPT reveal a more realistic potential distribution with less overprediction (Fig. 3A). The current ENM (both thresholds; Fig. 3A, B) shows a barrier of unsuitable climate fragmenting the populations of *A. maurus* from the Rif and Middle Atlas Mountains, whereas another barrier exists between the Middle Atlas and the Debdou Massif in the east.

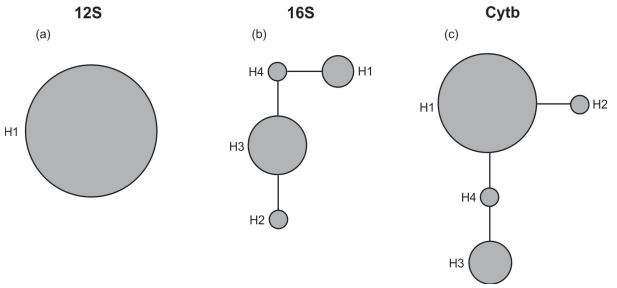


**Fig. 2.** Median–joining network inferred for three separate mtDNA fragments (a=12S, b=16S, c=Cytb). Grey circles represent different haplotypes and have been drawn proportional to the frequency of each haplotype. Information on the samples included is shown in Fig. 1 and Table 1.

The LGM model under the LPT (Fig. 3D) shows an increase in comparison to the present potential distribution with a continuous and unfragmented suitable area towards most parts of central, southwest and northeast Morocco, and northwest Algeria. The TPT map (Fig. 3C), however, reveals that the Moulouya River basin remained a barrier between the Middle Atlas and the northeastern region.

Clamped areas can be identified in most parts of the Rif, Middle and High Atlas Mountains, and also MESS and MoD pictures reveal that these regions have a non-analogue climate. Therefore, the predictions in these regions should be treated with caution (unpublished results, available upon request).

The result of the mtDNA analysis is unexpected, especially considering that many other Moroccan herpetofauna species show much higher levels of genetic variation, which may often indicate the presence of cryptic species or species complexes (e.g., Pinho et al., 2008; Rato & Harris, 2008; Perera & Harris, 2010). On the other hand, several species of North African amphibians (e.g., Stöck et al., 2008; Harris & Perera, 2009) also present low levels of genetic variability, which suggests that the amphibians in this region have higher vagility than expected or benefited from optimal climatic periods in the past such as the LGM. The intraspecific genetic variation of the other Moroccan endemic species such as *Pelobates varaldii*, *Discoglossus scovazzi* and *Barbarophryne brongersmai* is currently under study (PdP, pers. data) and the existence of genetic structuring in these species remains to be tested.

The low level of genetic variability detected in *A. maurus* is likely to be the result of a much broader climatic suitability during the LGM that allowed for the connection among populations and subsequent gene flow. The mtDNA network reveals that the geographically most distant specimens (Tetouan and Boulblane) share the same haplotype for both the 12S and cytb genes. The increased potential distribution of *A. maurus* during the LGM is a result of wetter and cooler annual climatic conditions in North Africa (Rognon, 1987; Wengler & Vernet, 1992), whereas expanded forest cover (e.g., Lubell, 2001) and an increase in shrubby vegetation (e.g., Fletcher & Sánchez-Goñi, 2008) might have also facilitated the species' dispersal and subsequent gene flow, as was also reported in a recent study (de Pous et al., 2011a). Populations in the Rif and Middle Atlas Mountains, that show isolation under current climatic conditions, could migrate north and south and were not affected by the present unsuitable barrier between these populations.

The present potential distribution of *A. maurus*, as predicted by the ENM, is larger than the currently known species range under both thresholds. The Moroccan herpetofauna has been the subject of investigation by many researchers in the last decades, resulting in a multitude of new distribution records, often extending

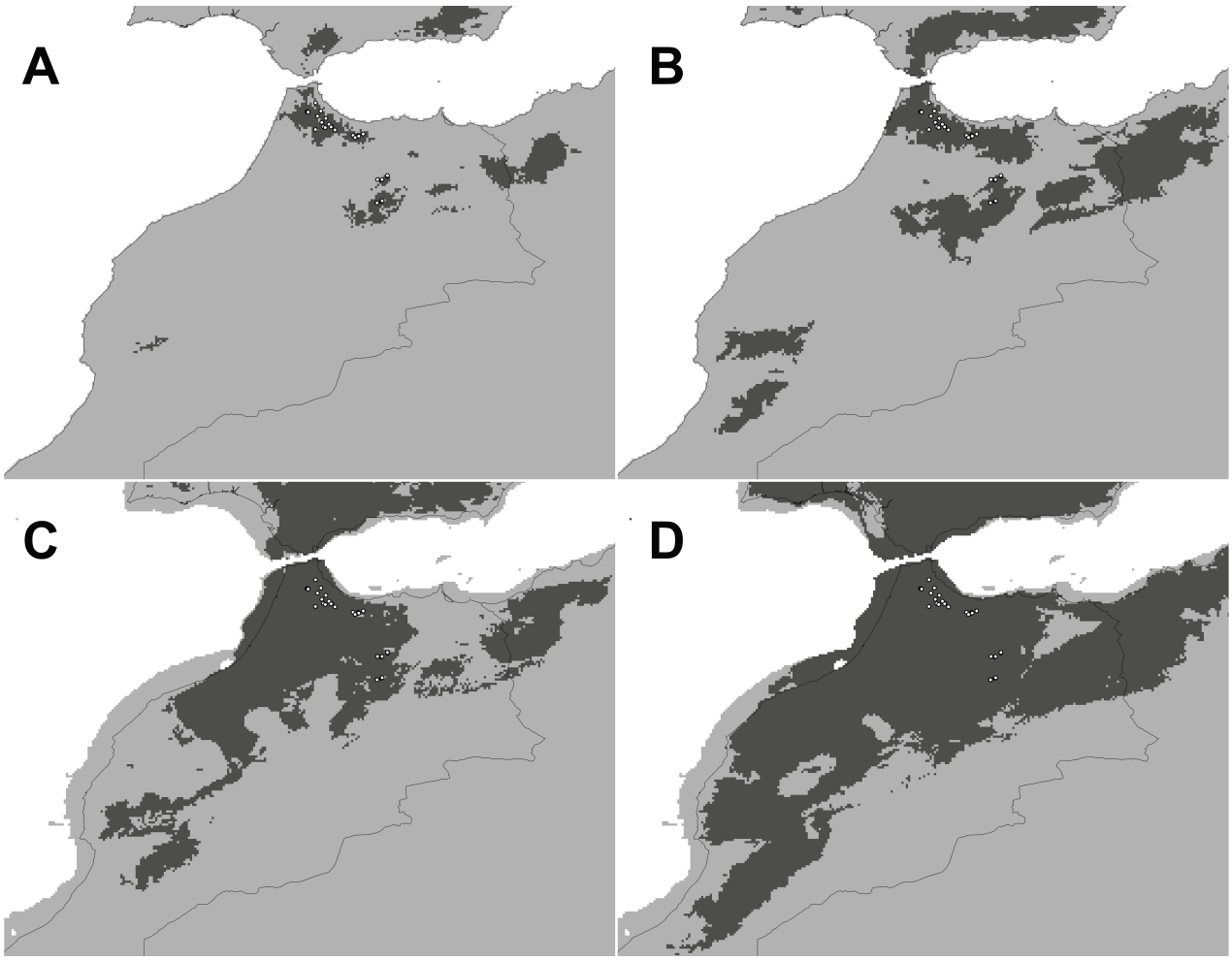


**Fig. 3.** Potential distribution models of *Alytes maurus* for the present (A and B) and Last Glacial Maximum (C and D) based on the TPT (A and C) and LPT (B and D) thresholds. The 26 records with precise locality data used for the ecological niche modelling (ENM) are also indicated (white dots).

the distribution ranges of species significantly (e.g., Bons & Geniez, 1996; Barata et al., 2011). The possible presence of the species in other well communicated and hence explored parts of the Middle Atlas region (e.g., Azrou and Ifrane) therefore seems unrealistic, although the species might occur in remote areas more southwards of Bou Iblane. *Alytes maurus* is typically associated with montane karst and escarpment areas in forests or shrubby vegetated areas at an altitude ranging from 200–2050 m a.s.l. The distribution of these environmental conditions might well limit the distribution of the species as seen in *Salamandra algira* (Beukema et al., 2010), which presents a similar distribution pattern. The populations of *S. algira* from the Rif and Middle Atlas Mountains have, however, diverged approximately 0.7 mya and also show phenotypic changes likely as a result of different ecological conditions (Beukema et al., 2010).

The LGM model of *A. maurus* reveals a much wider potential distribution that continuously extends to the south and southwest in Morocco and under the LPT into northeast Algeria. The species has never been recorded outside the Rif Mountains and the Middle Atlas region, although it might have had a much larger distribution in the past. Hossini (2001) described the fossil of a member of the genus *Baleaphryne* from the Lower Pleistocene that was found in a quarry at Jebel Irhoud between Marrakech and Safi. This fossil is likely attributable to *A. maurus* but might as well be an extinct species of *Alytes*, although this seems unlikely. The presence of a *Baleaphryne* sp. in southern Morocco during the Lower Pleistocene coincides more or less with the beginning of a period of documented cyclic fluctuations in vegetation and climate in north-western Africa, which occurred between 3.7 and 1.7 mya (Leroy & Dupont, 1994). During this time the vegetation fluctuated between humid phases that were characterized by tropical forests, and drier phases, characterized by grasslands (Thompson & Fleming, 1996). The shift towards a colder and drier climate that occurred during the upper Pliocene (2.5–1.8 mya; Webb & Bartlein, 1992) may have resulted in the extinction of the species in this region. There are currently no known fossils from the late Pleistocene or Holocene known from Morocco. The continued presence of *A. maurus* in a wider area therefore remains speculative even though optimal climatic conditions oscillated but remained present until at least the start of the cold and dry Younger Dryas (e.g., Lubell, 2001).

At present, *A. maurus* remains a relatively elusive and understudied species, despite the realistic threats that occur in the form of habitat fragmentation and destruction, pollution, climatic change and the globally spreading chytrid fungus. Furthermore, de Pous et al.

(2011b) found that only 6.96% of the total distribution range is covered by the existing protected area network. The use of additional genetic markers such as nuclear loci (Pinho et al., 2010) or microsatellites (Agata et al., 2011) to study the effects of population fragmentation on this species would therefore be advisable. Furthermore, the application of forecast ENM to assess the effects of climate change on the distribution range and wide-range sampling to detect the presence of *B. dendrobatidis* would be important steps to ensure the species' survival over time.

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# Chapter 2

## New insights on phylogeography and distribution of Painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula

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Painted frogs (*Discoglossus*) contain five to six species of Western Palearctic anurans that are mainly distributed in allopatry. We here provide the first comprehensive assessment of the phylogeography of the Moroccan species *D. scovazzi* and geographically characterize its contact zone with *D. pictus* in Eastern Morocco. *Discoglossus scovazzi* shows, in general, a weak phylogeographic structure across Morocco on the basis of mitochondrial DNA sequences of the cytochrome b gene, with only populations centered in the Atlas Mountains characterized by the presence of slightly divergent haplotypes. In eastern Morocco, all populations east of the Moulouya River were clearly assignable to *D. pictus*. This species was also found along the Mediterranean coast west of the Moulouya, in the cities of Nador and Melilla, suggesting that not the river itself but the wide arid valley extending along much of the river (except close to the estuary) acts as a possible distributional barrier to these frogs. No sympatry of *D. scovazzi* with *D. pictus* was observed, and all specimens were concordantly assigned to either species by DNA sequences of cytochrome b and of the nuclear marker RAG1. Species distribution models of the two taxa show largely overlapping areas of suitable habitat, and the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur. Comparative data are also presented from the southern Iberian contact zone of *D. galganoi galganoi* and *D. g. jeanneae*. These taxa showed less clear-cut distributional borders, extensively shared RAG1 haplotypes, and had instances of sympatric occurrence on the basis of cytochrome b haplotypes, in agreement with the hypothesis of a yet incomplete speciation. In this wide

contact zone area we found mitochondrial sequences containing double peaks in electropherograms, suggesting nuclear pseudogenes or (less likely) heteroplasmy, possibly related to the ongoing admixture among the lineages.

**Author contribution**: Second authorship reflects that I was the second main contributor to the paper. I have helped develop the concept, contributed to the lab work, geographical sampling design and fieldwork. I have also conducted all the spatial modelling. I have written all spatial modelling related parts and contributed and corrected several earlier drafts.

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# New insights on phylogeography and distribution of painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula

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**Abstract.** Painted frogs (*Discoglossus*) contain five to six species of Western Palearctic anurans that are mainly distributed in allopatry. We here provide the first comprehensive assessment of the phylogeography of the Moroccan species *D. scovazzi* and geographically characterize its contact zone with *D. pictus* in Eastern Morocco. *Discoglossus scovazzi* shows, in general, a weak phylogeographic structure across Morocco on the basis of mitochondrial DNA sequences of the cytochrome *b* gene, with only populations centered in the Atlas Mountains characterized by the presence of slightly divergent haplotypes. In eastern Morocco, all populations east of the Moulouya River were clearly assignable to *D. pictus*. This species was also found along the Mediterranean coast west of the Moulouya, in the cities of Nador and Melilla, suggesting that not the river itself but the wide arid valley extending along much of the river (except close to the estuary) acts as a possible distributional barrier to these frogs. No sympatry of *D. scovazzi* with *D. pictus* was observed, and all specimens were concordantly assigned to either species by DNA sequences of cytochrome *b* and of the nuclear marker RAG1. Species distribution models of the two taxa show largely overlapping areas of suitable habitat, and the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur. Comparative data are also presented from the southern Iberian contact zone of *D. galganoi galganoi* and *D. g. jeanneae*. These taxa showed less clear-cut distributional borders, extensively shared RAG1 haplotypes, and had instances of sympatric occurrence on the basis of cytochrome *b* haplotypes, in agreement with the hypothesis of a yet incomplete speciation. In this wide contact zone area we found mitochondrial sequences containing double peaks in electropherograms, suggesting nuclear pseudogenes or (less likely) heteroplasmy, possibly related to the ongoing admixture among the lineages.

*Keywords*: Alytidae, Amphibia, biogeography, heteroplasmy, Morocco, niche overlap, NUMTs, species distribution models.

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## Introduction

Painted frogs (*Discoglossus* Otth, 1837) are representatives of an ancient anuran clade, the Alytidae Fitzinger, 1843, with an exclusively Western Palearctic distribution. Among extant frogs, the sister taxon of *Discoglossus* is *Latonia nigriventer* (Mendelssohn and Steinitz, 1943) from Israel (Biton et al., 2013), and the *Discoglossus-Latonia* clade is sister to the midwife toads, *Alytes* Wagler, 1830 (Roelants and Bossuyt, 2005; Roelants et al., 2007; Biton et al., 2013). *Discoglossus* currently contains 5-6 extant species: *D. montalentii* Lanza, Nascetti, Capula and Bullini, 1984 from Corsica, *D. sardus* Tschudi, 1837 from Corsica, Sardinia, and some smaller Mediterranean islands; *D. pictus* Otth, 1837 from Sicily, Malta, Tunisia, Algeria, and eastern Morocco, introduced to southern France and eastern Spain (Catalonia Region); *D. scovazzi* Camerano, 1878 from Morocco; and *D. galganoi* Capula, Nascetti, Lanza, Bullini and Crespo, 1985 and *D. jeanneae* Busack, 1986 from Iberia. The taxon *jeanneae* is often considered a subspecies of *D. galganoi* (Zangari, Cimmaruta and Nascetti, 2006; Speybroeck, Beukema and Crochet, 2010; Pabijan et al., 2012; Vences and Grossenbacher, 2012). We herein follow this view which however might be challenged by future detailed analysis of gene flow among these taxa across their contact zone.

The phylogeny and phylogeography of *Discoglossus* has been subject of numerous studies (Lanza et al., 1984, 1986; García-Paris and Jockusch, 1999; Martínez-Solano, 2004; Real et al., 2005; Zangari, Cimmaruta and Nascetti, 2006; Velo-Antón et al., 2008; Pabijan et al., 2012; Biton et al., 2013). The available evidence strongly suggests *D. montalentii* being the sister taxon of all other species of *Discoglossus*, and a sister group relationship of *D. g. galganoi* and *D. g. jeanneae*. Most probably, *D. pictus* and *D. sardus* are sister to each other, and *D. scovazzi* is sister to the *galganoi-jeanneae* clade (Pabijan et al., 2012).

Besides the broadly sympatric *D. montalentii* and *D. sardus* in Corsica, the distribution areas of distinct *Discoglossus* taxa abut in two regions: on one hand, the ranges of *D. g. galganoi* and *D. g. jeanneae* contact each other across central Spain (García-Paris and Jockusch, 1999; Martínez-Solano, 2004; Real et al., 2005; Velo-Antón et al., 2008), and on the other hand, those of *D. pictus* and *D. scovazzi* abut in eastern Morocco, roughly in the area of the Moulouya River (Zangari, Cimmaruta and Nascetti, 2006). The geographical distribution of the two taxa in this region is at present poorly understood, because *D. scovazzi* has long been considered to be a subspecies of *D. pictus* (e.g., Lanza et al., 1986). Therefore natural history and distributional information of North African *Discoglossus* populations have usually been recorded under a single species name, *D. pictus* (e.g., Salvador, 1996), and the species identity of most records in eastern Morocco is so far uncertain (Beukema et al., 2013; Reques et al., 2013). Although a number of karyological (Odierna et al., 1999; Amor et al., 2007) and molecular data (Lanza et al., 1986; Zangari, Cimmaruta and Nascetti, 2006) have become available from North African *Discoglossus*, their phylogeography is so far understudied, especially compared with the Iberian taxa.

Here, we present the first phylogeographic analysis of *D. scovazzi* across its entire range, focusing on eastern Morocco where its range contacts that of *D. pictus*, and provide new data on the distribution and phylogeography of *D. g. galganoi* and *D. g. jeanneae* in southern Iberia. Our results are based on a large number of newly determined sequences of one mitochondrial and one nuclear gene from these four taxa, and on modelling environmental niches of *D. pictus* and *D. scovazzi* based on a newly compiled locality database for these species.

## Materials and methods

### *Field work and sampling*

Sampling was carried out from 2010-2013 in southern Spain and in Morocco, targeting specifically the putative contact zones of *galganoi-jeanneae* and *pictus-scovazzi* following

data of Zangari, Cimmaruta and Nascetti (2006). Additional tissue samples or extracted DNA were available from different collections, or were collected oportunistically by collaborators in the framework of other projects. Tissue samples included femur muscle of roadkills or preserved voucher specimens (in particular from Morocco), toe clips, and tadpole fin clips. See online Supplementary Material: Table S1 for a complete list of localities and geographical coordinates.

*Molecular methods*

Samples were extracted using a standard salt extraction protocol (Bruford et al., 1992). Fragments of one mitochondrial and one nuclear gene were amplified using newly developed specific primers: a fragment of the mitochondrial cytochrome *b* (COB) gene was amplified with the primers CytbA-Disco (CCCTGAGGACAGATATCRTTTTGAGG) and CytbC-Disco (CTACTGGTTGRCCCCCGATCCAGG T) with a PCR protocol consisting of an initial step of 90 seconds at 94°C, followed by 35 steps of 94°C (30 s), 53°C (45 s), 72°C (90 s) and a final elongation step of 10 min at 72°C. A fragment of the nuclear recombination-activating gene I (RAG1) was amplified using Disco-Rag1-F1 (ATCCAGTGGAAGCAATTTCG) and Disco-Rag1-R1 (CTCAGTGTGGCACCTGGTTA) with 120 seconds at 94°C, followed by 40 steps of 94°C (20 s), 58°C (50 s), 72°C (180 s) and a final elongation step of 10 min at 72°C. For a subset of individuals, an additional segment of COB (extending in the 5′ direction and partly overlapping with the first fragment) was amplified using Cytb-DiscoF (ATTGTTAATAACTCATTTATTG) and Cytb-DiscoR (ACTTTCTCTAAGTTTGAGT). In these cases sequences submitted to Genbank were contigs of the two segments. Because the second segment was only available for few specimens it was not included in the further analyses. From a few samples and to verify the presence of double peaks in electropherograms of mtDNA sequences (see below) we also amplified a segment of the mitochondrial Cytochrome Oxidase Subunit I (COX1) gene using primers COI-VertF1 and COI-VertF2; primer sequences in Vences et al. (2012).

PCR products were treated with Exonuclease I (New England Biolabs) and Shrimp Alkaline Phosphatase (Promega) to inactivate remaining primers and dNTPs, and then were cycle-sequenced using dye-labeled terminators (Applied Biosystems) with the amplification primers. All RAG1 amplicons were sequenced in both directions while the majority of COB amplicons were sequenced with the forward primer only. A selection of COB amplicons characterized by double peaks (see below) were ligated into the pCR Blunt vector included in the Zero Blunt PCR Cloning Kit (Invitrogen) and transformed into competent *E. coli* cells, following manufacturer's protols. After plasmid isolation via alkaline lysis, plasmids were sequenced using M13 primers. For this analysis we chose three samples with particularly clear double-peak signal in the COB sequences obtained through direct sequencing of PCR products.

Sequences were resolved on an ABI 3130XL automated DNA sequencer (Applied Biosystems). Chromatographs were checked and sequences were edited and assembled using Codon-Code Aligner (v2.0.6, Codon Code Corporation). All newly determined sequences were submitted to GenBank (accession numbers KF644587-KF645288).

*DNA sequence analysis*

Sequences were aligned using the Clustal algorithm in MEGA, vs. 5 (Tamura et al., 2011). RAG1 haplotypes of nuclear gene sequences were inferred using the PHASE algorithm (Stephens, Smith and Donnelly, 2001), implemented in DnaSP v5 (Librado and Rozas, 2009), and the same program was used to calculate values of genetic diversity per population. In some cases, haplotype reconstruction was not unambiguously possible; we nevertheless decided to include these haplotypes (the pair with the highest score for each individual) in the network analysis because a wrongly inferred haplotype would only slightly alter the number of haplotypes and their placement in the haplotype network, and thus would probably not influence our analyses (which relies mainly on identifying major haplogroups and their geographical distribution) in a relevant way.

During our analysis of COB sequences, we noted distinct double peaks in the electropherograms, i.e., overlapping peaks of about half the height of normal peaks. In some cases these were less distinct so that the total number of "heterozygous" individuals could not be determined with full certainty, although it certainly exceeded 50 individuals and mainly affected those from the South of the Iberian Peninsula. This phenomenon suggested occurrence of nuclear copies of mitochondrial DNA (NUMTs) and/or heteroplasmy. Clarifying this issue with full reliability was outside the scope of the present study and would require intensive additional molecular work. We here only performed a few experiments to obtain preliminary information on the possible causes of the observed double peaks. For three of the "heterozygous" specimens with particularly obvious double peaks, we sequenced the different sequences from the heterogeneous amplicons via cloning (see above), but for the others, in order to include these sequences in the haplotype network reconstruction, we used the PHASE algorithm to separate them. We are aware that this procedure strictly speaking is invalid, as more than two variants might be present per sample. This restriction in particular applies to 61 *D. galganoi* sequences for which we scored more than 2 heterozygote positions. Yet, we consider this approach to be more accurate than simply using the raw sequences for reconstruction of a phylogenetic tree or haplotype network, by either using IUPAC ambiguity codes (tree only) or excluding the heterozygous sites (tree or network).

Previous studies (e.g., Fromhage, Vences and Veith, 2004; Zangari, Cimmaruta and Nascetti, 2006) have shown that phylogenetic analyses based on one or two short gene fragments are of insufficient resolving power to reliably infer interspecific relationships among *Discoglossus* species. Concatenated and species tree analyses of multigene DNA sequence data sets have provided concordant reconstructions of the evolutionary history of this genus (Pabijan et al., 2012; Biton et al., 2013), and we therefore refrained from phylogenetic analyses of our COB and RAG1 data set and

instead opted for visualizing patterns of differentiation in the form of haplotype networks.

Haplotype network reconstructions for COB and RAG1 were performed under statistical parsimony (Templeton, Crandall and Sing, 1992) using the software TCS v1.21 (Clement, Posada and Crandall, 2000). To analyse the relationship between genetic (mean number of pairwise nucleotide differences) and geographical distance (km) between populations (often referred to as isolation-by-distance), we performed a Mantel's tests (Mantel, 1967) using ARLEQUIN v3.11 (Excoffier, Laval and Schneider, 2005). The geographical distances between localities were calculated from GPS coordinates in the software GENALEX v6 (Peakall and Smouse, 2006). Data were permuted 1000 times to estimate the 95% upper tail probability of the matrix correlation coefficients.

*Species distribution modelling*

A total of 456 distribution records of North African *Discoglossus* were assembled from literature, museum collections and own fieldwork (see online Supplementary Table S2). The distribution records went through a process of filtering that removed duplicate records within unique grid cells in ENMtools 1.3 (Warren, Glor and Turelli, 2010) and also reduced sampling bias by using a kernel density grid as implemented in the Java program OccurrenceThinner v1.0.4 (Verbruggen et al., 2013). After filtering, the final dataset used for modelling consisted of 218 distribution records (*D. scovazzi*: $n = 119$, *D. pictus*: $n = 99$; see online Supplementary Tables S3-S4).

All available bioclimatic variables were downloaded from the WorldClim database version 1.4 (http://www.worldclim.org; Hijmans et al., 2005) at a resolution of 2.5 arc minutes (nearly $5 \times 5$ km). Past climate data for the Last Glacial Maximum (LGM; ca. ~21 000 years BP) was obtained from the WorldClim database at the same resolution. Two general atmospheric circulation models (GCM) were used to generate the LGM scenarios: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The two GCMs were averaged using ArcGIS 10 (ESRI). Collinearity of the initial variables was measured by Pearson's correlation coefficient in ENMtools v1.3 (Warren, Glor and Turelli, 2010). A total of seven variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient) were retained. The final set of environmental predictor variables used for the species distribution models (SDM) consisted of: Annual Temperature (BIO1), Temperature Seasonality (BIO4), Maximum Temperature of Warmest Month (BIO5), Mean Temperature of Driest Quarter (BIO9), Annual Precipitation (BIO12), Precipitation Seasonality (BIO15) and Precipitation of Warmest Quarter (BIO18).

The SDMs were generated by the presence/background algorithm implemented in Maxent, version 3.3.3k (Phillips, Anderson and Shapire, 2006). Maxent was used with default settings (convergence threshold = 0.00001, maximum number of iterations = 500 and $\beta$j = 1), partitioning the geographical records between training and test samples (default settings). We defined a background for each species by drawing a 200 km buffer around all distribution records and subsequently projected the models onto a larger area.

The average of ten pseudo-replicated models with randomly selected test samples was used to produce SDMs, which were plotted in logistic format. The final models were reclassified in ArcGIS 10 (ESRI) into binary presence-absence maps based on the assumption that ten percent of the records were either wrongly identified or georeferenced, meaning, that the 10% of model outputs with the lowest predicted probabilities fall into the 'absence' region of the threshold model, and 'presence' regions include the 90% of distribution records with the highest model values (Raes et al., 2009). All models were tested with receiver operating characteristics (ROC) curve plots, and the area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model.

Additionally, we used null-models to test for significance of the SDMs. We generated 100 null distributions of random points in the study area using ENMtools (Warren, Glor and Turelli, 2010) for each of the two species, with the number of random points equal to the actual number of distribution records used for SDM. The null-models were created and assessed following Raes and ter Steege (2007).

*Quantifying niche overlap*

In order to quantify the degree of ecological differentiation between *D. pictus* and *D. scovazzi*, we employed a multivariate analysis framework proposed by Broennimann et al. (2012) implemented in R (R Development Core Team, 2008), using the same climate variables, distribution records and background as for SDM. Following this framework we computed multivariate environmental niche overlaps between *D. pictus* and *D. scovazzi* employing the two best performing ordination techniques (Broennimann et al., 2012): (1) Principal Component Analysis (PCA) calibrated on the entire environmental space of the study area (termed PCA-env; Broennimann et al., 2012), and (2) Ecological Niche Factor Analysis (ENFA) (Hirzel et al., 2002). The framework by Broennimann et al. (2012) implements a modified niche similarity and niche equivalency tests *sensu* Warren, Glor and Turelli (2008) and calculates niche overlap for pairs of species using Schoener's *D* (Schoener, 1970).

## Results

### *Genetic analyses*

We determined new COB sequences from 395 samples of the four target taxa and RAG1 sequences from 374 samples. All of these 769 sequences (not counting those obtained by cloning) were used to identify lineages and determine distribution ranges, but for further analysis only subsets of sequences were used. Numerous sequences were exceedingly short or

had longer sections with missing data and were therefore excluded from the set submitted to Genbank (a total of 702 sequences submitted: 359 COB and 343 RAG1). For final analyses, all sequences were cut to remove sections with missing data at the end or beginning. Because for haplotype phasing, alignments must not contain such missing data, we additionally excluded a few sequences so that the final alignments used for analysis contained 357 sequences of 430 bp for COB, and 342 sequences of 361 bp for RAG1. Geographical distribution of samples and their assignment to species according to the molecular data is shown in fig. 1. The haplotype networks for the different taxa are shown in figs 2-4, and numbers of sequences per locality are listed in Supplementary Table S1.

Both COB and RAG1 congruently separated haplotypes of *D. scovazzi* (fig. 2) and *D. pictus* (fig. 3) into two networks. In no case did a population share haplotypes belonging to the two species, no individual had a COB haplotype of one and RAG1 haplotypes of the other species, and no heterozygotes for RAG1 haplotypes of the two species were found. *Discoglossus g. galganoi* and *D. g. jeanneae* were separated by COB into two unconnected networks, whereas the RAG1 sequences of both taxa were

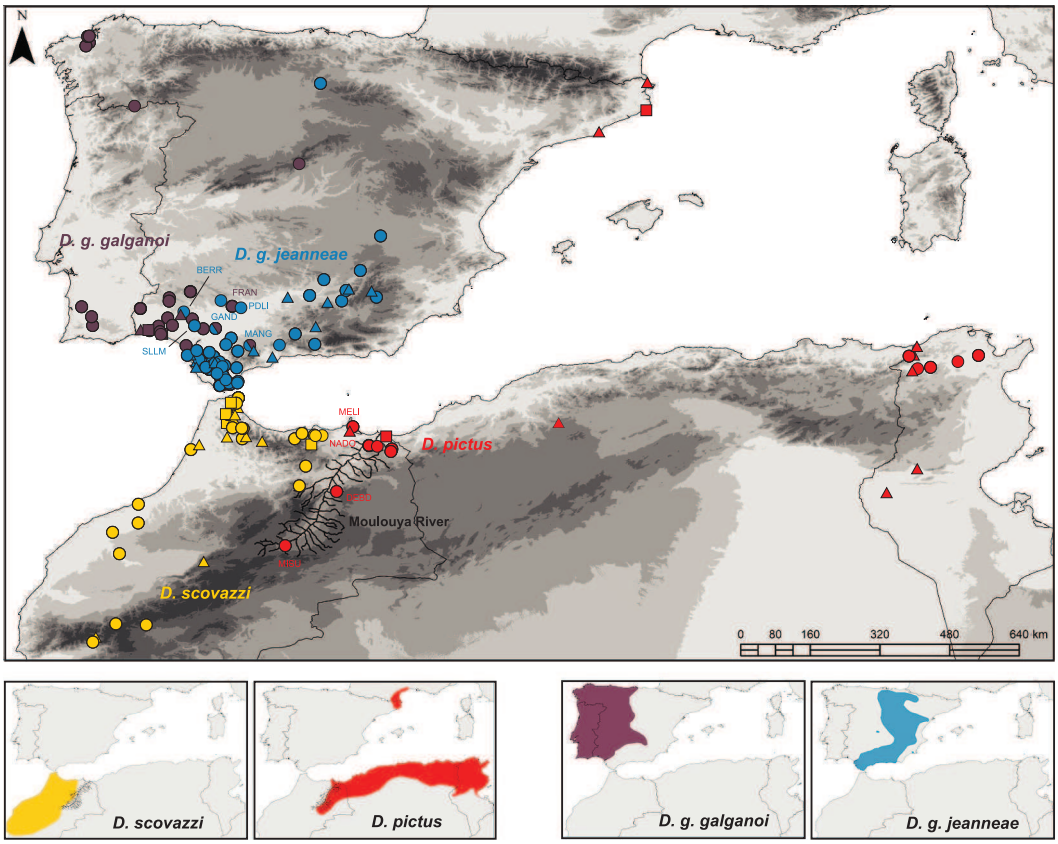


**Figure 1.** Distribution of sampling sites of *Discoglossus* taxa included in this study, with taxon assignment based on COB and RAG1 sequences. Circles: COB and RAG1 sequences available; triangles, only RAG1; squares, only COB. *D. g. galganoi* and *D. g. jeanneae* could not be distinguished based on RAG1 alone. Therefore, the taxon assignment of individuals from Iberia, for which only RAG1 sequences were available, was based only on geography. Two bicolored circles in Iberia indicate locations were mtDNA haplotypes of both taxa was observed in syntopy. Localities discussed in the text are marked with their four-letter codes (as explained in the text and/or in Table S1). The lower row of inset maps shows the approximate distribution of the four taxa according to the IUCN Red List (www.redlist.org, accessed April 2014); note that the distribution of *D. pictus* extends further into Malta, Gozo, and Sicily (not shown in inset map). This figure is published in colour in the online version.

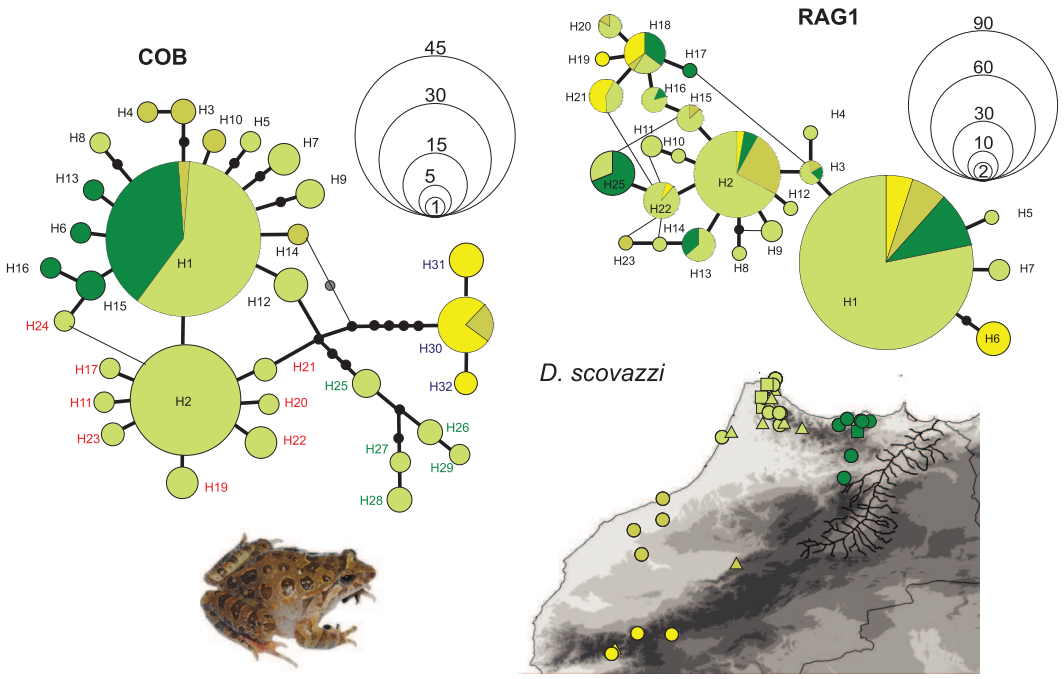


**Figure 2.** Haplotype networks for *Discoglossus scovazzi*, based on COB and RAG1 sequences. Colors represent ad-hoc definitions of major geographic areas, to visualize the distribution of the haplotypes. This figure is published in colour in the online version.

arranged in a single network with only five haplotypes, three of which were shared (fig. 4).

The isolation-by-distance analysis, excluding *D. pictus* for which our sampling is too patchy for reliable interpretation, revealed a significant correlation for *D. scovazzi*, and *D. g. galganoi*, but not for *D. g. jeanneae*, in COB, and for none of these lineages in RAG1 (details in Supplementary Material).

A closer look at the *D. scovazzi* COB network (fig. 2) suggests the presence of a somewhat distinct haplotype cluster in the High Atlas populations (colored in yellow in the network), differing by a minimum of seven mutational steps from other haplotypes. Of the three haplotypes we determined in the High Atlas, the most common one (H30), however, was also found in the lowlands between Marrakech and Casablanca. *Discoglossus pictus* showed a clear structure between populations from Tunisia, the population from Catalonia (Spain) (PALA), and the Moroccan populations (fig. 3), and this is in agreement with previous data indicating at least three distinct mitochondrial lineages within this species (Zangari, Cimmaruta and Nascetti, 2006).

Our intensive sampling in eastern Morocco roughly confirmed the basin of the Moulouya River constituting the barrier between *D. pictus* (east) and *D. scovazzi* (west). In the coastal areas, all populations sampled east of the Moulouya had *D. pictus* haplotypes only, and we also only found this species in the isolated Debdou Massif (locality DEBD) and in the upper Moulouya at Saïda, south of Missour (locality MISU). At the coast, however, we found haplotypes of *D. pictus* also in Melilla (locality MELI; several COB and RAG1 sequences) and probably in Nador (NADO; only a short poor-quality RAG1 sequence available, not included in Table S1), both located west of the Moulouya River (see fig. 1). About 30 km west from these localities, in the hills and mountains between Midar and Al Hoceima, all populations sampled were identified as *D. scovazzi*, while no *Discoglossus* were found in between Nador and Midar.

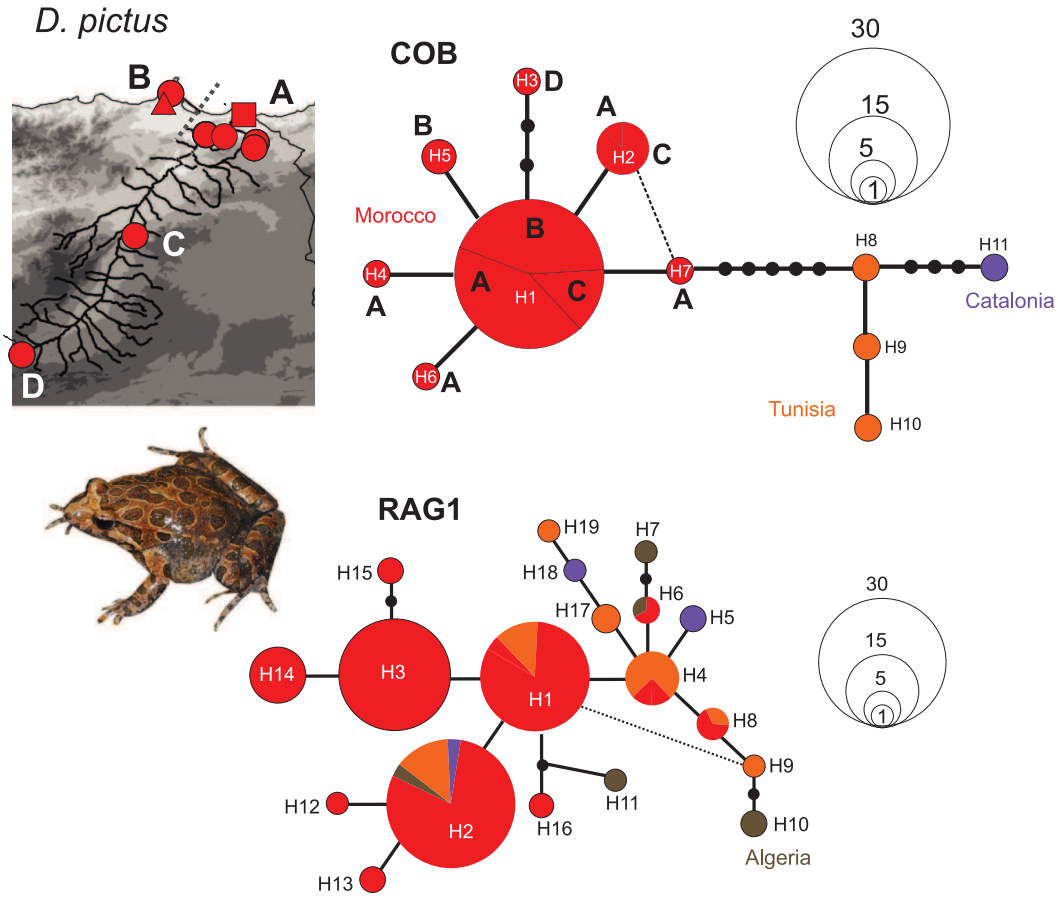


**Figure 3.** Haplotype network for *Discoglossus pictus* based on COB and RAG1 sequences. Colors represent ad-hoc definitions of major geographic areas, to visualize the distribution of haplotypes; i.e., in Morocco (red) Tunisia (orange) and Catalonia region, Spain (purple), of which only the Moroccan sites are shown on the map. Letters A-D on the map correspond to the letters in the COB network above, indicating that populations east (A, C, D) and west (B) of the Moulouya River share the same main COB haplotypes H1. This figure is published in colour in the online version.

In southern Spain, we found COB haplotypes of both *D. g. galganoi* and *D. g. jeanneae* in two populations: at Gandul near Alcala de Guadaira (locality GAND), close to an area where syntopy of the two lineages had been observed before (Zangari, Cimmaruta and Nascetti, 2006), and about 26 km north of Ronda (locality MANG). Furthermore, in several areas, single samples assigned to either of the two lineages occurred in close proximity and apparently without clear-cut range borders between them, for instance in the area between Constantina and Palma del Rio (locality PDLI assigned to *jeanneae*, FRAN to *galganoi*) or at Berrocal (BERR) and Sanlucar la Mayor (SLLM), where *jeanneae* haplotypes occurred largely surrounded by localities with *galganoi* haplotypes (see Supplementary Table S1 for locality codes and geographical coordinates; not indicated in map due to the very dense sampling in this area).

To obtain a first understanding of the pattern and underlying causes for the observed double peaks in the mitochondrial COB sequences, we first amplified an additional mitochondrial gene (COX1) from a series of specimens and found that double peaks were present also in those sequences. Furthermore, we carefully re-extracted DNA from a series of freshly collected samples to exclude sample contamination. Re-

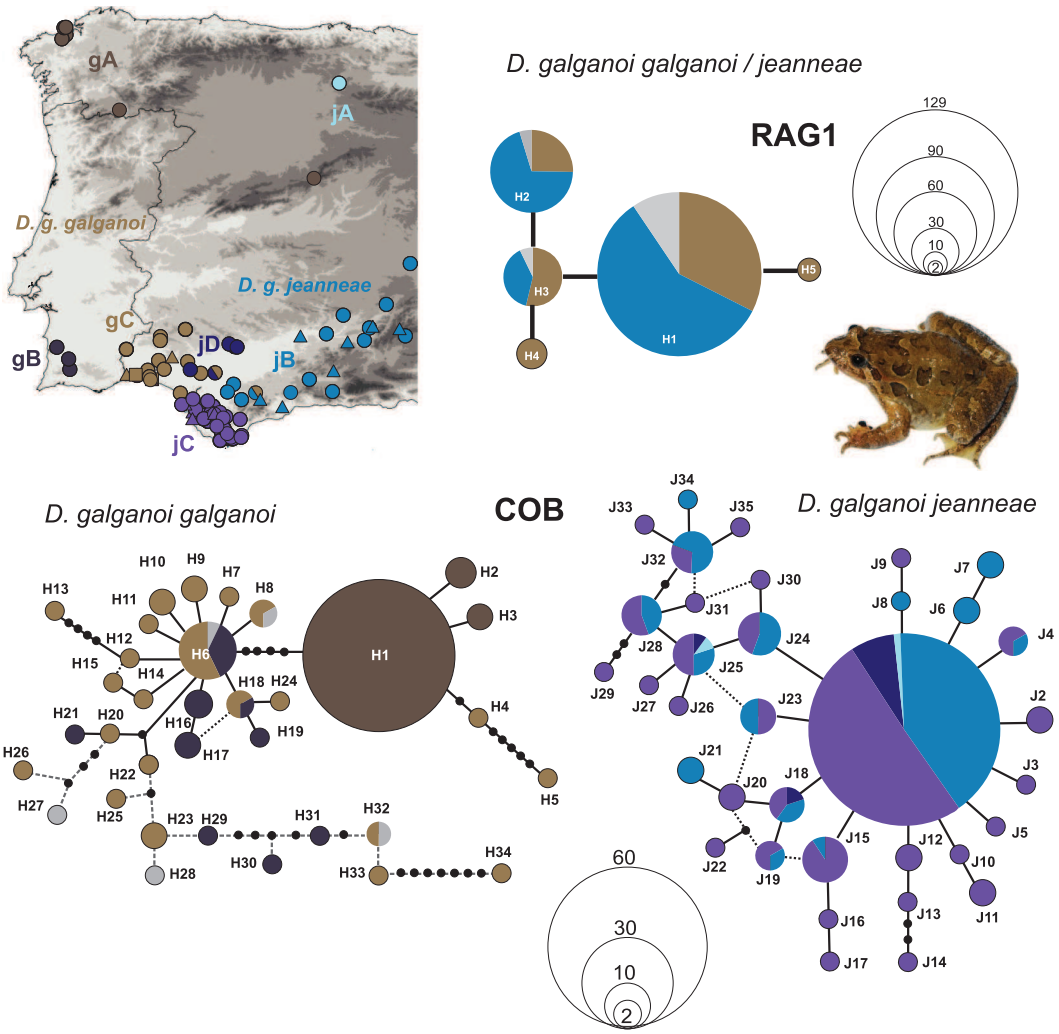


**Figure 4.** Haplotype network for *Discoglossus g. galganoi* and *D. g. jeanneae*, based on RAG1 (above) and COB sequences (below). Letters gA-gC and jA-jD denote populations from ad hoc defined geographical regions which are color coded in the map and in the COB networks, to indicate the geographical distribution of the haplotypes. In the RAG1 network, colors (brown vs. blue) denote the proportion of *D. g. galganoi* vs. *D. g. jeanneae* individuals sharing a particular haplotype; grey denotes individuals for which no assignment to either of the main mtDNA lineages is possible due to lack of COB sequences. Note that sampling of these taxa is concentrated on the southern parts of the species' ranges. This figure is published in colour in the online version.

sequencing from these new extractions yielded the identical sequences with double peaks. After using the Phase algorithm to separate the obtained "haplotypes", these always clustered with the same lineage (either both with *galganoi*, or both with *jeanneae*). When we examined a large number of samples of *D. g. galganoi* from localities in Galicia (north-western Spain), far from the contact zone with *D. g. jeanneae*, we could not find double peaks in any of the COB sequences from this region.

We then re-amplified COB from three specimens from southern localities characterized by a particular intensity of such double peaks. The PCR products were firstly sequenced directly and secondly also cloned into plasmids. Four to five isolated plasmids per sample were sequenced. In two cases (MV2894, a speci-

men without reliable locality information included because of its particularly obvious double peaks, but not further considered for the phylogeographic analysis, and DG_GAND02 from Gandul) we found clones clustering with both *jeanneae* and *galganoi*. For DG_GAND02, three of the four clones clustered with *galganoi* and one with *jeanneae*. This individual initially had been placed in *galganoi*, when the taxon assignment was based on the sequence obtained by direct sequencing of the PCR product and this sequence contained many double peaks. For the third individual, DG_SDMO01, all four sequences derived from clones, as well as the regularly obtained one, clustered with *jeanneae*. All COB sequences obtained from clones translated into amino acids without gaps or stop codons.

### *Species distribution modelling and niche overlap*

Maxent produced SDMs of moderate predictive accuracy (following Swets, 1988), according to the average test AUC for the present and past models (*D. pictus*: average AUC = 0.770 ± 0.057; *D. scovazzi*: average AUC = 0.801 ± 0.050). All SDMs (fig. 5) performed statistically significantly better than random. The main predictor variables differed between *D. pictus* (BIO4 = 54.1%, BIO12 = 14.9%) and *D. sco-*

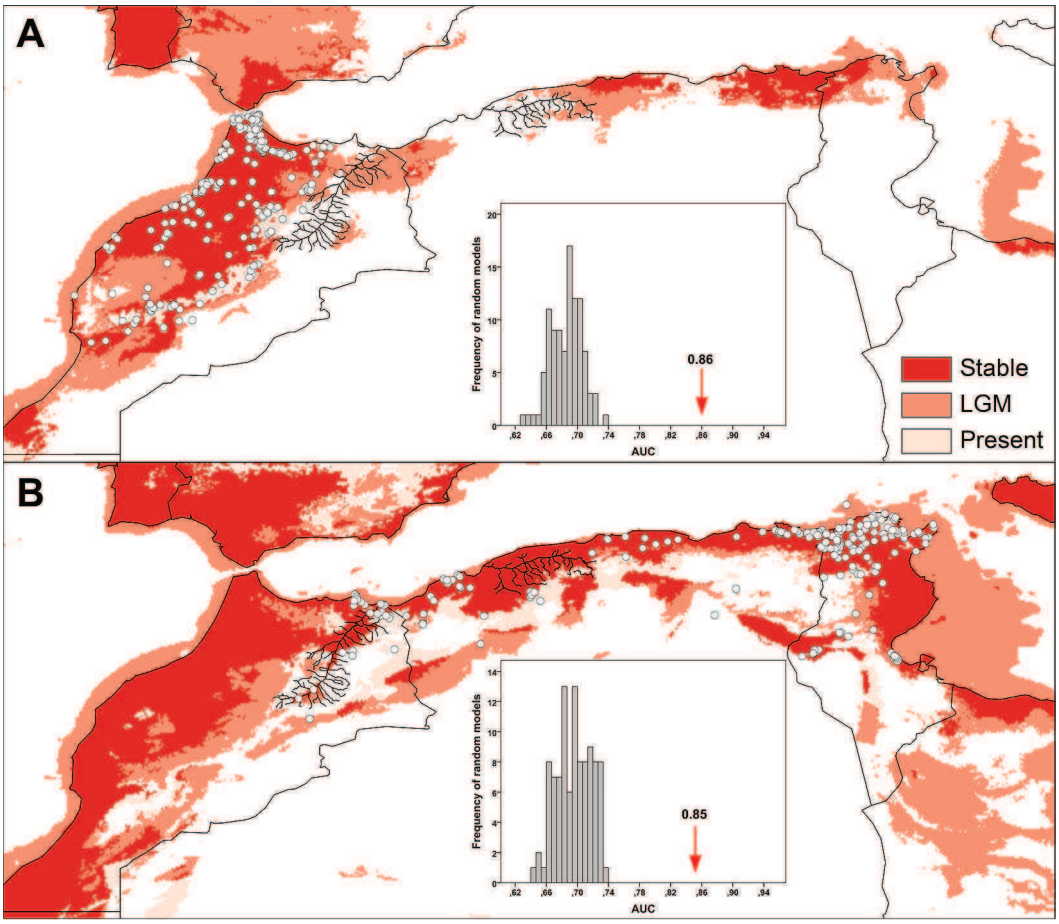


**Figure 5.** Potential species distribution models of *Discoglossus scovazzi* (A) and *D. pictus* (B) for the present and Last Glacial Maximum based on the TPT threshold. Results of null-models to test for significance of the SDMs and the available distribution data for both species are indicated (white dots). This figure is published in colour in the online version.

*vazzi* (BIO12 = 63.4%, BIO15 = 14.6%). The SDMs reveal climate stability in large parts of the species' distributions. The present and past SDMs for both *D. pictus* and *D. scovazzi* reveal large suitable areas in northern Africa extending well beyond the current distribution ranges of the species (fig. 5). The present SDMs for *D. scovazzi* indicate unsuitability of the Mouloya River Basin as well as in the Jbilets region north of Marrakech, whereas the present and past SDMs of *D. pictus* indicate a continued suitability in the Moulouya River Basin extending into most areas in Morocco, but excluding parts of the High Atlas.

The environmental space occupied by *D. pictus* and *D. scovazzi* as determined by PCA-env and ENFA is shown in Supplementary figs S10 and S11, respectively. Niche overlap between the species ($D = 0.452$) is limited. The niche equivalency hypothesis was rejected ($p = 0.02$), revealing significant differences between the two species' niches. The result of the randomization test of background similarity for PCA-env, however, shows that the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur (PCA-env, $p = 0.02$ and ENFA in a single direction, $p = 0.02$). These results are consistent with the large overlap predicted by the SDMs.

## Discussion

We provide a first comprehensive assessment of the phylogeographic structure in *D. scovazzi*, given that the sole previous study (Zangari, Cimmaruta and Nascetti, 2006) included mtDNA sequences of only six individuals from two sites, and allozyme data of 28 individuals from four sites. The data suggest an overall weak phylogeographic structure of the species; only the populations from high elevations in the Atlas Mountains appear to be characterized by slightly divergent haplotypes, suggesting these populations originated from an ancestral population that diverged in a refugial area or in a sanctuary located in southern Morocco, possibly in the Atlas.

A pattern of generally low phylogeographic structure is also found in several other Moroccan amphibians, such as *Alytes maurus* Pasteur and Bons, 1962, *Amietophrynus mauritanicus* (Schlegel, 1841), *Bufotes boulengeri* (Lataste, 1879), and *Pelophylax saharicus* (Boulenger, 1913), all of which lack a clear phylogeographic structure in Morocco (Harris, Batista and Carretero, 2003; Batista et al., 2006; Stöck et al., 2008a; Harris and Perera, 2009; de Pous et al., 2013), whereas the treefrog *Hyla meridionalis* Boettger, 1874 is subdivided into three deep geographically structured mtDNA lineages in this country (Recuero et al., 2007; Stöck et al., 2008b).

While our data confirm the Moulouya River Basin largely coinciding with the range boundaries of *D. pictus* and *D. scovazzi*, the dense sampling herein also provided some surprising results. The area of Melilla and Nador appear to be populated by pure *D. pictus*, as we did not find any mitochondrial or nuclear alleles of *D. scovazzi* in the 17 specimens analyzed from this area. Hence, the species inventory of the Spanish territory of Melilla should include *D. pictus* rather than *D. scovazzi*. We cannot exclude that the presence of *D. pictus* in this area might be due to introduction. If however, the species is indeed native to this area, it provides evidence for the capacity of these frogs to cross big rivers, such as the Moulouya, even in the proximity of the estuary where the river is widest. Our study provides necessary data so that future efforts to characterize the contact zone between these two taxa can be focused more narrowly. Namely the area between Nador and Midar should be sampled in more detail to understand whether the two species might occur sympatrically at some sites within this region.

Although in this coastal area suitable habitat for *Discoglossus* does exist, it needs to be taken into account that large parts of the Moulouya River Basin further south are very arid and prob-

ably not suitable for these frogs, while for numerous arid-adapted taxa this valley has served as a dispersal corridor to the north (Bons and Geniez, 1996). The barrier effect for *Discoglossus* might be caused by the aridity of the valley rather than by the river itself. Given that *D. scovazzi* and *D. pictus* are not sister species (Pabijan et al., 2012), it is unlikely that the Moulouya River Basin played a role as a barrier triggering their primary vicariant divergence; more likely, this basin acts as a secondary barrier.

The present and LGM-SDMs of *D. pictus* and *D. scovazzi* show a large potential distribution in northern Africa extending well beyond the current known distribution ranges. Interestingly, the models predict that only *D. pictus* occurs in the Moulouya River Basin and that *D. scovazzi* is limited to the mountainous and wetter areas to the west. Also, in general, the area currently occupied by *D. scovazzi* appears to be fully suitable for *D. pictus* (except for high areas in the Atlas) while suitable areas for *D. scovazzi* within the *D. pictus* range are patchy. The LGM models show that the Moulouya River Basin remained largely unsuitable for *D. scovazzi*, while the extent of suitability for *D. pictus* increased in comparison with the present. The potential distribution during the LGM is larger than the present for both species. This is in agreement with previous studies (de Pous et al., 2011, 2013) and likely results from wetter and cooler annual climatic conditions in North Africa (Rognon, 1987; Wengler and Vernet, 1992). In northern Africa, *D. pictus* and *D. scovazzi* inhabit largely similar habitats with the exception that the former occurs in much drier areas and the latter occurs at high altitudes (up to 2650 m a.s.l.) in the Atlas Mountains. These environmental conditions are absent from the historical ranges of the other species respectively and this has likely contributed to rejection of the niche equivalency hypothesis. Both the SDMs and the niche overlap tests indicate that the niches of *Discoglossus* in northern Africa are conserved. The SDMs indicate the potential existence of a contact zone in several regions west of the Moulouya River Basin and this requires additional fieldwork in these areas.

Combining data from distribution ranges (*D. pictus* extending west of the Moulouya River Basin) and SDMs (large parts of the Moulouya valley and of the *D. scovazzi* range suitable for *D. pictus*, but less so vice-versa) suggests a scenario in which *D. scovazzi* had a historical range largely restricted to Morocco, possibly limited by the Moulouya River Basin, while *D. pictus* expanded its range westwards and crossed the Moulouya River near its estuary, leading to the currently observed contact zone largely coinciding with the Moulouya River Basin.

While the distribution of species of *Discoglossus* is predominantly allopatric, in three cases the distribution areas of species or major lineages of the genus overlap or abut. The first case is in Corsica, where the two species *D. montalentii* and *D. sardus* are broadly sympatric (Lanza et al., 1984, 1986; Zangari, Cimmaruta and Nascetti, 2006). *Discoglossus montalentii* mainly inhabits montainuous areas and breeds inside puddles of fast-flowing streams, while *D. sardus* is distributed mainly in temporary waters in the lowlands. Yet, several sites of close syntopy exist (Lanza et al., 1984, 1986; Vences, Glaw and Hirschberger, 1996) and so far, no indication of hybridization has been published. In this case, the taxa concerned are phylogenetically distant (Lanza et al., 1984, 1986; Fromhage, Vences and Veith, 2004; Pabijan et al., 2012; Biton et al., 2013), and differentiated both morphologically (Lanza et al., 1984; Clarke and Lanza, 1990; Capula and Corti, 1993; Clarke, 2007) and bioacoustically (Glaw and Vences, 1991). The other two cases are the contact zones which herein are characterized geographically in some detail. The ranges of *D. pictus* and *D. scovazzi* in Morocco appear to be characterized by a sharp boundary, without broad overlap. Although we cannot exclude isolated sympatric occurrence of the two taxa, it seems clear that such instances will be exceptional. The concordance between mitochondrial and nuclear markers in

our analysis suggests that instances of ongoing gene flow between these taxa are probably rare if they even exist. These two species are morphologically and bioacoustically similar (Clarke and Lanza, 1990; Capula and Corti, 1993; Vences and Glaw, 1996; Clarke, 2007) but do not seem to be sister taxa (Pabijan et al., 2012), and show a moderate genetic differentiation. The third example is the contact zone between two main Iberian lineages, which are considered distinct species by some (e.g., Busack, 1986; García-París and Jockusch, 1999; Martinez-Solano, 2004; Real et al., 2005) and subspecies by others (Lanza et al., 1986; Vences and Glaw, 1996; Zangari, Cimmaruta and Nascetti, 2006; Speybroeck, Beukema and Crochet, 2010; Pabijan et al., 2012; Vences, 2012; Sillero et al., 2014). These two mitochondrial lineages clearly are sister to each other (Pabijan et al., 2012); they show no consistent divergence in the nuclear markers investigated so far, and only comparatively weak divergence in mtDNA (e.g., Zangari, Cimmaruta and Nascetti, 2006; Velo-Antón, Martínez-Solano and García-Paris, 2008; Pabijan et al., 2012; data herein). Their uncorrected pairwise divergence in the 16S rRNA gene (as sequenced for instance by Fromhage et al., 2004) is only about 2%, and thus below the minimum threshold of 3% that characterizes many well-differentiated species of amphibians from their closest relatives (e.g., Fouquet et al., 2007). Although they might show weak morphological differentiation (Capula and Corti, 1993), it is uncertain whether this variation is clinal. Bioacoustically, no obvious differences have been observed (Vences and Glaw, 1996). In this example, mtDNA suggests a somewhat broader contact zone with syntopic occurrence of the two lineages in at least some locations, and either incomplete lineage sorting or extensive gene flow in the nuclear genes taken into account to date.

In a nutshell, we here propose the hypothesis that these three examples might represent different stages of the speciation and differentiation process: *D. montalentii* and *D. sardus* are strongly divergent genetically, and also have diverged in morphology, ecology and bioacoustics, allowing them to occur in sympatry and sometimes in close syntopy, without apparent admixture. *Discoglossus pictus* and *D. scovazzi* are less divergent genetically and do not differ clearly in morphology, ecology and bioacoustics, yet the genetic divergence might be strong enough to avoid admixture across their contact zone in eastern Morocco. Finally, the two Iberian lineages show the weakest divergence from a mitochondrial perspective and extensive sharing of nuclear alleles. These findings agree with considering *jeanneae* as subspecies of *D. galganoi* following the rationale of Speybroeck, Beukema and Crochet (2010) at least until more detailed analysis of the contact zone using highly variable nuclear markers may indicate absence of gene flow among them.

This study mainly aimed at characterizing the geographical ranges of *Discoglossus* lineages in Iberia and Morocco, and providing a first asssessment of the phylogeographic differentiation of *D. scovazzi*. It is outside our scope to comprehensively analyze and understand the evolutionary and molecular dynamics in the contact zones of these frogs, and the limited molecular data provided herein can only help defining hypotheses that will require future testing. We propose that the double peaks observed in the chromatograms of a large number of individuals are related to the admixture of *jeanneae* and *galganoi* in their contact zone. Whether these additional copies are all nuclear pseudocopies (NUMTs) or are partly caused by heteroplasmy can only be inferred by more extensive molecular work. In two individuals, we could show that they indeed contain COB variants of both *jeanneae* and *galganoi*, which may have resulted from hybridization and admixture between these two taxa. However, by direct sequencing and phasing, the two sequences per individual always clustered with the same lineage (either both with *galganoi*, or both with *jeanneae*), suggesting that one of the copies present in the respective individuals

might occur more commonly or amplify preferentially. Whether all double-peak individuals bear a combination of *jeanneae* and *galganoi* sequences is therefore completely unclear; in some cases, double peaks are only at single or few positions which do not constitute diagnostic differences between the two lineages. Altogether this suggests that the use of mtDNA as marker for a clear-cut distinction of *jeanneae* and *galganoi* might not always yield reliable results. Future studies of this phenomenon could benefit from next-generation sequencing methods to identify all underlying sequences present in amplicons. Similar situations have been found in other taxa, including bristletails (Baldo et al., 2011) and lizards (Podnar et al., 2007; Miraldo et al., 2012). In all these cases, hybridization between species or lineages led to the presence of more than one mitochondrial genome in the individuals concerned. Usually, the variation was located in NUMTs, but low levels of heteroplasmy were also detected (Miraldo et al., 2012). It is a fascinating perspective for future studies to test whether selective advantages of particular mitochondrial variants, and/or genomic conflict might be responsible for these bizarre patterns, and whether such genomic conflict might aid in characterizing the divergence process, or absence thereof, between the lineages involved (see Crespi and Nosil, 2013). Our study suggests that the contact zone of *D. g. galganoi* and *D. g. jeanneae* might be a suitable model for such future studies.

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# New insights on phylogeography and distribution of painted frogs (*Discoglossus*) in northern Africa and the Iberian Peninsula

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## Supplementary Material in this file

**Supplementary Figures S1-S9**: Isolation-by-distance plots showing genetic vs. geographic distances.

**Supplementary Figures S10-S11**: Niches of *Discoglossus pictus* and *D. scovazzi* based on PCA-env. and ENFA.

**Supplementary Table S1.** Sampling locations, geographical coordinates, and numbers of sequences per location.

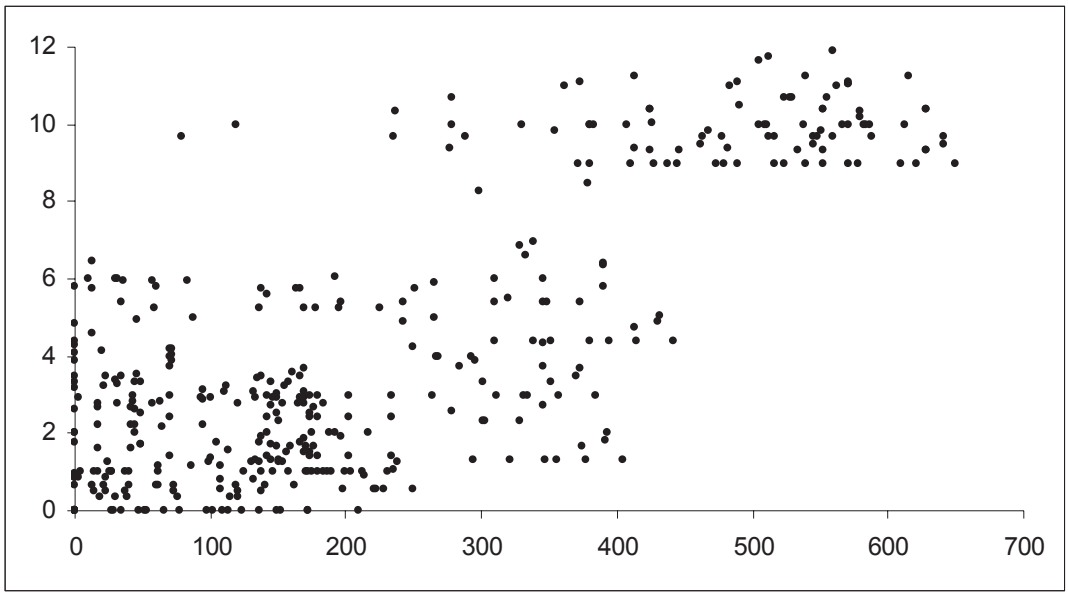
**Supplementary References** (as used in Table S2).

## Additional Supplementary Material in separate Text files

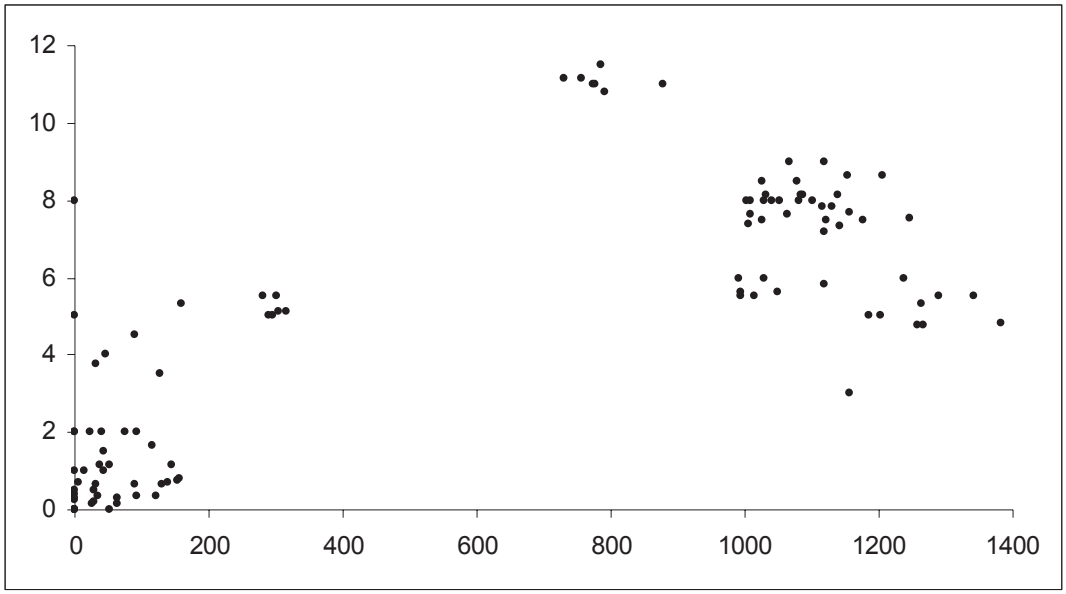
**Supplementary Table S2.** Distribution database with georeferenced locality records of *Discoglossus pictus* and *D. scovazzi.*

**Supplementary Table S3.** List of localities (geographical coordinates: subset of locations listed in Table S2) of *D. pictus* used for MaxEnt modelling.

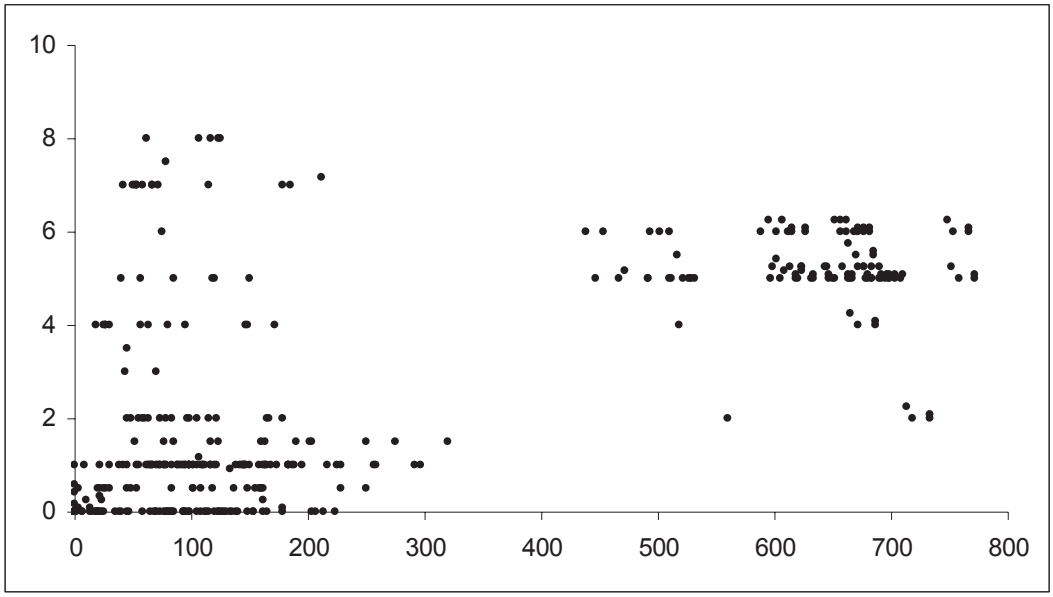
**Supplementary Table S4.** List of localities (geographical coordinates: subset of locations listed in Table S2) of *D. scovazzi* used for MaxEnt modelling.



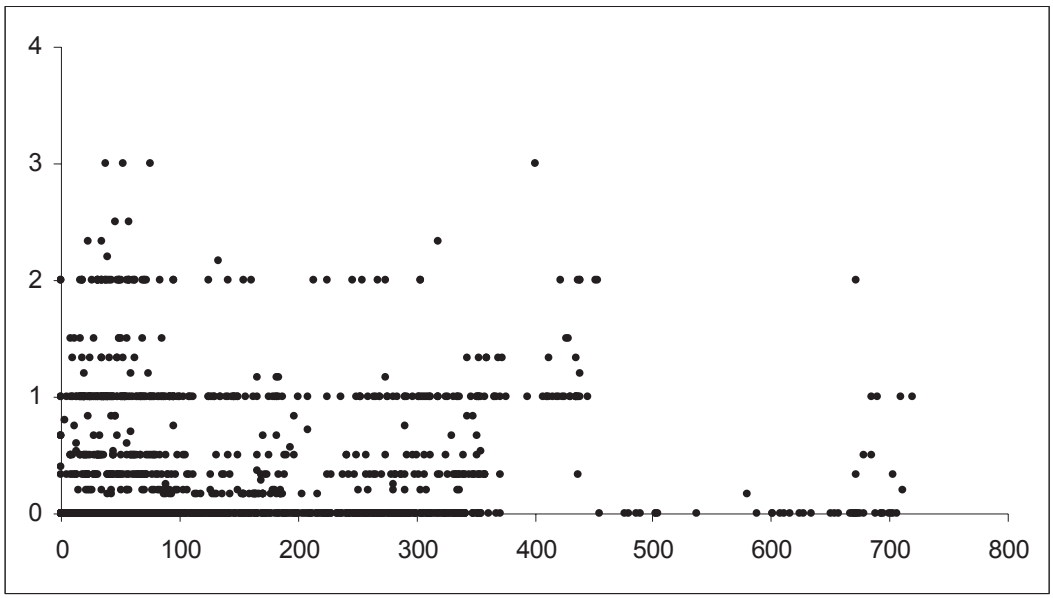

**Supplementary Figure S1.** Graph showing COB genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus scovazzi.*
Associated test statistics:
Regression coefficient (bY1): 0.015215
Correlation coefficient (rY1): 0.771430
Determination of Y by X1(%): 0.595104
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.000000
No. of smaller rand rY1: 1000
No. of equal rand rY1: 0
No. of larger rand rY1: 0



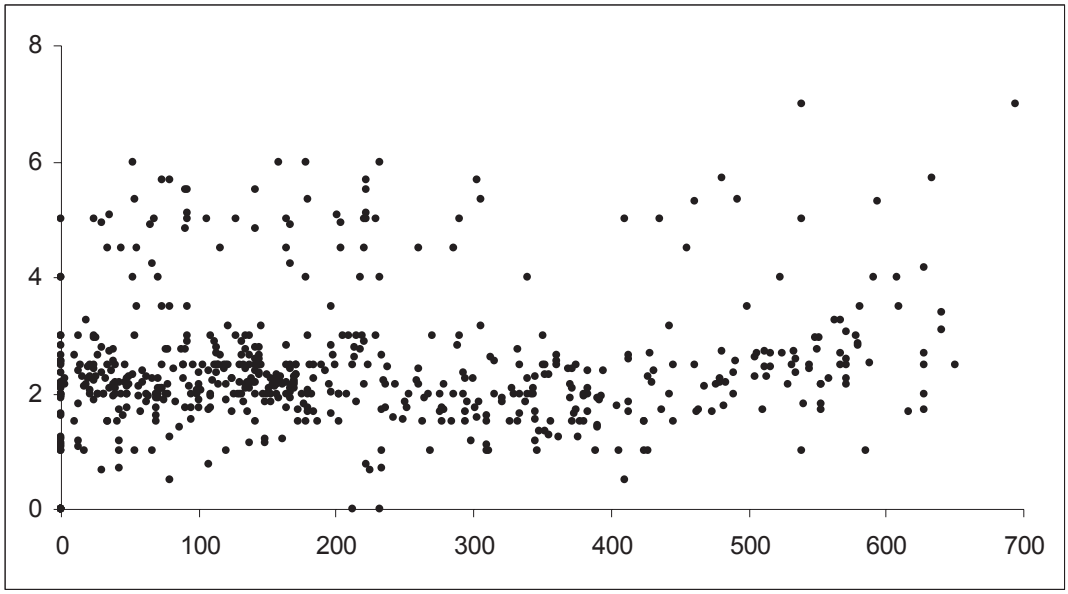

**Supplementary Figure S2.** Graph showing COB genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus pictus*. Note that sampling density of this species is very patchy and results need to be seen with caution.
Associated test statistics:
Regression coefficient (bY1): 0.005065
Correlation coefficient (rY1): 0.750793
Determination of Y by X1(%): 0.563690
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.000000
No. of smaller rand rY1: 1000
No. of equal rand rY1: 0
No. of larger rand rY1: 0



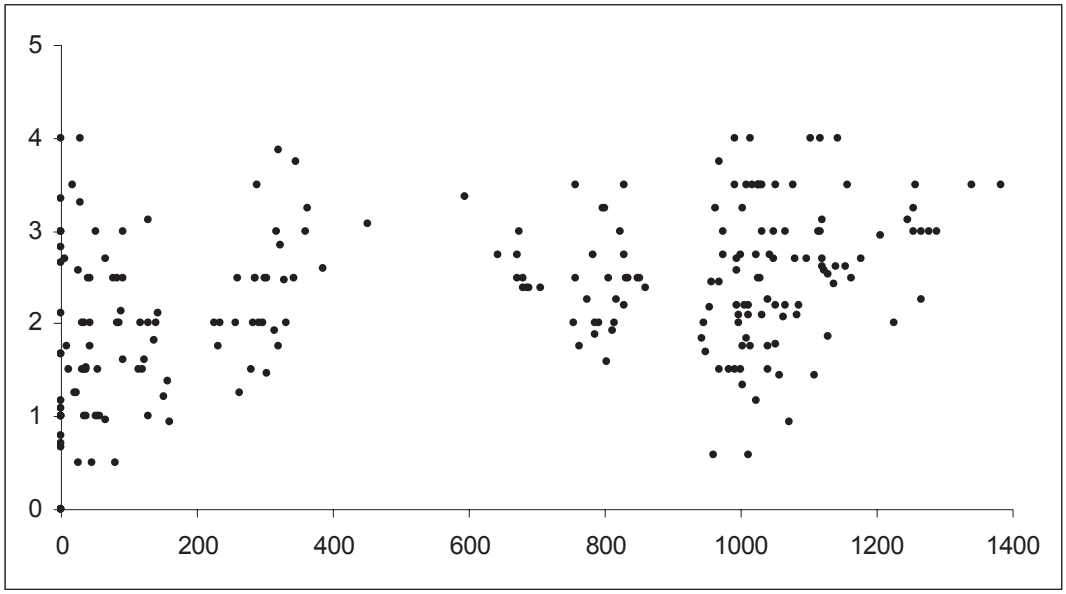

**Supplementary Figure S3.** Graph showing COB genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus g. galganoi.* Note that sampling density of this taxon is somewhat patchy and results need to be taken with caution.
Associated test statistics:
Regression coefficient (bY1): 0.006234
Correlation coefficient (rY1): 0.654720
Determination of Y by X1(%): 0.428658
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.000000
No. of smaller rand rY1: 1000
No. of equal rand rY1: 0
No. of larger rand rY1: 0



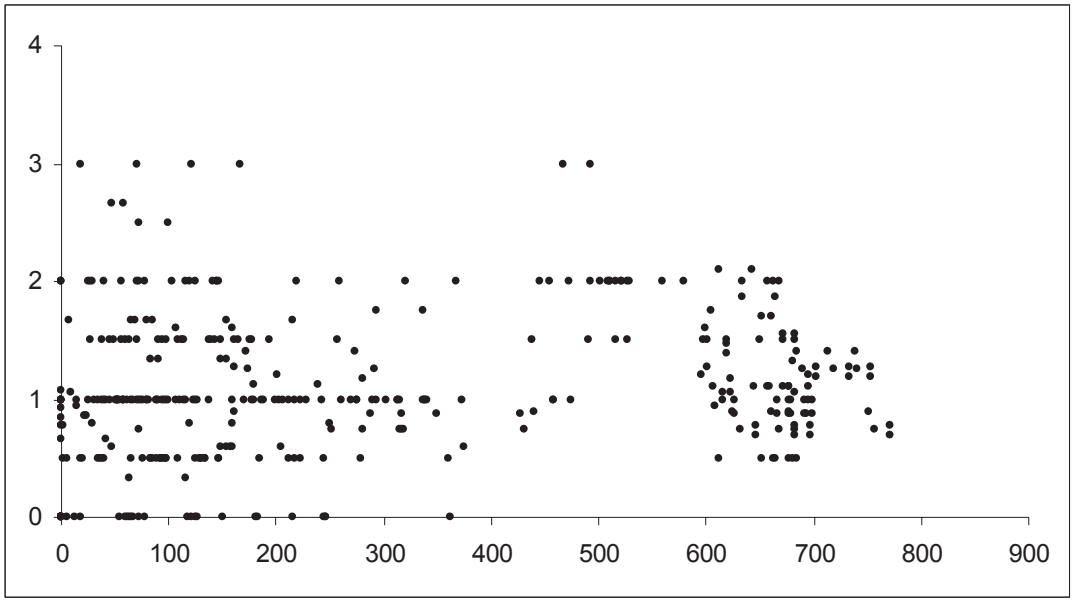

**Supplementary Figure S4.** Graph showing COB genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus galganoi jeanneae.* Note that sampling density of this taxon is somewhat patchy and results need to be taken with caution.
Associated test statistics:
Regression coefficient (bY1): -0.000213
Correlation coefficient (rY1): -0.054907
Determination of Y by X1(%): 0.003015
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.627000
No. of smaller rand rY1: 373
No. of equal rand rY1: 0
No. of larger rand rY1: 627



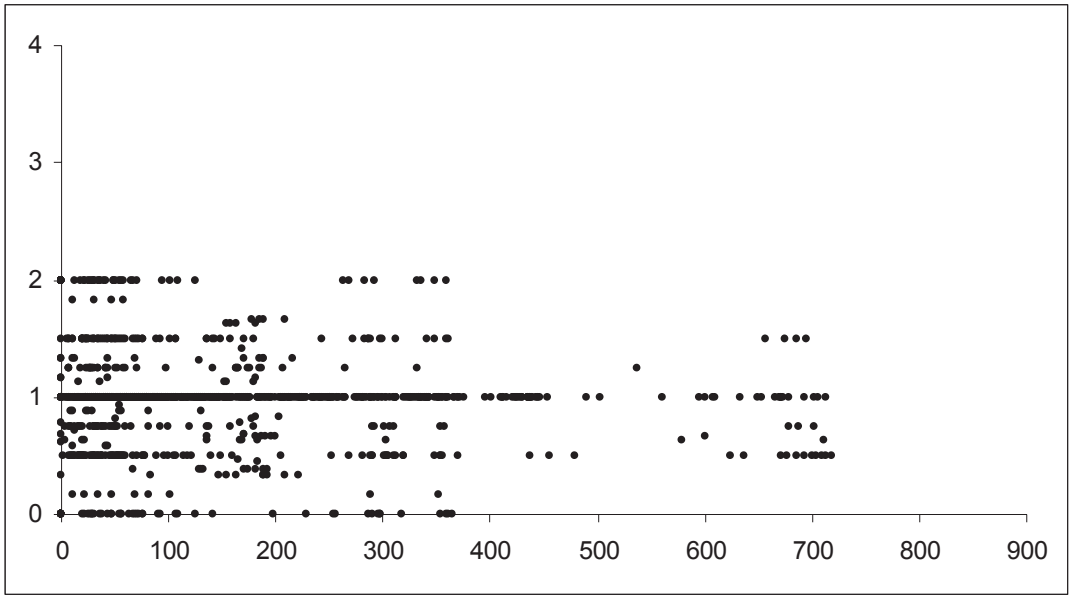

**Supplementary Figure S5.** Graph showing RAG1 genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus scovazzi.*
Associated test statistics:
Regression coefficient (bY1): 0.000297
Correlation coefficient (rY1): 0.046216
Determination of Y by X1(%): 0.002136
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.283000
No. of smaller rand rY1: 717
No. of equal rand rY1: 0
No. of larger rand rY1: 283



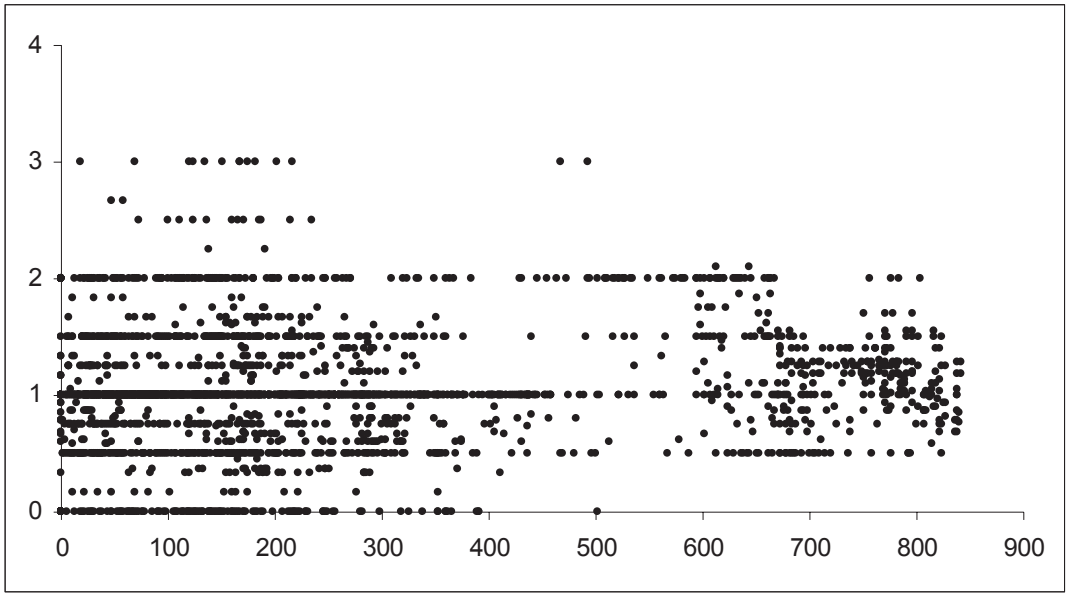

**Supplementary Figure S6.** Graph showing RAG1 genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus pictus*. Note that sampling density of this species is very patchy and results need to be seen with caution.
Associated test statistics:
Regression coefficient (bY1): 0.000668
Correlation coefficient (rY1): 0.367976
Determination of Y by X1(%): 0.135406
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.000000
No. of smaller rand rY1: 1000
No. of equal rand rY1: 0
No. of larger rand rY1: 0




**Supplementary Figure S7.** Graph showing RAG1 genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus g. galganoi.* Note that sampling density of this taxon is somewhat patchy and results need to be taken with caution.
Associated test statistics:
Regression coefficient (bY1): 0.000317
Correlation coefficient (rY1): 0.129880
Determination of Y by X1(%): 0.016869
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.158000
No. of smaller rand rY1: 842
No. of equal rand rY1: 0
No. of larger rand rY1: 158

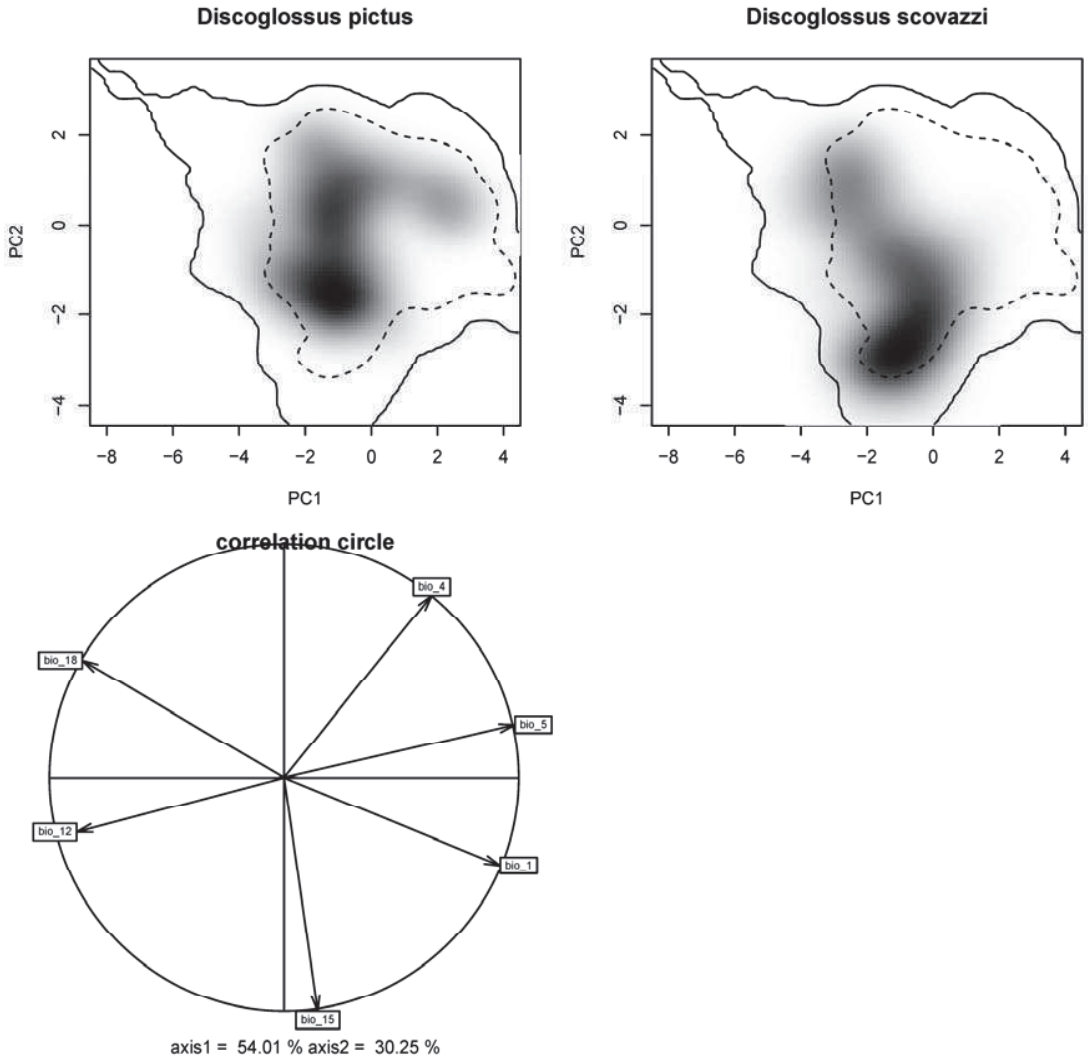



**Supplementary Figure S8.** Graph showing RAG1 genetic distance (number of mutations) vs. geographic distance (km between locations) for *Discoglossus galganoi jeanneae.* Note that sampling density of this taxon is somewhat patchy and results need to be taken with caution.
Associated test statistics:
Regression coefficient (bY1): -0.000150
Correlation coefficient (rY1): -0.061964
Determination of Y by X1(%): 0.003840
Significance testing (1000 permutations for Mantel test):
P(rY1 rand >= rY1 obs): 0.893000
No. of smaller rand rY1: 107
No. of equal rand rY1: 0
No. of larger rand rY1: 893

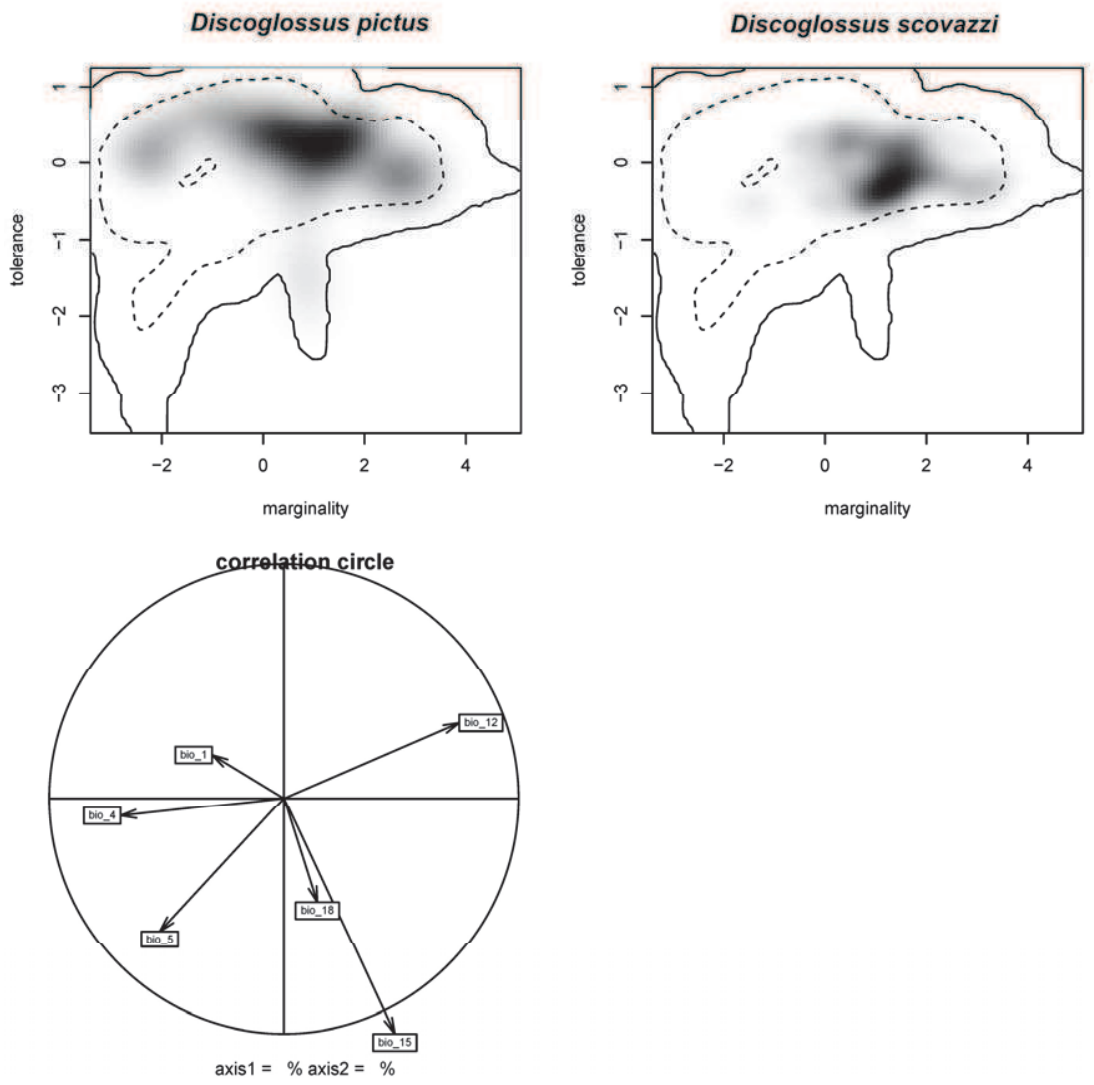



**Supplementary Figure S9.** Graph showing RAG1 genetic distance (number of mutations) vs. geographic distance (km between locations) for the merged dataset of *D. g. galganoi* and *D.* g. *jeanneae.*

Associated test statistics:
Regression coefficient (bY1): 0.000248
Correlation coefficient (rY1): 0.114878
Determination of Y by X1(%): 0.013197
Significance testing (1000 permutations for Mantel test)
P(rY1 rand >= rY1 obs): 0.054000
No. of smaller rand rY1: 946
No. of equal rand rY1: 0
No. of larger rand rY1: 54




**Supplementary Figure S10.** Niches of *Discoglossus pictus* and *D. scovazzi* based on PCA-env. The niches of both species are displayed on a multi-dimensional scale represented by the first two axes of a principal component analyses (PCA) summarizing the entire study area, with the grey shadings reflecting the density of the occurrences of each species by cell. The dashed and solid contour lines indicate 50% and 100% of the available background environment. The correlation circle (bottom left) shows climatic variables contribution on the two axes of the PCA as well as the percentage of inertia explained by the two axes.




**Supplementary Figure S11**. Niches of *Discoglossus pictus* and *D. scovazzi* based on ENFA. The x-axis shows marginality and the y-axis specialization. The grey shadings reflect the density of the occurrences of each species by cell. The dashed and solid contour lines indicate 50% and 100% of the available background environment. The correlation circle (bottom left) shows the contribution of the climatic variables contribution to the two axes

**Supplementary Table S1.** Sampling locations, geographical coordinates, numbers of sequences obtained per population, and numbers of COB sequences for which more than 2 double-peaks were scored as nucleotide ambiguities. Detailed geographical coordinates refer to own GPS readings; in other cases, approximative coordinates were determined by locating sites on Google Earth.

| Code Locality final | N-COB | N-RAG1 | N-COB with >1 ambiguity scores | Country | Latitude | Longitude |
|---|---|---|---|---|---|---|
| ***D. galganoi* (subspecies uncertain as only RAG1 sequence available))** | | | | | | |
| DG_ECAR | 0 | 1 | | Spain: Estacion De Cártama | 36.74 | -4.61 |
| DG_EPEC | 0 | 1 | | Spain: Escorrentia Pasado El Coto | 36.17 | -5.58 |
| DG_ESTR | 0 | 1 | | Spain: Ermita Na Sa de la Estrella | 37.98 | -4.29 |
| DG_PDLF | 0 | 1 | | Spain: La Fontanilla, Palos de la Frontera | 37.22 | -6.90 |
| DG_PVBL | 0 | 1 | | Spain: Villablanca | 37.30 | -7.34 |
| DG_SSCR | 0 | 1 | | Spain: Sierra San Cristobal, Jerez | 36.64 | -6.18 |
| DG_TRDR | 0 | 1 | | Spain: Junto Alberca Cerrada En El Tiradero | 36.17 | -5.58 |
| DG_VNCA | 0 | 1 | | Spain: Villanueva De Cauche | 36.95 | -4.44 |
| ***D. g. galganoi* and *D. g. jeanneae*** | | | | | | |
| DG_MANG | 2* | 0 | 2 | Spain: Arroyo de las Pilas,Cjo. Del Fraile, La Manga | 36.98 | -5.08 |
| DG_GAND | 1** | 1 | 1 | Spain: Gandul | 37.33 | -5.79 |
| ***D. g. galganoi*** | | | | | | |
| DG_AZNA | 1 | 1 | | Spain: Aznalcollar | 37.52 | -6.27 |
| DG_BSCL | 1 | 1 | | Portugal: Barragem Sta Clara | 37.57 | -8.36 |
| DG_CART | 2 | 1 | 2 | Spain: Cartaya | 37.28 | -7.15 |
| DG_CATE | 1 | 1 | 1 | Spain: Calera/Tentudia | 38.09 | -6.31 |
| DG_CDTE | 1 | 1 | | Spain: Cabezo del Tesoro (Valverde del Camino) | 37.55 | -6.78 |
| DG_CELA | 2 | 2 | | Spain: Celas-Culleredo | 43.26 | -8.40 |
| DG_CERC | 8 | 5 | | Spain: Cerceda | 43.18 | -8.47 |
| DG_COLO | 6 | 5 | 3 | Portugal: Campo Redondo, Colos | 37.78 | -8.56 |
| DG_DONA | 1 | 1 | 1 | Spain: Reserva Biologica Donana | 36.97 | -6.40 |
| DG_EDRU | 1 | 0 | | Spain: Estero Domingo Rubio | 37.22 | -6.90 |
| DG_FRAN | 1 | 1 | | Spain: Cortijo Las Franchas | 37.79 | -5.44 |
| DG_GBSB | 1 | 1 | 1 | Spain: Gibraleon, San Bartolome De La Torre | 37.37 | -6.97 |
| DG_JELV | 1 | 1 | 1 | Spain: Gelves-Mairena | 37.32 | -6.04 |
| DG_LCOL | 1 | 1 | 1 | Spain: Las Colonias | 37.26 | -6.94 |
| DG_PAYM | 1 | 1 | 1 | Spain: Paymogo | 37.74 | -7.35 |
| DG_ROME | 1 | 1 | | Spain: Los Romeros | 37.89 | -6.75 |
| DG_SPED | 12 | 9 | | Spain: Acantilado San Pedro | 43.38 | -8.44 |
| DG_TOHE | 11 | 8 | | Spain: Torre de Hercules, La Coruna | 43.39 | -8.41 |
| DG_TQJO | 2 | 0 | | Spain: Arroyo Tariquejo | 37.30 | -7.18 |
| DG_UNIR | 1 | 1 | 1 | Spain: Universidad de la Rabida | 37.21 | -6.92 |
| DG_VERI | 1 | 1 | | Spain: Vilaza-Verin | 41.94 | -7.47 |
| ***D. g. jeanneae*** | | | | | | |
| DG_ACRA | 1 | 1 | 1 | Spain: Acra | 37.90 | -3.17 |
| DG_ALGA | 1 | 1 | | Spain: Las Algamitas | 36.31 | -5.65 |
| DG_ALGV | 1 | 1 | | Portugal: Algarve | 37.39 | -8.33 |
| DG_ALIN | 1 | 1 | 1 | Spain: Alcaidesa, La Linea | 36.23 | -5.32 |
| DG_ARCO | 1 | 1 | | Spain: Arcos de la Frontera | 36.74 | -5.82 |
| DG_BARB | 1 | 1 | 1 | Spain: Cola del Embalse de Barbate | 36.37 | -5.64 |
| DG_BERR | 1 | 0 | | Spain: El Berrocal | 37.61 | -6.54 |

| | | | | | | |
|---|---|---|---|---|---|---|
| DG_BLCV | 2 | 2 | 1 | Spain: near Benalup | 36.30 | -5.68 |
| DG_CACA | 1 | 1 | | Spain: Near Casas de la Carera | 36.59 | -6.04 |
| DG_CALZA | 1 | 1 | | Spain: Canada Del Calzadillo | 39.24 | -2.37 |
| DG_CBEC | 3 | 1 | 2 | Spain: Cuevas Del Becerro | 36.88 | -5.05 |
| DG_CDPO | 1 | 0 | 1 | Spain: Collado del Pocico | 38.12 | -3.08 |
| DG_CHAP | 1 | 1 | | Spain: Charca Cruce Chaparrito, Cadiz | 36.63 | -5.86 |
| DG_CHOR | 2 | 2 | | Spain: Near El Chorreadero | 36.56 | -5.87 |
| DG_CLAJ | 3 | 1 | | Spain: Casa De La Laja | 36.56 | -5.60 |
| DG_DFAD | 1 | 1 | 1 | Spain: La Puebla de Don Fadrique | 37.98 | -2.45 |
| DG_EGAN | 1 | 1 | 1 | Spain: Estacion De Gaucin | 36.51 | -5.35 |
| DG_FACI | 1 | 1 | 1 | Spain: Facinas | 36.14 | -5.70 |
| DG_GARC | 1 | 0 | | Spain: Garciez | 37.87 | -3.47 |
| DG_GAST | 2 | 2 | | Spain: Gastor | 36.86 | -5.32 |
| DG_GFTE | 1 | 1 | 1 | Spain: Gibalbin Fuente Teneria | 36.83 | -5.92 |
| DG_HOCE | 6 | 1 | | Spain: Las Hoces | 38.53 | -2.79 |
| DG_HURO | 1 | 1 | 1 | Spain: Embalse Hurones | 42.41 | -3.61 |
| DG_IZNA | 1 | 0 | | Spain: Iznatoraf | 38.15 | -3.03 |
| DG_JAUT | 1 | 1 | | Spain: Under bridge, South of Jautor (close to type locality) | 36.3534 | -5.6320 |
| DG_JDLF | 1 | 1 | | Spain: Jerez de la Frontera | 36.69 | -6.13 |
| DG_LAME | 5 | 4 | 5 | Spain: Los Barrios, La Menacha | 36.17 | -5.48 |
| DG_LARA | 1 | 1 | | Spain: Poblado Del Lara | 38.01 | -3.84 |
| DG_LBAR | 1 | 1 | 1 | Spain: Near Los Barrios | 36.20 | -5.51 |
| DG_MESI | 2 | 2 | 2 | Spain: Near Medina-Sidonia | 36.48 | -5.91 |
| DG_MHCS | 6 | 4 | 6 | Spain: Huerta Canada,Canada Del Secretario | 37.21 | -4.14 |
| DG_MORO | 1 | 1 | | Spain: Moron de la Frontera | 37.13 | -5.46 |
| DG_MOSI | 1 | 1 | 1 | Spain: Jerez, Montesierra | 36.67 | -6.11 |
| DG_PDLI | 2 | 2 | | Spain: Near Puebla de los Infantes | 37.78 | -5.40 |
| DG_PEDR | 1 | 1 | | Spain: El Pedroso | 36.52 | -5.99 |
| DG_PUER | 1 | 0 | | Spain: Puerto Real | 36.53 | -6.19 |
| DG_RGRA | 7 | 6 | 4 | Spain: Cabecera Rio Granada,Loma Del Padron | 37.00 | -3.73 |
| DG_SALN | 1 | 1 | 1 | Spain: San Lucar, El Navazo | 36.77 | -6.37 |
| DG_SALU | 1 | 1 | | Spain: Near San Lucar (Trebujena) | 36.87 | -6.18 |
| DG_SDMO | 3 | 3 | 2 | Spain: Las Lagunillas, Sierra de Montecoche | 36.26 | -5.57 |
| DG_SEGU | 1 | 0 | | Spain: Segura | 38.10 | -2.56 |
| DG_SELE | 2 | 5 | 2 | Spain: Santa Elena | 38.31 | -3.58 |
| DG_SEVM | 1 | 1 | 1 | Spain: Sevilla - Montellano | 36.99 | -5.57 |
| DG_SJDV | 3 | 2 | 2 | Spain: San Jose del Valle | 36.63 | -5.71 |
| DG_SJPE | 2 | 2 | 1 | Spain: San Jose Del Pedroso | 36.52 | -5.99 |
| DG_SJVC | 1 | 0 | 1 | Spain: San Jose del Valle Cadiz | 36.61 | -5.80 |
| DG_SJVE | 1 | 0 | | Spain: San Jose Velle | 36.62 | -5.80 |
| DG_SLLM | 1 | 1 | 1 | Spain: Rio Guadiamar, Sanlucar la Mayor | 37.39 | -6.22 |
| DG_TNUE | 2 | 1 | 2 | Spain: Torre Nueva | 36.20 | -5.33 |
| DG_UTMD | 1 | 1 | 1 | Spain: 10km NE of Alcalá de los Gazules | 36.54 | -5.65 |
| DG_VENT | 1 | 1 | | Spain: Near Casa Ventisquero | 36.40 | -5.76 |
| DG_VGPL | 1 | 2 | 1 | Spain: Vega Del Plantonar,Colomera, Granada | 37.37 | -3.71 |
| ***D. pictus*** | | | | | | |
| DP_AIND | 0 | 2 | | Tunisia: Near Ain Draham | 36.78 | 8.69 |
| DP_BARC | 0 | 1 | | Spain: Barcelona | 41.41 | 2.16 |
| DP_BENS | 2 | 2 | | Morocco: Beni Snassen | 34.83 | -2.13541 |
| DP_BERK | 1 | 1 | | Morocco: Berkane province | 34.90 | -2.61 |
| DP_BOUS | 1 | 1 | | Tunisia: 10 km from Bou Salem | 36.53 | 9.03 |
| DP_DEBD | 11 | 12 | | Morocco: Debdou | 33.9470 | -3.27731 |

| | | | | | |
|---|---|---|---|---|---|
| DP_GIRO | 0 | 2 | Spain: Port Bou, Girona | 42.43 | 3.16 |
| DP_HAMM | 2 | 2 | Tunisia: Hammam | 36.76 | 8.59 |
| DP_JEND | 2 | 2 | Tunisia: Jendouba | 36.49 | 8.77 |
| DP_MABA | 1 | 1 | Tunisia: Majaz al Bab | 36.64 | 9.60 |
| DP_MADA | 1 | 1 | Morocco: Oued Sheraa | 34.88 | -2.43 |
| DP_MANA | 0 | 1 | Tunisia: Near Mashtá al Anad | 36.45 | 8.65 |
| DP_MELI | 15 | 15 | Spain: Melilla, Rio de Oro | 35.2923 | -2.9388 |
| DP_MISU | 2 | 2 | Morocco: near Misura | 32.8267 | -4.34098 |
| DP_PALA | 1 | 0 | Spain: Palamos near King s Camping | 41.8519 | 3.1402 |
| DP_SAID | 7 | 6 | Morocco: Saidia | 35.0912 | -2.2531 |
| DP_SEFR | 5 | 5 | Morocco: Oued Sefru | 34.7780 | -2.15323 |
| DP_TABA | 0 | 1 | Tunisia: Tabarka | 36.96 | 8.75 |
| DP_TIAR | 0 | 4 | Algeria: Tiaret | 35.38 | 1.33 |
| DP_TOZE | 0 | 1 | Tunisia: Tozeur City | 33.93 | 8.12 |
| DP_TUNI | 3 | 3 | Tunisia: Tunis | 36.77 | 10.03 |
| ***D. scovazzi*** | | | | | |
| DS_ALKS | 0 | 1 | Morocco: Alksrquivir | 35.08 | -5.55 |
| DS_ANOU | 4 | 3 | Morocco: near Anoual | 35.1104 | -3.578266 |
| DS_BENI | 1 | 1 | Morocco: Beni Hadifa | 35.04 | -4.15 |
| DS_BERR | 3 | 3 | Morocco: Berrechid | 33.30 | -7.39 |
| DS_BEYD | 2 | 2 | Morocco: Beni Yder | 35.46 | -5.42 |
| DS_BOUH | 4 | 4 | Morocco: Bouhachem | 35.27 | -5.44 |
| DS_CEUC | 5 | 5 | Spain: Ceuta, stream near Calamocarro | 35.91 | -5.36 |
| DS_CEUP | 5 | 5 | Spain: Ceuta, dam | 35.89 | -5.32 |
| DS_CEUV | 3 | 3 | Spain: Ceuta, Virgen de Aranguren | 35.90 | -5.37 |
| DS_CHIA | 0 | 2 | Morocco: Bou Chia | 34.99 | -4.82 |
| DS_ELKI | 33 | 32 | Morocco: El Khizana | 35.0425 | -5.2336 |
| DS_ESPA | 1 | 1 | Morocco: Espada | 35.55 | -5.52 |
| DS_HIGH | 0 | 1 | Morocco: High atlas | 30.93 | -8.27 |
| DS_HOCE | 3 | 3 | Morocco: Al Hoceima | 35.1528 | -3.995036 |
| DS_IMZO | 6 | 6 | Morocco: between Imzourne and Tamsamane | 35.1180 | -3.741479 |
| DS_JBAR | 1 | 1 | Morocco: Had Jbarna | 34.4743 | -3.916952 |
| DS_KATA | 0 | 1 | Morocco: Kasba-Tadla | 32.4989 | -6.0356 |
| DS_MDIQ | 0 | 1 | Morocco: Just North of M Diq | 35.6935 | -5.3285 |
| DS_MEBE | 1 | 1 | Morocco: Mechra-Benabbou | 32.6583 | -7.7787 |
| DS_MEZE | 28 | 27 | Morocco: Merja Zerga | 34.8159 | -6.2971 |
| DS_MLET | 1 | 1 | Morocco: R Mlet | ?? | ?? |
| DS_MOHA | 1 | 1 | Morocco: Mohammedia | 33.6838 | -7.3891 |
| DS_OUKA | 12 | 12 | Morocco: Oukaimeden | 31.2075 | -7.853987 |
| DS_REST | 8 | 6 | Morocco: Restinga, Negron | 35.7807 | -5.3545 |
| DS_ROUA | 1 | 0 | Morocco: Roualam | 34.9242 | -3.804092 |
| DS_ROUE | 11 | 11 | Morocco: Roueda | 35.2541 | -5.2155 |
| DS_SAAD | 0 | 1 | Morocco: Saadi | 34.9207 | -6.1282 |
| DS_SETT | 6 | 6 | Morocco: Settat | 33.1037 | -7.9232 |
| DS_TAMS | 6 | 6 | Morocco: 15 km before Tamsamane | 35.1053 | -3.715716 |
| DS_TANG | 1 | 0 | Morocco: 16 km S Tanger | 35.5532 | -5.5739 |
| DS_TARS | 1 | 1 | Morocco: Tarsoute | 35.2617 | -5.2432 |
| DS_TAZA | 1 | 1 | Morocco: South of Taza | 34.0660 | -4.0512 |
| DS_TETU | 0 | 1 | Morocco: Hills just south of Tetouan | 35.5420 | -5.3856 |
| DS_TICH | 5 | 5 | Morocco: Tizi-n-Tichka | 31.1827 | -7.22304 |
| DS_TIZI | 1 | 1 | Morocco: Tizi n  Test | 30.8295 | -8.3305 |

*3 specimens genotyped for COB but only 2 included in analysis.

** 2 specimens genotyped for COB but only 1 included in analysis.

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# Chapter 3

## Molecular phylogeny of grass snakes (*Natrix*) with emphasis on the biogeography of northern African (*N. natrix*) populations

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The Western Mediterranean region provides one of the most interesting areas to study historical biogeography. In addition to the divergence patterns found for many species on both sides of the Strait of Gibraltar, multiple studies have recovered high degrees of intra-specific genetic divergence between eastern and western populations of species occurring in the Maghreb, but the possible biogeographic scenarios explaining such a pattern remain understudied. In this study, we used an updated time-calibrated phylogeny of the genus *Natrix* (77 individuals from the entire range sequenced for ND4 and cytochrome *b*) to explore its diversification patterns. We specifically aimed at exploring the historical biogeography of *N. natrix* in the Western Mediterranean region. We also used paleodistribution modelling to explore the role of past climatic conditions and the potential existence of dispersal barriers on contemporary genetic structuring of the North African *N. natrix* populations.

According to the obtained genealogy, *N. natrix* specimens from North Africa and the Iberian Peninsula form a well-supported clade that includes three deep divergent lineages (Iberia, Morocco and Tunisia). The separation between the North African and the Iberian clade of *N. natrix* dates back to 2.5 Ma, and between the eastern and western North African populations to 2.2 Ma. *Natrix natrix* likely reached North Morocco through transmarine dispersal from southern Spain and rapidly expanded its range along the Mediterranean coast towards Tunisia. Quaternary climate stability in the eastern and western Maghreb and the presence of dispersal barriers in the form of the Moulouya and Chelif River basins that prevented gene flow likely contributed to the observed genetic diversity between the North African populations.

**Author contribution**: First authorship reflects that I was the main contributor to the paper. I have developed the concept, written the first draft and conducted all the spatial analyses.

# Molecular phylogeny of *Natrix* (Reptilia; Serpentes) with emphasis on the biogeography of North African *Natrix natrix* populations

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**Abstract**

The Western Mediterranean region provides one of the most interesting areas to study historical biogeography. In addition to the divergence patterns found for many species on both sides of the Strait of Gibraltar, multiple studies have recovered high degrees of intra-specific genetic divergence between eastern and western populations of species occurring in the Maghreb, but the possible biogeographic scenarios explaining such a pattern remain understudied. In this study, we used an updated time-calibrated phylogeny of the genus *Natrix* (77 individuals from the entire range sequenced for ND4 and cytochrome *b*) to explore its diversification patterns. We specifically aimed at exploring the historical biogeography of *N. natrix* in the Western Mediterranean region. We also used paleodistribution modelling to explore the role of past climatic conditions and the potential existence of dispersal barriers on contemporary genetic structuring of the North African *N. natrix* populations.

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**Keywords:** Colubridae; Maxent; dispersal barrier; Maghreb; transmarine dispersal/colonization; paleodistribution modelling

## Introduction

Considered one of the global biodiversity hotspots (Myers et al. 2000), the Mediterranean Basin is one of the most geologically complex areas in the world but at the same time one of the better studied in terms of its geological history, geography, morphology and natural history (Blondel et al. 2010). Situated in the crossroad of Europe, Africa and Asia, the Mediterranean Basin has been an area of contact and interaction of faunas of diverse origin, being renowned for its high reptile endemism and species richness (Bons and Geniez 1996; Cox et al. 2006; Sillero et al. 2014; Sindaco and Jeremčenko 2008; Sindaco et al. 2013). Although its origin dates back 200 ma, in the Mesozoic Era, the active geological dynamics of the last 35 million years, with the northwards migration of the African plate and its posterior contact with Eurasia, produced the isolation and movement of several micro plates, the formation of thousands of islands and the main mountain ranges that surround the Basin, and the almost complete desiccation of the Mediterranean sea in the Messinian (see Blondel et al. 2010 and references therein). All these complex and, at the same time, well studied and well dated geological events have had important consequences on endemism and differentiation of plants and animals and are responsible for the relevance and interest of the Mediterranean Basin as a model for phylogeographic and evolutionary studies, especially in reptiles (e.g. de Jong 1998; Carranza et al. 2006a, 2008; Busack and Lawson 2008; Kyriazi et al. 2008; Fritz et al. 2009; Kornilios et al. 2010).

One of the main events that had a great influence on the origin and genetic structure of many contemporary reptile species was the opening of the Gibraltar land bridge which occurred 5.33 Ma and the subsequent refilling of the Mediterranean Basin at the end of the Messinian Salinity Crisis (MSC) (Hsü et al. 1977; Krijgsman et al. 1999; see also Pleguezuelos et al. 2008). The opening of the Strait of Gibraltar was the principal cause of vicariance events affecting the amphibian genera *Pelobates*, *Discoglossus*, *Alytes*, *Salamandra* and *Pleurodeles* on each side of the Strait of Gibraltar (Steinfartz et al. 2000; García-París et al. 2003; Carranza and Wade 2004; Martínez-Solano et al. 2004; Zangari et al. 2006), but for reptiles the overall pattern has been ambiguous, with scenarios varying widely among species (e.g. Albert et al. 2007; Fritz et al. 2006; Pinho et al. 2006; Carranza et al. 2004, 2006a, 2006b, 2008; Paulo et al. 2008; Santos et al. 2012; Velo-Antón et al. 2012; Stuckas et al. 2014). In addition to the divergence patterns found for many species on both sides of the Gibraltar Strait, multiple studies have recovered high degrees of intra-specific genetic divergence between eastern and western populations of species occurring in the northern African Maghreb (e.g. Garcia-Porta et al. 2012; Santos et al. 2012; Stuckas et al. 2014), but the possible causes of this biogeographical pattern have never been comprehensively investigated. The possible factors that have promoted genetic sub-structuring in this region include range shifts and vicariance processes resulting from climatic fluctuations such as the Pliocene aridification and the Pleistocene glaciations (e.g. Krijgsman et al. 1999; Duggen et al. 2003; Beukema et al. 2010; Jimenez-Moreno et al. 2010).

The snake genus *Natrix* comprises three species (*Natrix natrix*, *N. maura* and *N. tessellata*) that are widely distributed in the Western Palaearctic. The genus has received considerable attention from systematists (e.g. Hecht 1930; Mertens 1947; Thorpe 1979; Schätti 1982; Guicking et al. 2006; Kindler et al. 2013), but several issues regarding its taxonomy and historical biogeography remain unclear (Guicking et al. 2006; Fritz et al. 2012; Kindler et al. 2013). In North Africa, *N. natrix* occurs from Morocco eastwards to Tunisia and is considered very rare. The enigmatic *N. natrix* populations of North Africa have only been included in a phylogeny recently, represented by a single specimen from Tunisia (Kindler et al. 2013). Although the latter was revealed to group with the long-diverged Iberian clade (i.e. *N. n. astreptophora*, see also Guicking et al. 2006; Fritz et al. 2012), the position and systematics of North African *N. natrix* remain unresolved and, in particular, the lack of information on the

Moroccan populations currently hampers exploration of divergence patterns, while including these populations should provide important insights into Western Mediterranean biogeography (Husemann et al. 2014).

In this study, we used an updated time-calibrated phylogeny of the genus *Natrix* to explore its diversification patterns. We specifically aimed at exploring the historical biogeography of *N. natrix* in the Western Mediterranean region. Furthermore, we used paleodistribution modelling to explore the role of past climatic conditions and the potential existence of dispersal barriers on contemporary genetic structuring of the North African populations.

## Materials and methods

*Taxon sampling, DNA extraction and sequencing*

A total of 77 individuals of all three species of *Natrix* were included in the present study. Partial sequences of the cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (ND4) were used for the phylogenetic and divergence dating analyses. From these samples, 45 were sequenced for this work (33 *N. natrix*, 11 *N. maura* and 1 *N. tessellata*) (GenBank accession numbers: KC570222-KC570311) while sequences from the remaining specimens were downloaded from GenBank together with a specimen of *Nerodia fasciata* used as outgroup. All specimens included in the study are illustrated in Fig. 1 and listed in Supporting Information Table S1, together with their extraction codes, locality information and GenBank accession numbers. DNA was extracted using the DNeasy Tissue kit (Qiagen, Valencia, CA, USA). All primers used were specifically designed for this study: two overlapping fragments of cyt *b* were amplified and sequenced with primer pairs (cb1) CBF_nat (5´-GTAGGCCTAAATATTTCRACCTG-3') and inCBR_nat (5'-TCAGTGTGAAGAAGTATAATGTG-3'), and (cb2) inCBF_nat (5'-ACCCTCACAACCTGACTCTG-3') and CRB_nat (5'-GTTGTTATAAAAAATGTRAAGTA-3'), respectively. The two cyt *b* fragments were joined manually. The ND4 gene fragment was amplified and sequenced with primers ND4F_nat (5'-GGATCAATRGTACTAGCAGC-3') and ND4R_nat (5'-ATTCAGGTTTTATYGAGATAAG-3'). PCR conditions for amplification of the two fragments included a first step of 5' at 94ºC, followed by 35 cycles of 30'' at 94◦C, 45'' at 52ºC (cb1), 44ºC (cb2) or 50ºC (ND4) and 1' at 72ºC. A final step of 10' at 72ºC was also included. Sequencing was done on an Applied Biosystems 3730XL sequencer at Macrogen, Korea and

all amplified fragments were sequenced for both strands. Geneious v.5.3 (Drummond et al. 2010) was used for contig assembly and as a platform for exporting into different formats.

*Phylogenetic analyses and estimation of divergence times*

DNA sequences were aligned using the online version of MAFFT (Katoh and Toh 2008) applying default parameters (gap opening=1.53, offset value=0.0). Both fragments were translated into amino acids and no stop codons were observed. Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI) methods. The nucleotide substitution model GTR+I+G was selected as the best-fitting model under the Akaike information criterion (Akaike 1973) using jModelTest v.0.1.1 (Posada 2008). ML analysis was performed with RAxML v.7.4.2 (Stamatakis 2006), as implemented in raxmlGUI (Silvestro and Michalak, 2012) with 100 random addition replicates, and model parameters were calculated independently for each partition (cyt *b* and ND4). Reliability of the resulting tree was assessed by bootstrap analysis (Felsenstein 1985) with 1000 replications. MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) was used for the Bayesian analysis. Two independent runs of 5 x $10^6$ generations were carried out with sampling at intervals of 1000 generations, producing 5000 trees. Convergence was confirmed by ensuring that the standard deviation of the split frequencies between the two simultaneous runs was lower than 0.01 and by examining the Potential Scale Reduction Factor diagnostic. The first 1500 trees of each run were discarded as a burn-in fraction and a majority rule consensus tree was generated from the remaining trees.

Estimation of divergence times was done using a Bayesian approach as implemented in BEAST v.1.6.1 (Drummond and Rambaut 2007). A two-step methodology was followed in order to avoid mixing phylogenetic together with coalescent processes in the same dating analysis. A first dataset (Supporting Information Table S2) with published sequences of Colubroidea and including two newly sequenced *Natrix* samples was used in order to obtain an estimation for the age of the basal divergence of *Natrix*. This was done applying four calibration points retrieved from the recent literature: (1) the age of the basal divergence of Colubroidea, based on the youngest unambiguous colubroid fossils dated at approximately 40 Ma (Head et al. 2005; Wüster et al. 2008; Lukoschek et al. 2012) (lognormal prior, mean:2.0, stdev:1.2, offset:40.0); (2) the split between the Asian and the African clade of the genus *Naja*,

dated back to a minimum of 16 Ma ago according to the fossil record (Szyndlar and Rage 1990; Wüster et al. 2008). Although it is debatable whether this point should be used to calibrate the stem or the crown of the *Naja* clade, Lukoschek et al. (2012) mention that in the case of mitochondrial data, the crown placement was not identified as an outlier (see also Suppl. Material A of Lukoschek et al. 2012) (lognormal prior, mean:1.0, stdev:1.0, offset:16.0); (3) the time of divergence between the eastern and western species of *Hemorrhois*, assuming it coincides with the earliest land connection between Asia and Africa 16-18 Ma ago (Nagy et al. 2003) (normal prior, mean:18.0, stdev:2.04); (4) the split between *Malpolon monspessulanus* and *M. insignitus* 3.5-6 Ma ago (Carranza et al. 2006a) (lognormal prior, mean:1.5, stdev:1.0, offset:3.4). The rest of the prior specifications for this analysis were as follows (otherwise by default): GTR+G; Relaxed Uncorrelated Lognormal Clock (estimate); Random starting tree; Yule process of speciation; parameter values for both clock and substitution models unlinked across partitions. Additional analyses were performed removing one or two points from the calibration scheme, to test for consistency between results. The estimated age of the crown of the *Natrix* clade was obtained from this first step (Supporting Information Fig. S1A) and was used as a calibration point in the next step. In this second step, we used the dataset including all the *Natrix* samples, as in the reconstruction of the phylogenetic tree above (excluding the outgroup), and applied the calibration with a normal prior with mean: 16.3893 and stdev: 2.73. Since all *Natrix* samples were included in the analysis, a coalescent tree prior was applied, as more appropriate for intraspecific relationships (Drummond and Rambaut 2007). In this case, the rest of the prior specifications were as follows (otherwise by default): GTR+G; Relaxed Uncorrelated Lognormal Clock (estimate); Random starting tree; parameter values for both clock and substitution models unlinked across partitions.

*Climate data and variable selection*

A total of 27 distribution records of North African *N. natrix* were assembled from both literature and fieldwork (Fig. 3; Supporting Information Table S4). Bioclimatic variables were downloaded from the WorldClim database version 1.4 (Hijmans et al. 2005) at a resolution of

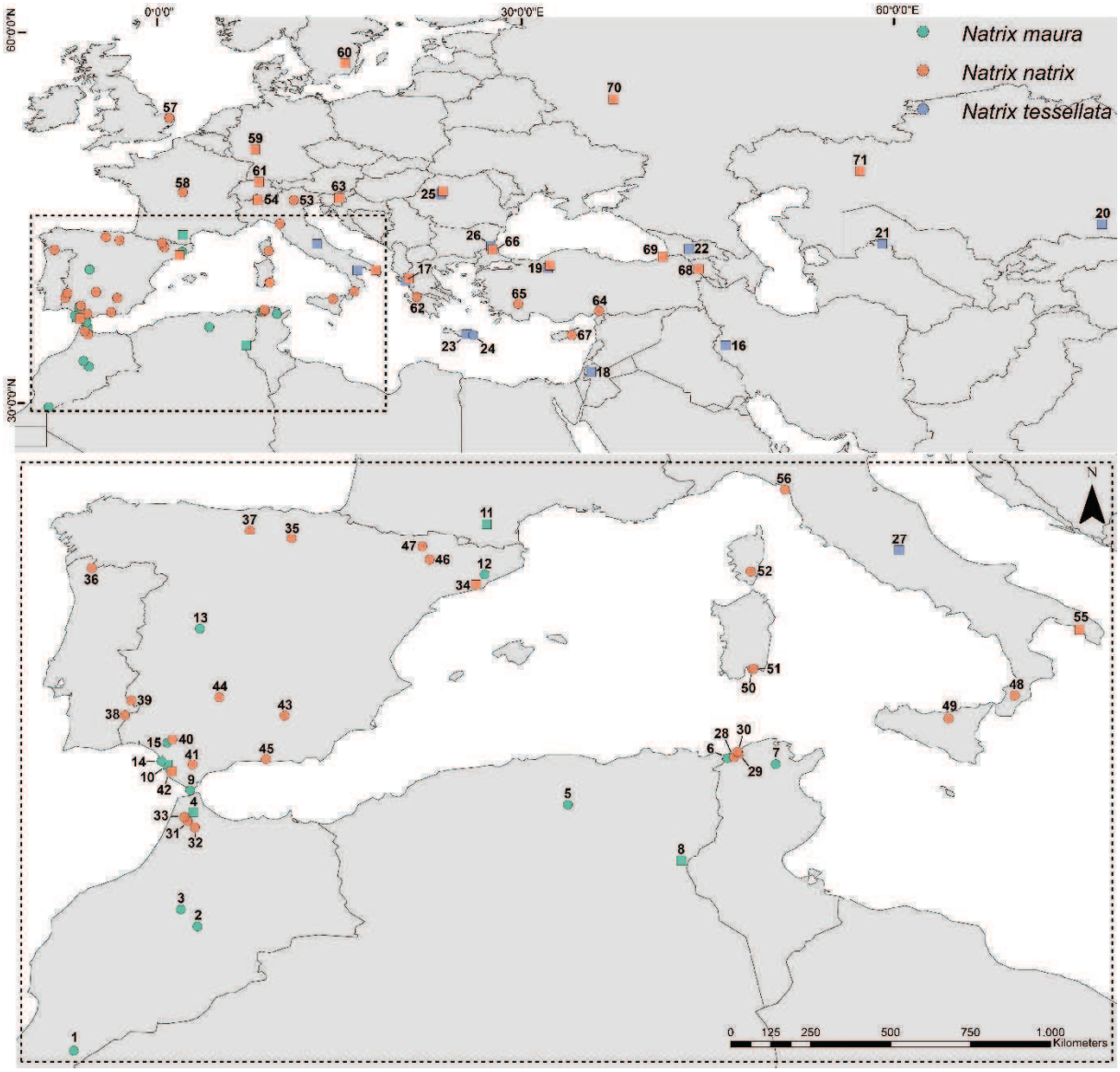


**Figure 1**. Map of the study area showing the Western Mediterranean region. Colours of locality dots correspond to different taxa. Circles indicate samples sequenced for the present study and squares indicate samples obtained from GenBank. Detailed information on sampling localities and specimens is found in Table S1 (Supporting information).

30 arc seconds (nearly 1x1 km). Past climate data for the Last Glacial Maximum (LGM) and Last Inter Glacial (LIG) were obtained from the WorldClim database and Otto-Bliesner et al. (2006), at the highest available resolutions (2.5. arc minutes and 30 arc seconds, respectively) Two general atmospheric circulation models (GCM) were used to generate the LGM scenarios: the Community Climate System Model (CCSM) and the Model for Interdisciplinary Research on Climate (MIROC). The two GCMs were averaged using ArcGIS 10 (ESRI). The ECHAM3

atmospheric GCM (DKRZ 1992) was used to obtain the Mid-Holocene climate data. Collinearity of the initial variables was measured by Pearson's correlation coefficient using ENMtools 1.3 (Warren et al. 2010). A total of five variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient) were retained. The final set of environmental variables used for SDM consisted of: Annual Temperature (BIO1), Temperature Seasonality (BIO4), Mean Temperature of Driest Quarter (BIO9), Annual Precipitation (BIO12) and Precipitation Seasonality (BIO15).

*Species distribution modelling*

The SDMs were generated by the presence/background algorithm Maxent, version 3.3.3k (Phillips et al. 2006). Maxent was used with default settings (Convergence threshold = 0.00001, maximum number of iterations = 500 and βj = 1) while partitioning the geographical records between training and test samples (default settings). We used two different backgrounds to calibrate our SDMs. First (A), we calibrated the models in a background defined by the Köppen-Geiger classes occupied by *N. natrix* in northern Africa, as extracted from the distribution records (Thompson et al. 2011; Webber et al. 2011). Köppen-Geiger polygons were obtained from the CliMond database (Kriticos et al. 2012). Second (B), we used a 200 km buffer around the distribution records as suggested by VanDerWal et al. (2009) and used by previous studies (e.g. Vences et al. 2014). Both these background regions encompass all known localities and include areas that have been accessible to the species via dispersal over relevant time periods. Subsequently, models were projected onto a larger area (Fig. 3). The average of ten pseudo-replicated models with randomly selected test samples was used to produce SDMs, which were plotted in logistic format. The final models were reclassified in ArcGIS 10 (ESRI) into binary presence-absence maps using the ten percentile threshold. All models were tested with receiver operating characteristics (ROC) curve plots, which plot the true-positive rate against the false-positive rate. The average area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model. The threshold dependent true skill statistic (TSS) was also used for model validation as this method is not influenced by prevalence (Allouche et al. 2006). Additionally, we used null-models to test for significance of the ENMs (Raes and ter Steege 2007). We generated 1000 null distributions of random points in the study area using ENMtools (Warren et al. 2010) for each

of the two backgrounds, with the number of random points equal to the actual number of distribution records used for SDM. The null-models were created and assessed following Raes and ter Steege (2007). Comparisons of the environmental variables used for projection to those used for training the model were made using visual interpretation of multivariate similarity surface (MESS) pictures and the most dissimilar variable (MoD) (Elith et al. 2010).

**Results**

*Phylogenetic analyses and estimation of divergence times*

A dataset of two concatenated mitochondrial gene fragments (cyt *b* and ND4) with a total alignment length of 1615 bp (982 and 633, respectively) was used to infer the phylogeny. The number of variable and parsimony informative positions was 372 and 307, respectively, for the cyt *b* and 239 and 212 for the ND4 gene. The results of the ML and BI analyses were almost identical and are summarized in Fig. 2. There is relatively low support (ML:65 / BI:0.93) over the sister relationship of *N. tessellata* and *N. natrix*, although this result is consistent among different analyses with exclusion or addition of specimens from the different species (results not shown). Within *N. natrix*, three major clades are observed: a first one including specimens from North Africa and the Iberian Peninsula, a second one grouping specimens from the Italian Peninsula (including Calabria), the islands of Sicily, Sardinia and Corsica, and from France, Germany and the U.K., and finally, a third clade comprising specimens from localities that extend all the way from Sweden and Denmark, across the Balkans and Turkey, to Russia and Kazakhstan. Within the North African-Iberian clade of *N. natrix*, three deeply divergent lineages belonging to Iberian, Moroccan and Tunisian specimens are observed. The specimens from Morocco and Tunisia are grouped together, yet with low support, and are fairly similar genetically within each country (see Fig. 2).

Convergence of the estimation of divergence times analysis was confirmed by examining the likelihood and posterior trace plots of the two independent runs combined in Tracer v.1.5, where effective samples sizes of parameters were estimated to be above 200, indicating a good representation of independent samples in the posterior. Additional analyses with alternative calibration schemes (see Materials and Methods) gave very similar age estimates. The estimated divergence times of the main clades within the phylogeny are illustrated in Fig. 2 and the chronogram is provided in Supporting Information Fig. S1B. Posterior mean rates for the cyt *b* and ND4 were estimated at 0.0113 (stderr of mean: 6.60 x

$10^{-5}$) and 0.0103 (stderr of mean: 6.01 x $10^{-5}$) substitutions per lineage per million years, respectively. Diversification within the genus *Natrix* started almost 15 Ma ago (95% HPD: 9.2-20.7), while for each one of the three species, *N. maura*, *N. tessellata* and *N. natrix* it is estimated at 3.1 Ma (95% HPD: 1.7-4.4), 5.9 Ma (95% HPD: 3.5-8.5) and 4.7 Ma (95% HPD: 2.8-6.8) ago, respectively. The separation between the North African and the Iberian clade of *N. natrix* dates back to 2.49 Ma ago (95% HPD: 1.4-3.6), whereas between the Moroccan and Tunisian clades 2.16 Ma (95% HPD: 1.2-3.2).

*Species distribution modelling*

For both backgrounds (A and B) Maxent produced SDMs of high predictive accuracy, according to the average test AUC for the present and past models (average AUC=0.894±0.063, range 0.746-0.99; see Supporting Information Fig. S2 and Table S3). The TSS values were lower compared to the AUC values (average TSS=0.654±0.149, range 0.094-0.806; see Supporting Information Fig. S2 and Table S2) but indicated accuracy well above the minimum evaluation score for a model to be considered useful (TSS=0.4; Engler et al. 2011). The SDMs also performed statistically significantly better than random (Fig. 3). The two different background approaches produced overall comparable results and revealed climate stability in parts of the Rif, Middle and High Atlas Mountains in Morocco, as well as in areas along the north-east Algerian and northern Tunisian Mediterranean coast (Fig. 3). Background B, however, produced a larger stability area in northeastern Algeria and northern Tunisia. The SDMs for both backgrounds predicted unsuitability of the Moulouya River Basin (MRB) and the Chelif River Basin (CRB) throughout all time periods, except the LGM for the CRB (see Supporting Information Fig. S3A-B). Areas most affected by variables being outside their training range (clamping) were minimal. The MESS and MoD pictures for the past SDMs revealed several regions with non-analog climate, indicating considerable climatic fluctuations (Supporting Information Fig. S4A-B).

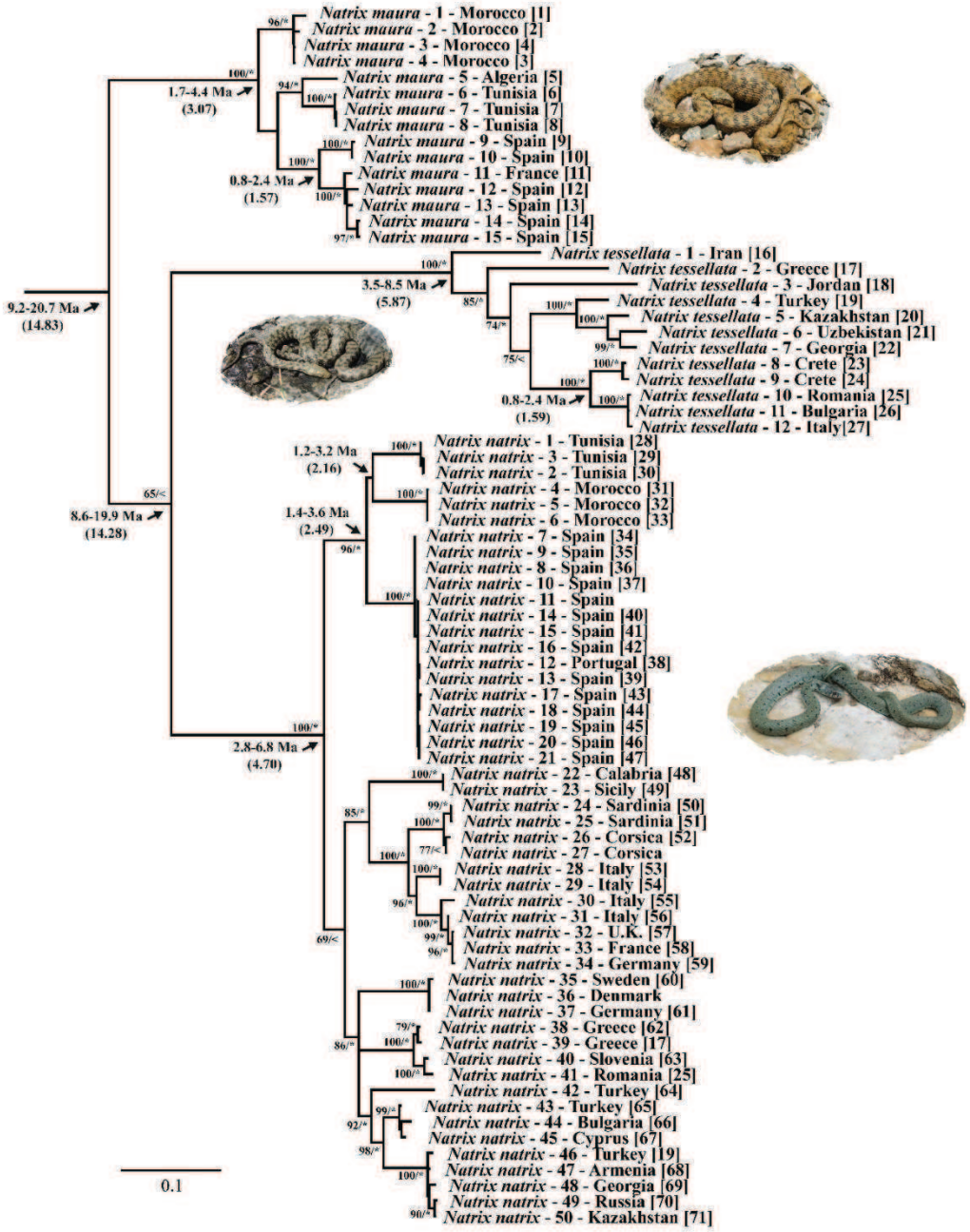


**Figure 2**. ML tree of the genus *Natrix* inferred using cyt*b* and ND4 mitochondrial gene fragments. Numbers next to the nodes indicate bootstrap support of the ML analysis (only values above 65 are shown) followed by an asterisk if posterior probability in the BI analysis is ≥0.95 (otherwise <). Ages of selected nodes are indicated with an arrow, with the mean age in brackets. The tree was rooted with *Nerodia fasciata*. Numbers in square brackets next to specimen codes refer to localities shown in Fig. 1. Information on the samples included in the analyses is shown in Supporting Information Table S1

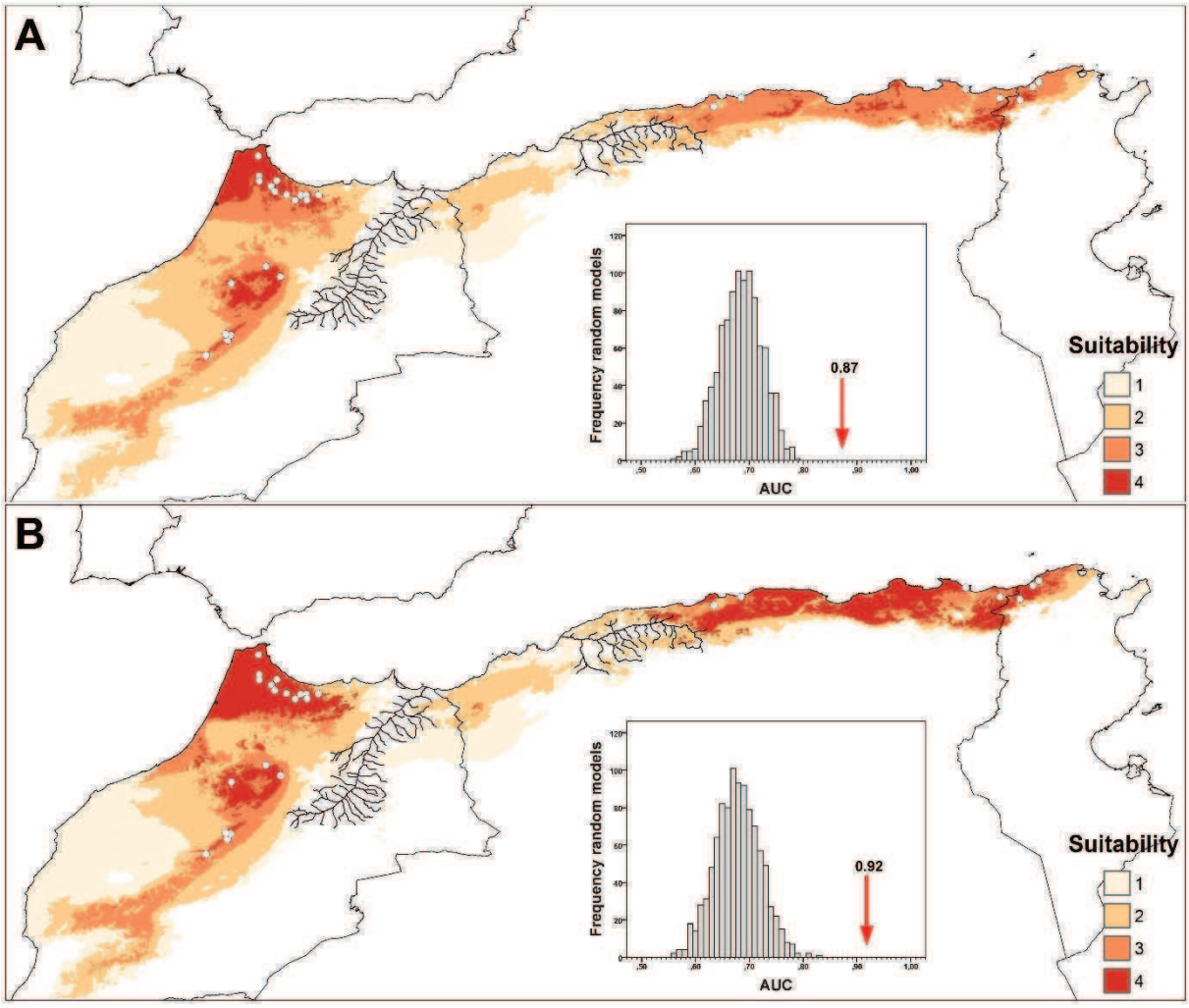


**Figure 3**. Potential species distribution models of *Natrix natrix* in the Maghreb for the present, Mid-Holocene, Last Glacial Maximum and Last Interglacial based on the TPT threshold using two different background approaches (A and B; see Materials and Methods). Red areas indicate areas stable throughout all time periods. Results of null-models to test for significance of the SDMs and the available distribution data are indicated (white dots). The Moulouya River and Chelif River are indicated.

## Discussion

Our study shows that the genus *Natrix* diversified during the Miocene, approximately 15 Ma, with the offset of intraspecific diversification estimated at the Miocene/Pliocene boundary. The topology recovered by our analyses is largely in agreement with results from previous phylogenetic and phylogeographic analyses on *Natrix* species (Guicking et al. 2006, 2008, 2009; Fritz et al. 2012; Kindler et al. 2013). The separation between the North African and the Iberian clade of *N. natrix* dates back to 2.5 Ma, and between the eastern and western North African populations to 2.2 Ma. Quaternary climate stability in the eastern and western

Maghreb and the presence of dispersal barriers in the form of river basins that prevented gene flow likely contributed to the observed genetic diversity between the North African populations.

*Phylogenetic analyses and estimation of divergence time*

According to our results, *N. maura* is sister to a poorly supported, yet consistent among analyses, clade grouping *N. tessellata* and *N. natrix*, corroborating the findings of Guicking et al. (2006) (Fig. 2). The results also confirm that *N. natrix* contains three major clades (Fritz et al. 2012; Kindler et al. 2013) and reveal three deep lineages in the clade that groups North African and Iberian samples, one from each main sampling area (Tunisia, Morocco, Iberian Peninsula). The sister relationship between the North African and the Iberian populations is in agreement with the results of Kindler et al. (2013).

The divergence estimation approaches applied in most of the recent literature are, however, different from the one followed in the present study, resulting in considerable differences in the dating estimations. In the first studies with age estimations (Guicking et al. 2006, 2008, 2009), calibration of the molecular clock for *Natrix* was done based on aminoacid distances using a series of fossils, the use of which has since been challenged (Lukoschek et al. 2012). Moreover, it has been shown that the extrapolation of molecular rates across different evolutionary timescales should be done with great caution as it can result in invalid estimations (Ho et al. 2005). An alternative dating strategy for *Natrix* followed by the same and posterior authors (Guicking et al. 2006; Fritz et al. 2012) was based on the use of the reopening of the Strait of Gibraltar as a calibration point, assuming the split of African and European *N. maura* to be 5.3 Ma old. However, it can be argued that with this assumption made, the potential role of the Messinian Salinity Crisis in the diversification of the other two species cannot be studied, because of obvious circularity. In the present study, none of the above calibration points were used, in an attempt to perform an independent estimation and be able to compare and contrast the results. We considered more appropriate to carry out the analysis in a two-step approach and applied multiple calibration points (see Materials and Methods).

In total, our analyses yielded much more recent age estimates than that in Fritz et al. (2012) and are more congruent with those by Guicking et al. (2006) regarding intraspecific differentiation in *N. natrix* and *N. tessellata*. Interestingly, we find that neither the split in *N. natrix* (see below) nor in *N. maura* between North African and Iberian clades are related to the opening of the Strait of Gibraltar, as previously proposed (Guicking et al. 2006; Fritz et al. 2012). In fact, it has been suggested that in *N. tessellata* the split between Crete island and the mainland was also posterior to that time (Guicking et al. 2009), and this is independently confirmed herein.

*Biogeography of North African populations*

The Strait of Gibraltar, which has separated the African and the Iberian plates since the onset of the Pliocene, is among the best studied biogeographical barriers. The opening of the Gibraltar land bridge and the subsequent refilling of the Mediterranean Basin at the end of the MSC (Krijgsman et al. 1999) is an important vicariant event that has helped shaping the observed biogeographical patterns in this region. Despite the fact that this event has been shown to be the principal cause of vicariance between several amphibian genera on each side of the Strait of Gibraltar, this pattern is less clear for reptiles (see Introduction). The estimated divergence between the African and Iberian *N. natrix* dates back to approximately 2.5 Ma ago, indicating a possible post-Messinian transmarine dispersal from Europe to Africa and matching with the arrival of *Bufo bufo* (Garcia-Porta et al. 2012; Recuero et al. 2012). This is not unexpected considering the reasonable salinity tolerance of *Natrix* species (van der Meijden and Chiari 2006; Galán 2012) and the relative short minimal distance (14.3 km) between both continents. As suggested by Garcia-Porta et al. (2012), Iberian rivers, such as the Guadalquivir, could have played an important role in projecting rafts into the open water, which afterwards could have reached the North African coast transported by the currents. Evidence for transmarine dispersal in the Mediterranean region has been reported for a large number of reptiles including the genus *Natrix* (e.g. Carranza et al. 2004, 2006a; Guicking et al. 2009; Kyriazi et al. 2012; Poulakakis et al. 2013).

A plausible scenario could be that *N. natrix* reached the Tingitana Peninsula in Morocco through transmarine dispersal from southern Spain and rapidly expanded its range along the Mediterranean coast towards Tunisia. The arrival of *N. natrix* during the Early Pleistocene

coincides with a period of vegetation- and climate oscillations in north-western Africa, which occurred between 3.7 and 1.7 Ma (Leroy and Dupont 1994). During this period, the vegetation fluctuated between humid phases that were characterized by tropical forests, and drier phases, characterized by grasslands (Thompson and Fleming 1996). The low support for the grouping of the Moroccan and Tunisian populations of *N. natrix* may represent the genetic signature of a rapid expansion of the species in the Maghreb. Meanwhile, the lack of genetic diversity within each country most probably results from the proximity of the sampling localities due to the difficulty of obtaining samples.

The large fluctuations in the potential distribution of *N. natrix* during the Late Quaternary are a direct result of the climatic oscillations in North Africa. For example, the increased potential distribution during the LGM is the result of relatively humid climatic conditions that transformed the lower elevation vegetation of North Africa into warm, mixed forests (Rognon 1987; Wengler and Vernet 1992; Jolly et al. 1998a,b; Lubell 2011). Likewise, the existence of mixed forests at intermediate elevations throughout the Mid-Holocene resulted in an increased suitable distribution area for *N. natrix* in the Maghreb. During the interglacial oscillations, *N. natrix* likely expanded its distribution to lower elevations during cooler phases and shifted to higher elevations during drier and warmer phases (Husemann et al. 2014).

The present study shows that several parts of the distribution of *N. natrix* in the Maghreb have remained stable throughout the Late Quaternary. These areas largely correspond to previously identified refugia for plant diversity (Médail and Diadema 2009). Furthermore, the Moulouya River Basin (MRB), and possibly the Chelif River Basin (CRB), acted as dispersal barriers that prevented gene flow and likely contributed to the observed genetic divergence between the eastern and western populations. The MRB has been previously suggested to act as a barrier against gene flow for some species (e.g. Arano et al. 1998; Álvarez et al. 2000; Zangari et al. 2006; Barata et al. 2008; Vences et al. 2014), but other studies have disputed this claim (Harris et al. 2003a,b; Paulo et al. 2008; de Pous et al. 2011; Santos et al. 2012). The CRB is one of the largest Messinian maginal basins in the Mediterranean (44.630 $km^2$) and is characterised by a semi-arid climate (average 400 mm annual rain and 20°C), high salinity conditions, and low benthic invertebrate and plant species richness (Arab et al. 2004; Rouchy et al. 2007; Ababou et al. 2013). However, the importance of both the MRB and CRB as a dispersal barrier remains speculative (but see e.g. Husemann et al. 2014). Our findings provide the first plausible explanation for the existence of a biogeographic pattern of eastern vs.

western genetic divergence in the Maghreb. This pattern has been previously reported for mammals (Cosson et al. 2005; Ben Slimen et al. 2006; Biollaz et al. 2010; Dool et al. 2013), plants (Magri et al. 2007;), birds (García et al. 2008), reptiles (Dimaki et al. 2008; Guicking et al. 2008; Santos et al. 2012; Stuckas et al. 2014) and amphibians (Garcia-Porta et al. 2012; Stöck et al. 2008). Future research using a denser sampling and multiple species is needed to fully understand the possible effects of these river barriers on the contemporary genetic structure of North African biodiversity.

*Taxonomic remarks*

Hecht (1930) described the North African populations as a subspecies (*N. n. algericus*) but this taxonomic treatment was not adopted by Thorpe (1979) following his elaborate range-wide review of internal and external morphological characters. Thorpe (1979) assigned the North African populations to the subspecies *N. n. helvetica*, whereas subsequent authors (i.e. Bons and Geniez 1996) treated them as part of the Iberian subspecies *N. n. astreptophora*. The North African populations of *N. natrix* are indeed more closely related to *N. n. astreptophora* but could well be taxonomically distinct (Kindler et al. 2013). Further research including a thorough morphological assessment, increased sampling effort and the use of nuclear genomic markers is therefore needed to resolve the taxonomic position of populations in North Africa.

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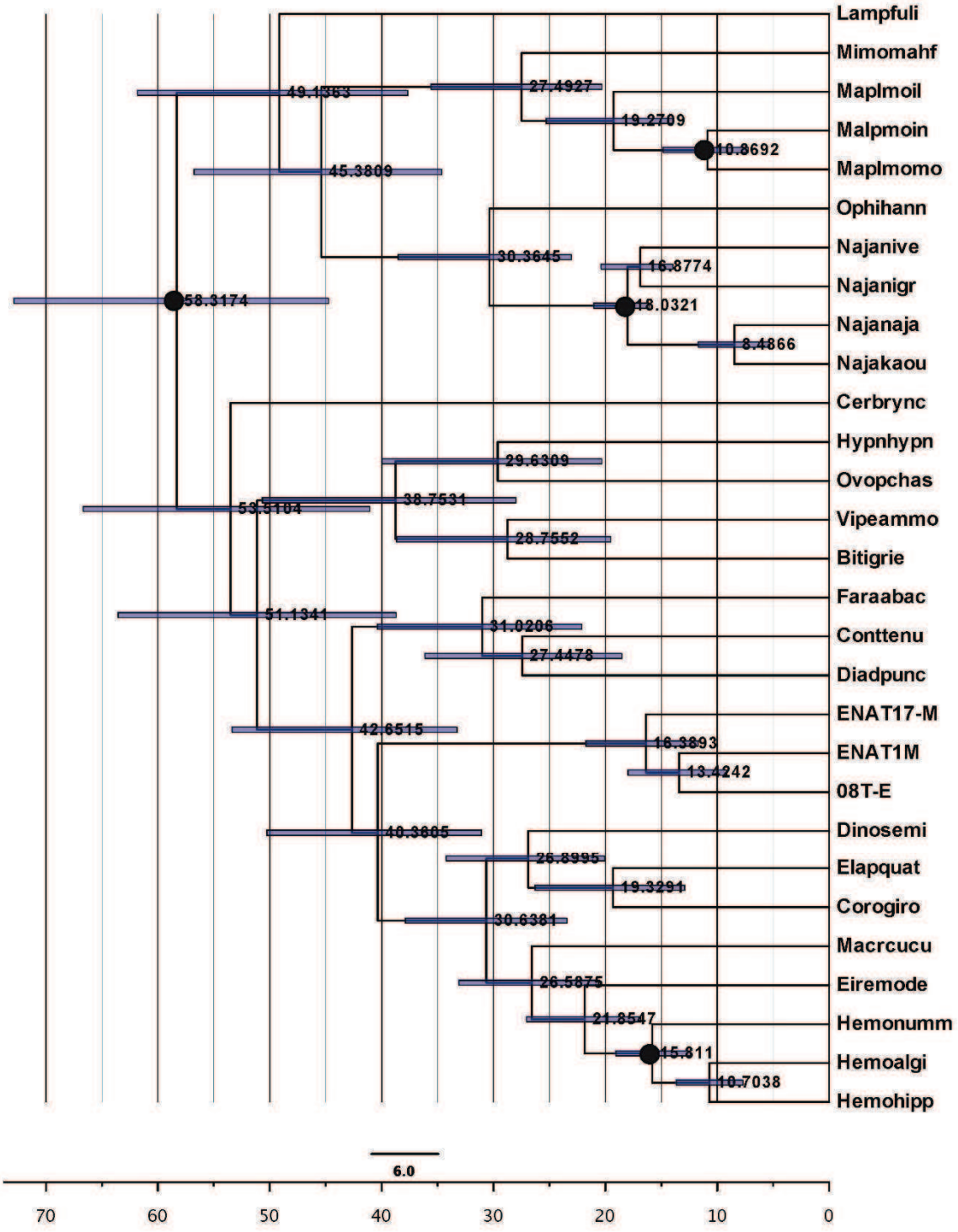
## Supporting information



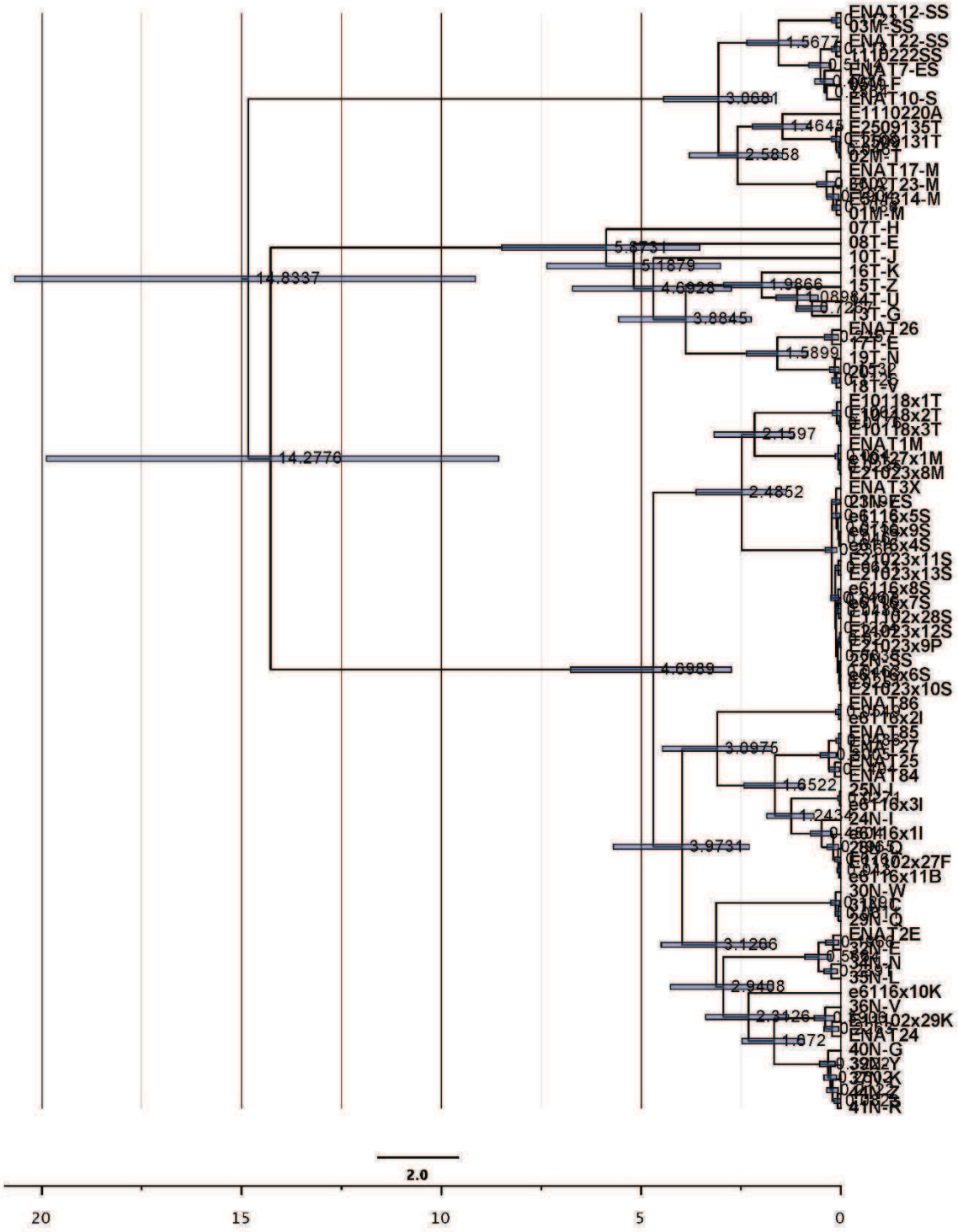



**Figure S1.** (A) Chronogram corresponding to the first analysis of the two-step approach for the estimation of divergence times (see Materials and Methods). Nodes marked with black circles indicate calibration points applied. (B) Ultrametric tree of the genus *Natrix*, calibrated at the root using the age estimated in the first step. In both trees, mean ages appear next to the nodes and horizontal bars correspond to 95%HPD.

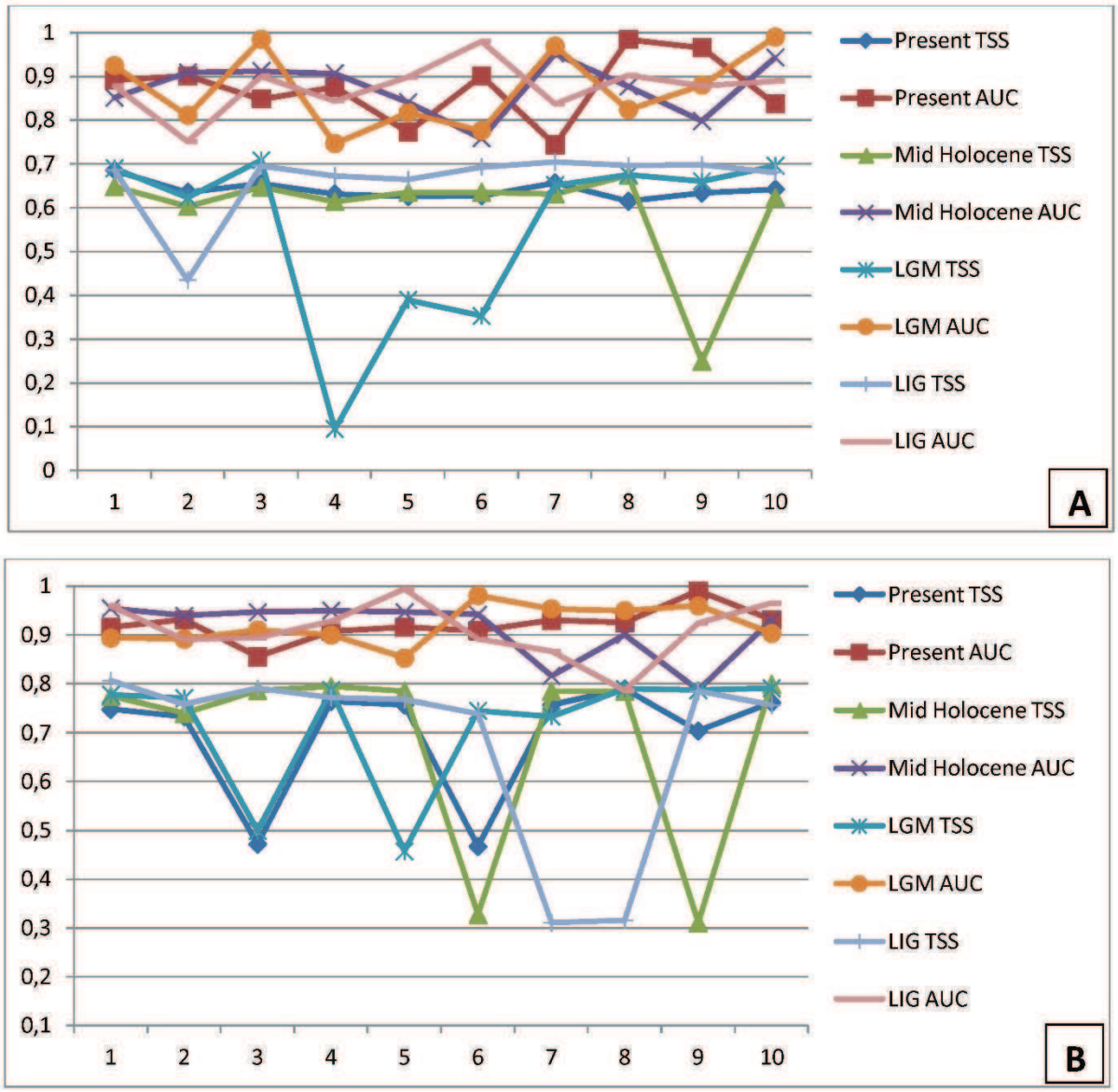
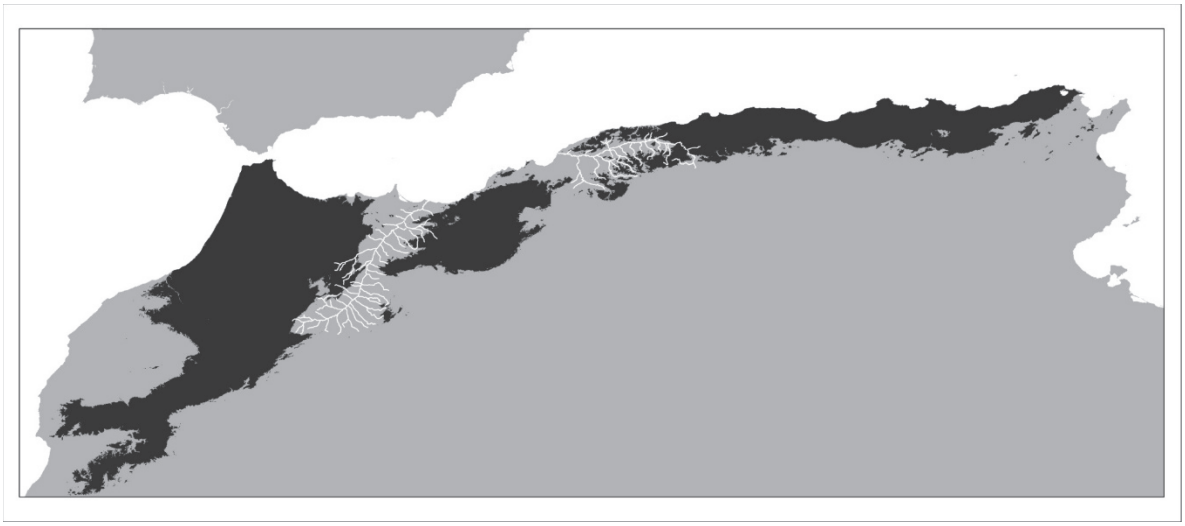



**Figure S2.** Test AUC and TSS values for the individual models calibrated using backgrounds A and B.

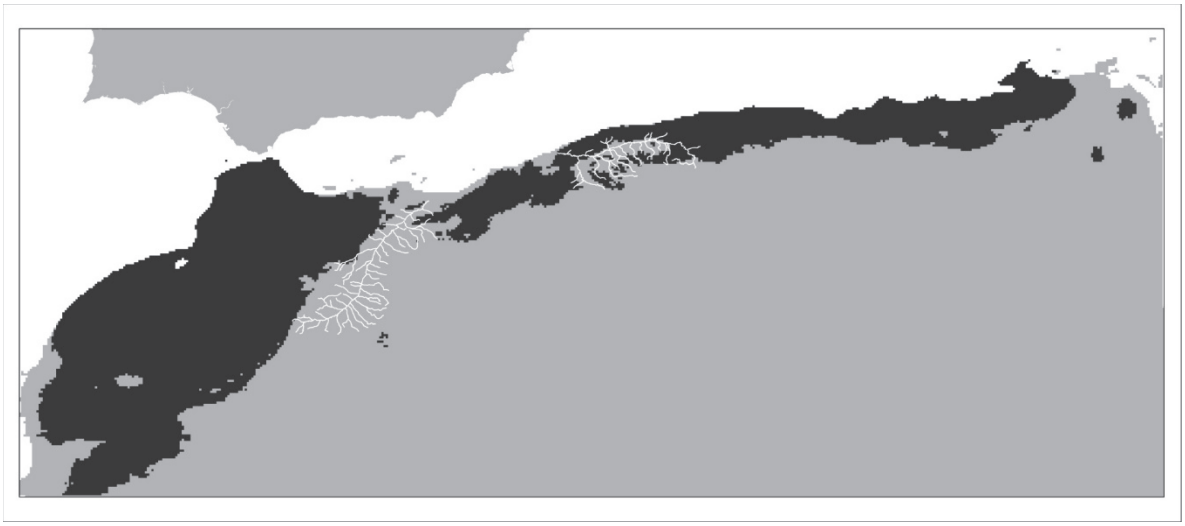
**Figure S3A**. The SDM of *Natrix natrix* in the Maghreb for each time period (Present, Mid-Holocene, Last Glacial Maximum and Last Inter Glacial) and background A under the ten percentile threshold. Dark grey areas indicate areas of suitability. The Moulouya River and Chelif River are indicated.

*Present*

*Mid-Holocene*



*Last  Glacial Maximum*

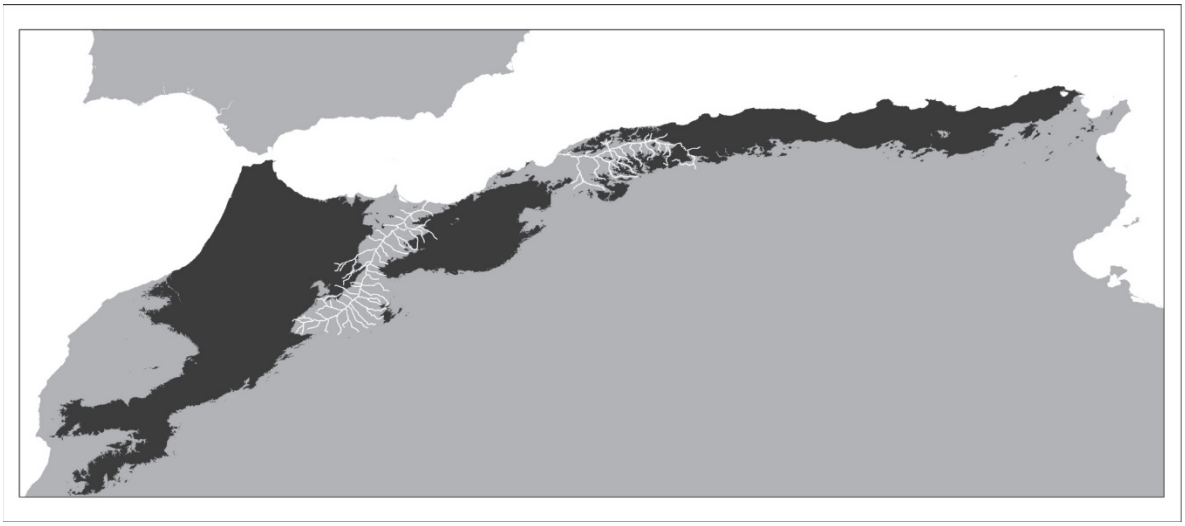


*Last Inter Glacial*

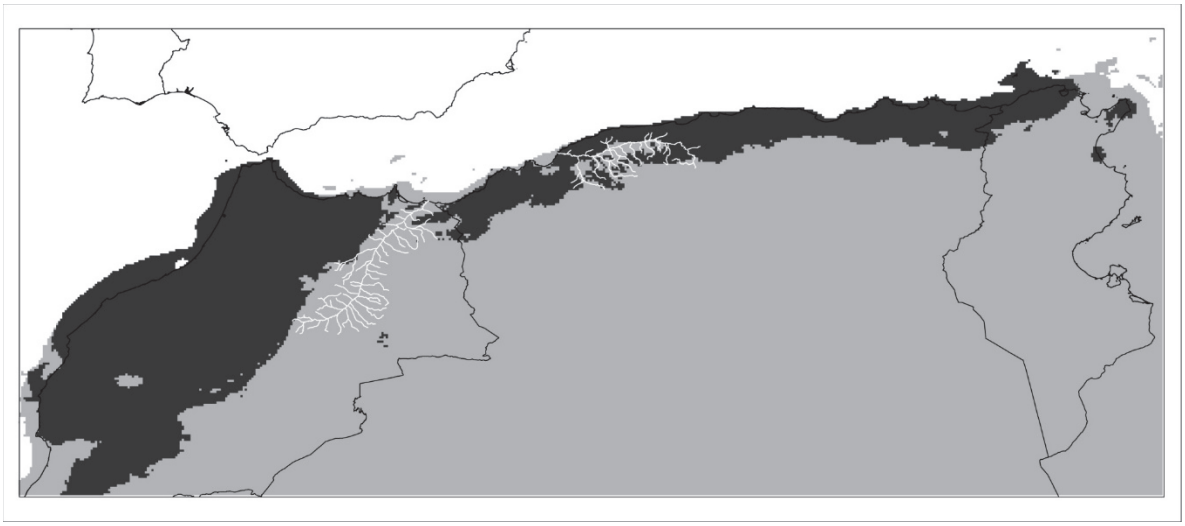
**Figure S3B**. The SDM of *Natrix natrix* in the Maghreb for each time period (Present, Mid-Holocene, Last Glacial Maximum and Last Inter Glacial) and background B under the ten percentile threshold. Dark grey areas indicate areas of suitability. The Moulouya River and Chelif River are indicated.

*Present*

*Mid-Holocene*



*Last Glacial Maximum*



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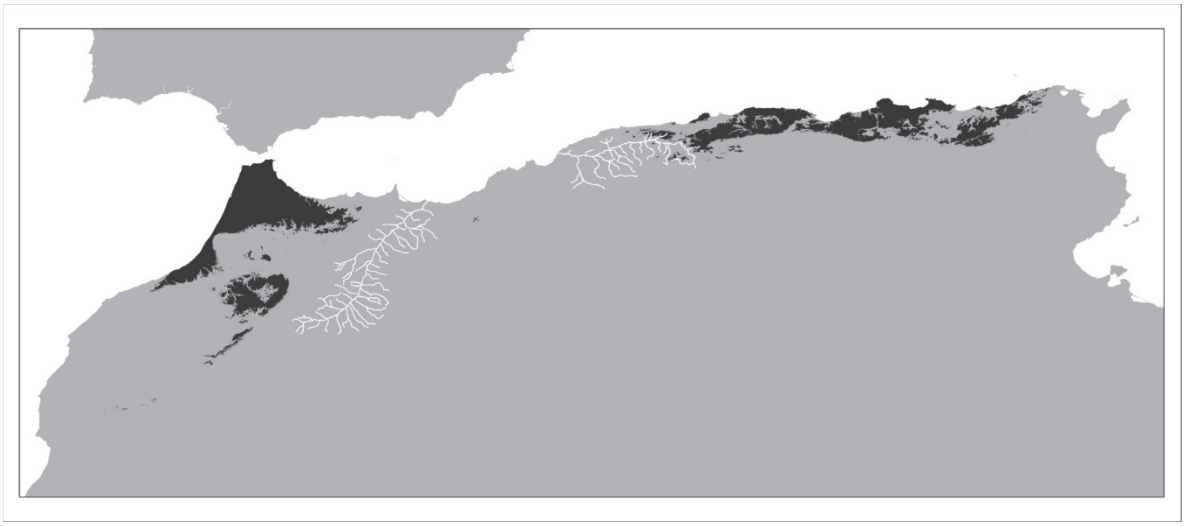
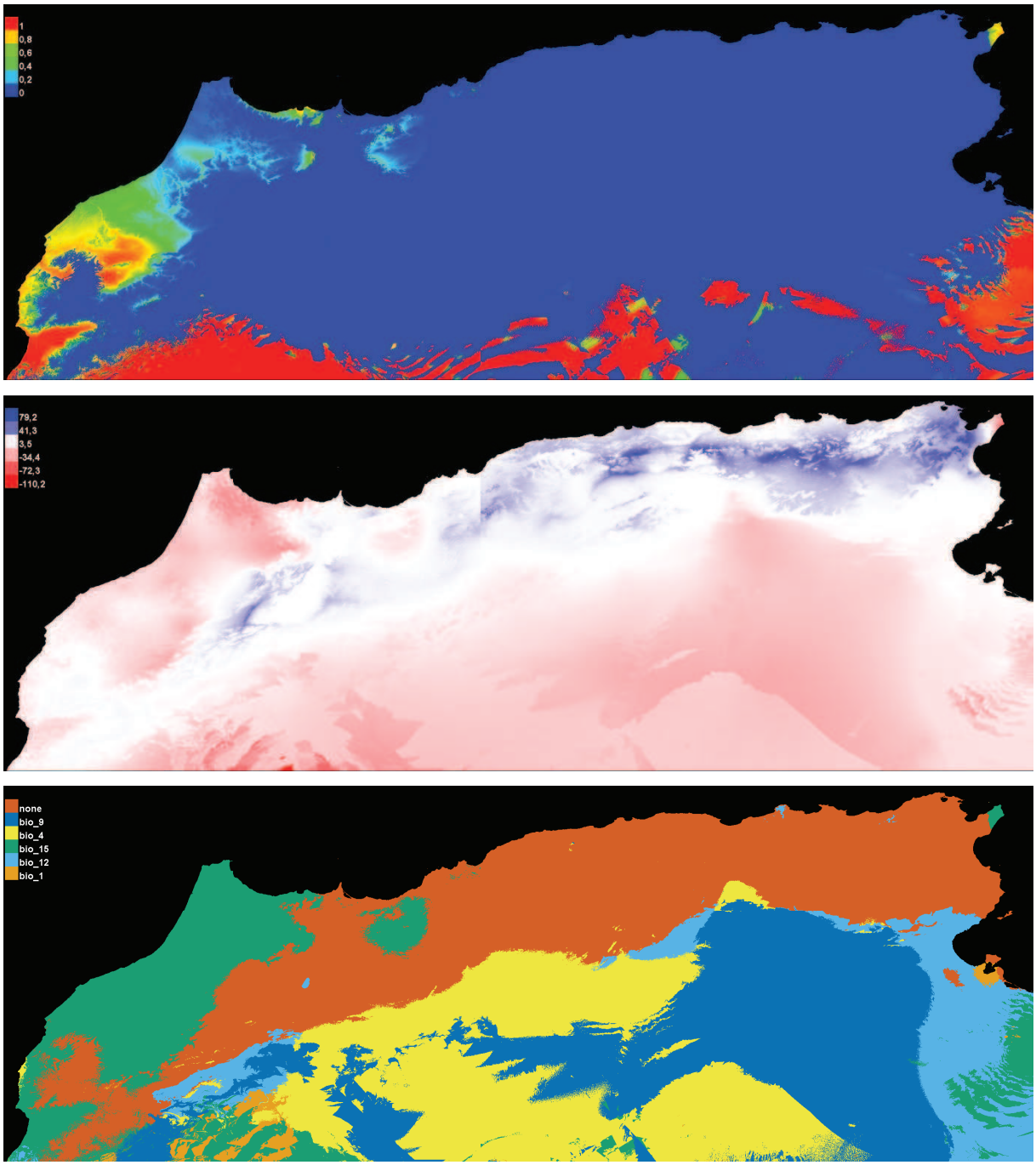


**Figure S4A.** Clamping (top), MESS (middle) and MoD (below) pictures of the projected SDMs for the (A) Mid-Holocene, (B) Last Glacial Maximum and (C) Last Inter Glacial calibrated using background A.

**A**



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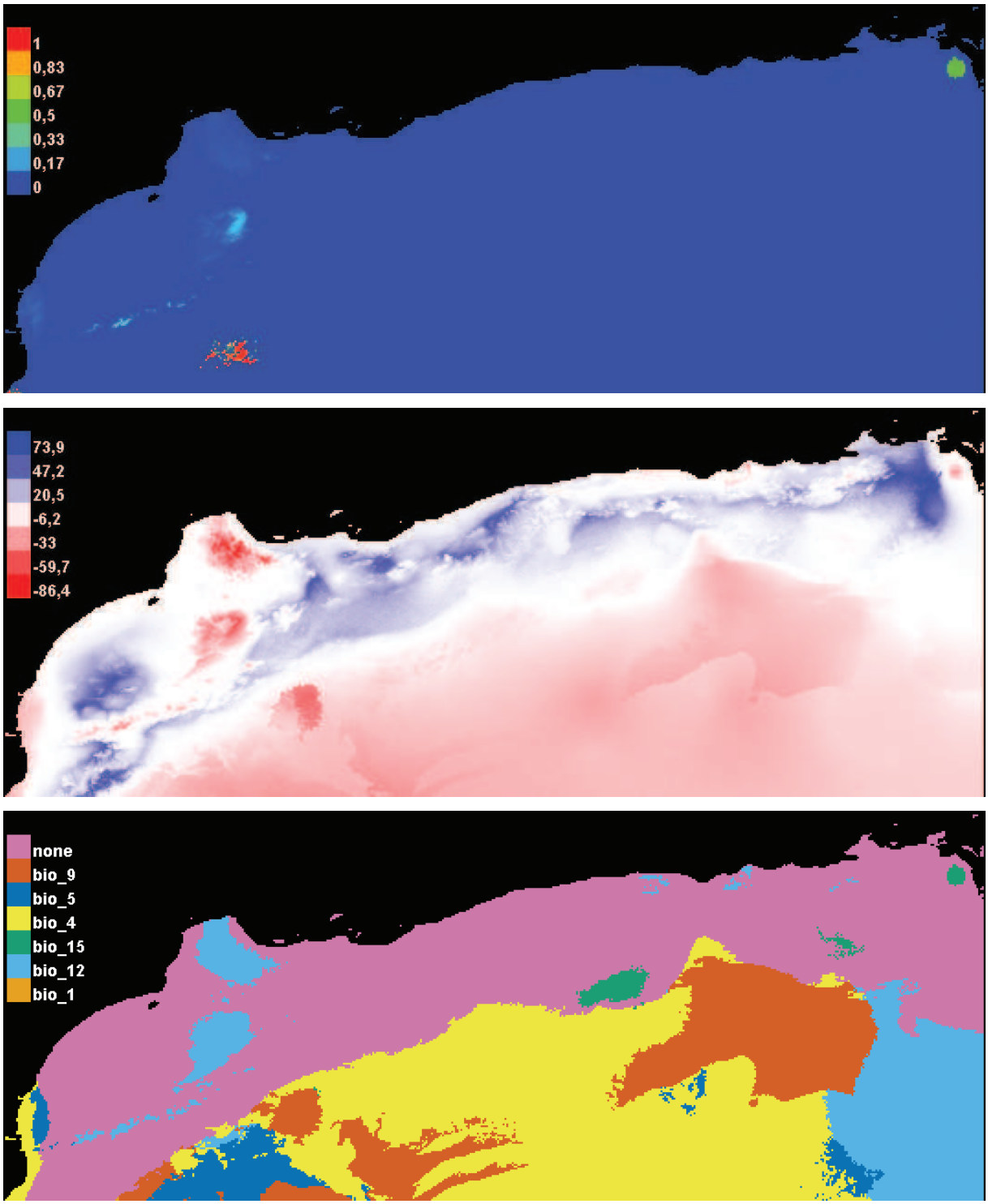
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bio_5
bio_4
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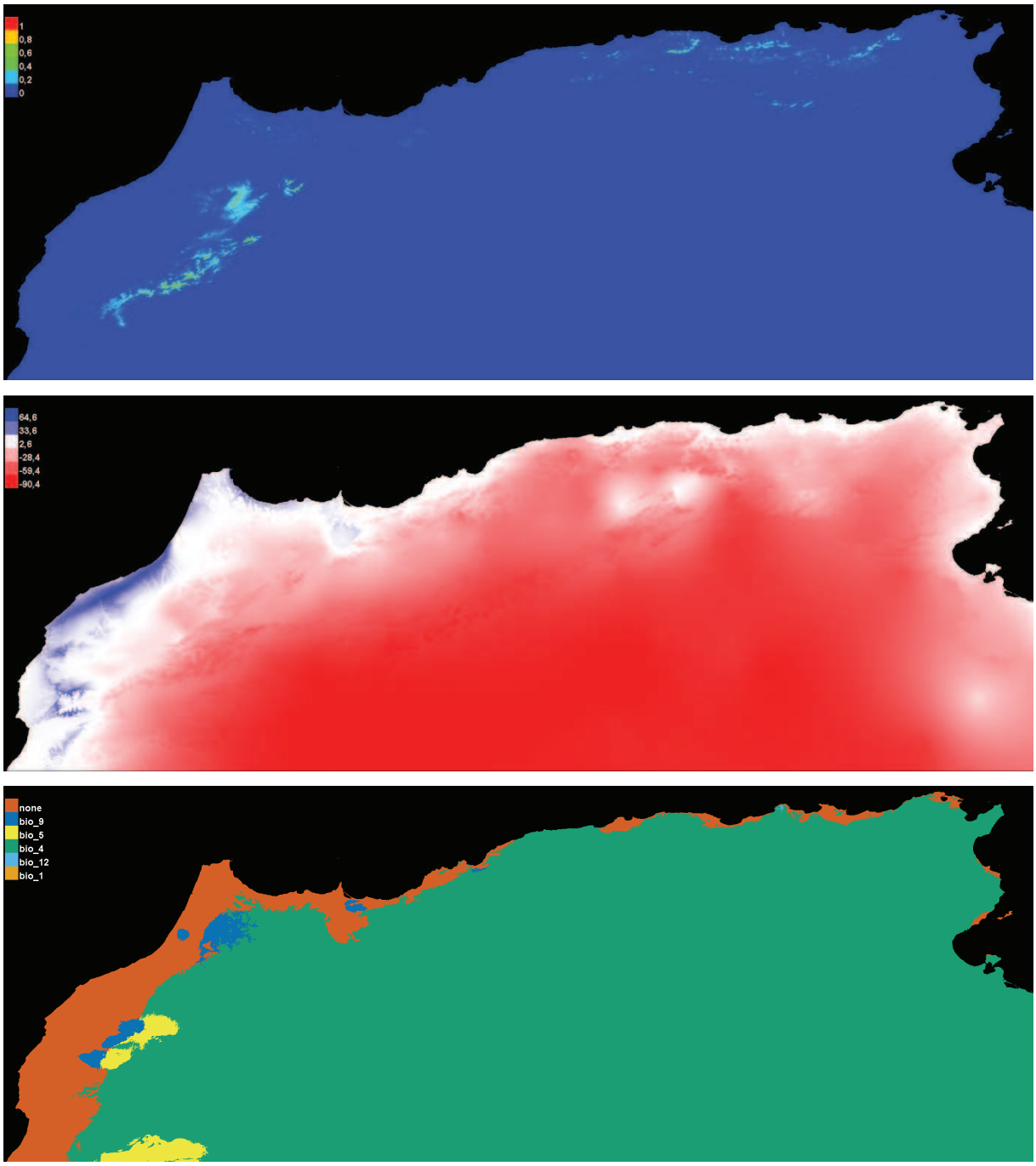
bio_12
bio_1

C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-90,4
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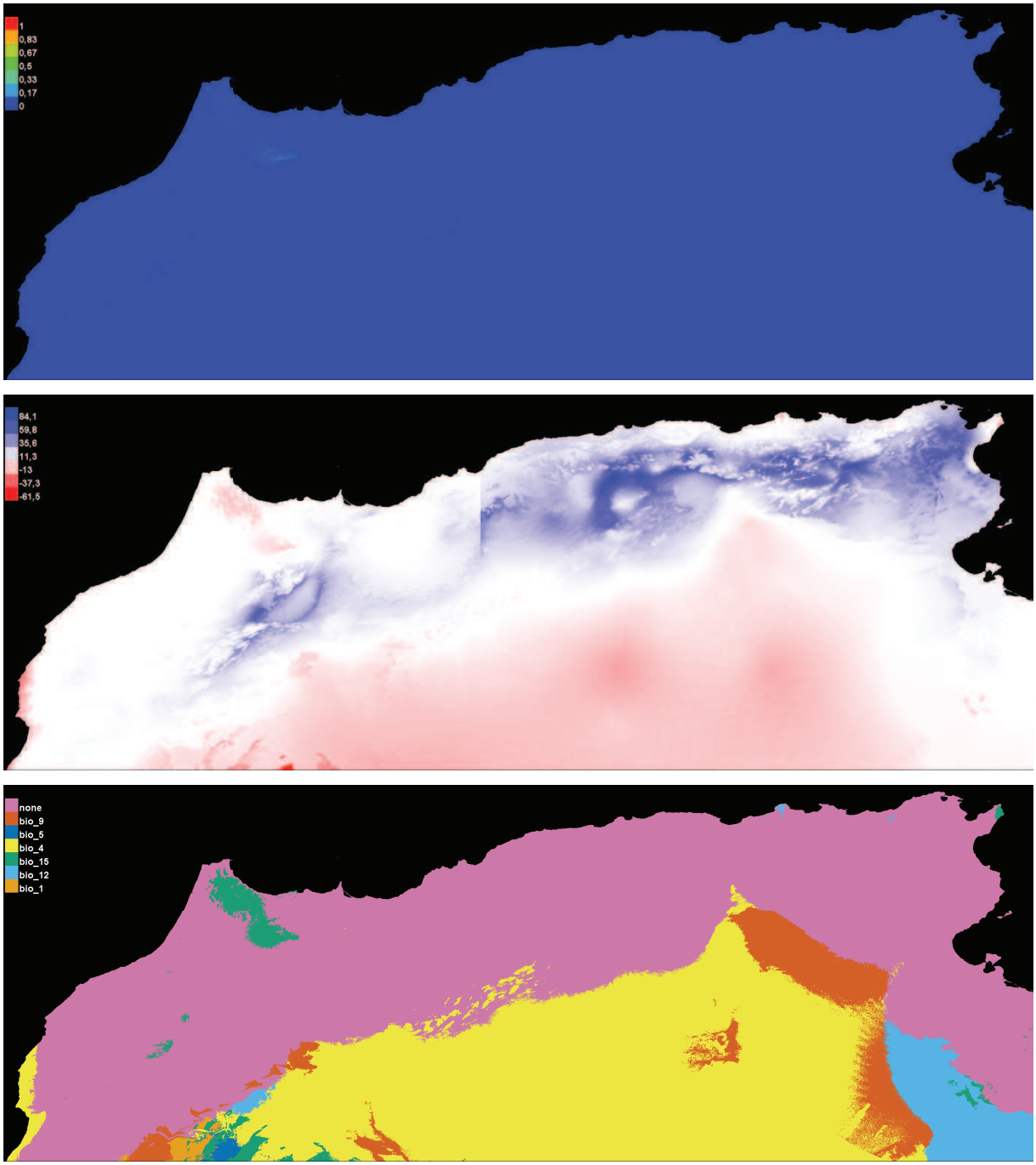
bio_9
bio_5
bio_4
bio_12
bio_1

**Figure S4B.** Clamping (top), MESS (middle) and MoD (below) pictures of the projected SDMs for the (A) Mid-Holocene, (B) Last Glacial Maximum and (C) Last Inter Glacial calibrated using background B.

**A**



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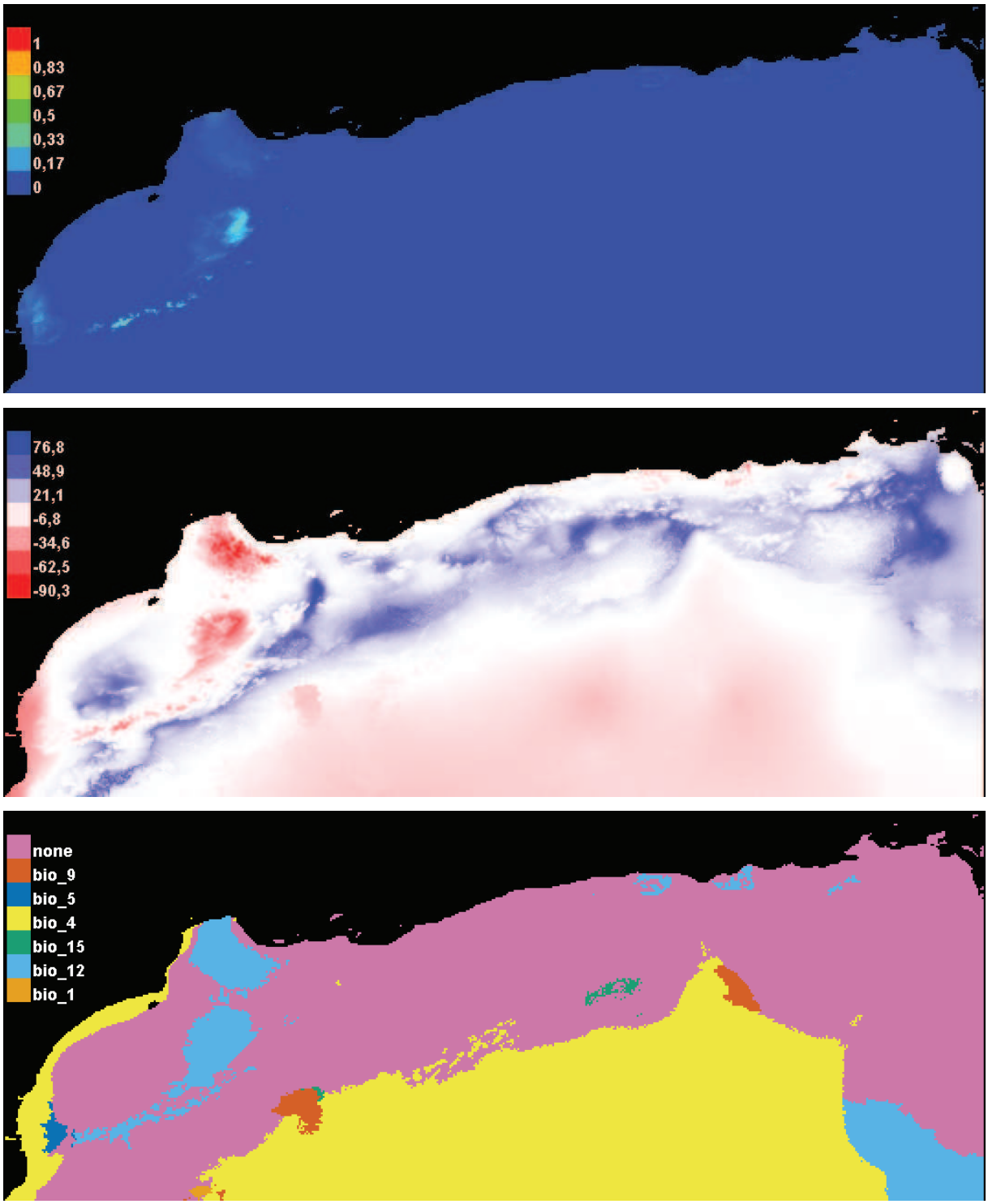
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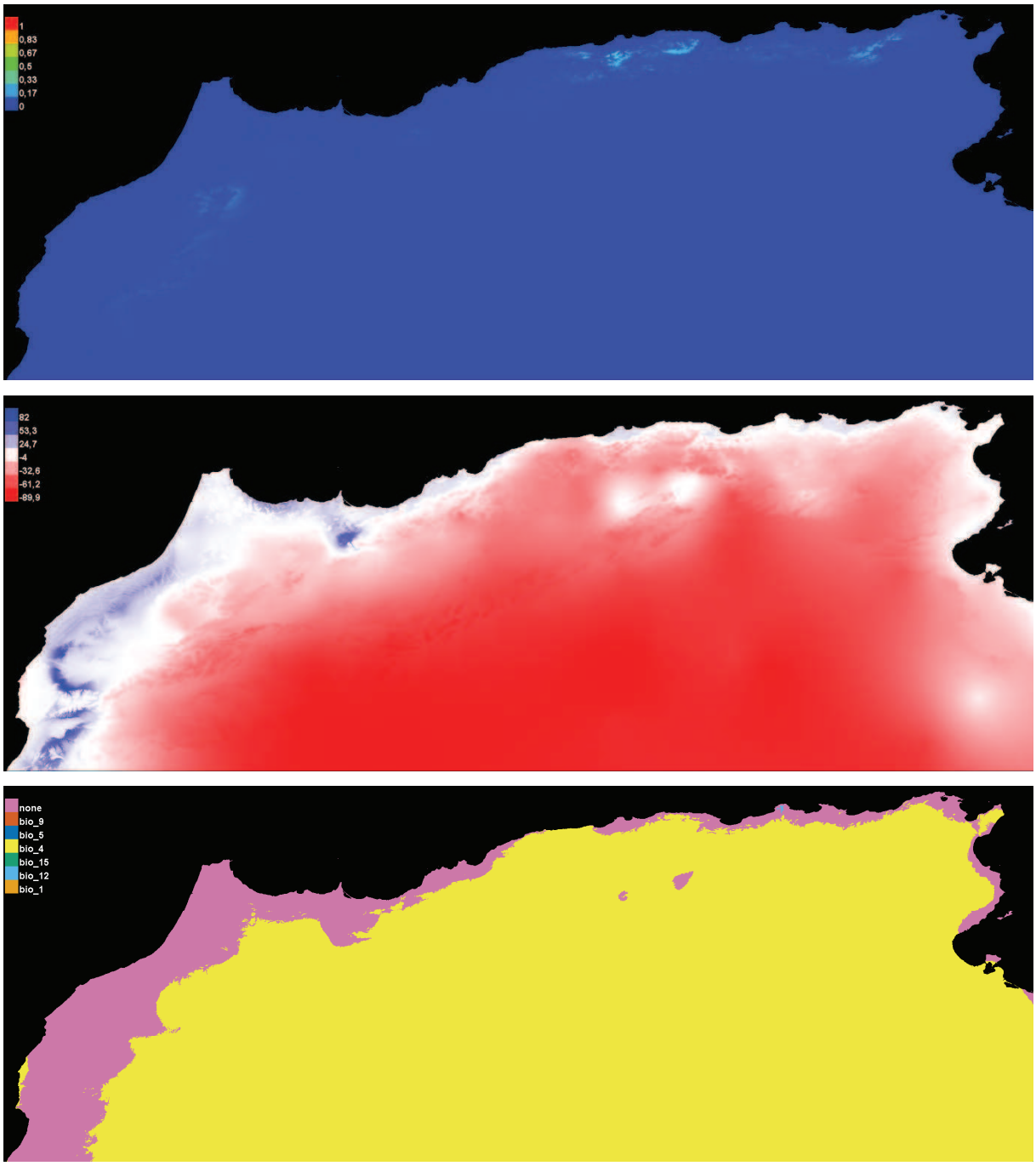
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**Table S1**. Information on the *Natrix* specimens and outgroup (Out) included in the genetic (DNA) analyses of the present study, with extraction code, country, locality data and GenBank accession codes (newly added sequences in bold). Numbers listed under the column "Locality" refer to localities shown in Fig. 1.

| Locality | Specimen | Extraction code | Country | Locality | Latitude | Longitude | Genbank (cytb) | Genbank (ND4) |
|---|---|---|---|---|---|---|---|---|
| 1 | *Natrix maura* -1 | ENAT17-M | Morocco | Guelta Tanzida. S Akka | 29.22 | -8.49 | **KC570269** | **KC570224** |
| 2 | *Natrix maura* -2 | ENAT23-M | Morocco | 3 Km North of Tounfite | 32.49 | -5.23 | **KC570268** | **KC570223** |
| 3 | *Natrix maura* -3 | E511314-M | Morocco | Khenifra | 32.94 | -5.67 | **KC570267** | **KC570222** |
| 4 | *Natrix maura* -4 | 01M-M | Morocco | Tetuan | 35.50 | -5.34 | AF420077 | AY873709 |
| 5 | *Natrix maura* -5 | E1110220A | Algeria | M'sila | 35.70 | 4.54 | **KC570277** | **KC570227** |
| 6 | *Natrix maura* -6 | E2509135T | Tunisia | 3Km S of Tabarca. Oued Quebir | 36.93 | 8.75 | **KC570276** | **KC570226** |
| 7 | *Natrix maura* -7 | E2509131T | Tunisia | 8Km W of Tunis | 36.77 | 10.03 | **KC570275** | **KC570225** |
| 8 | *Natrix maura* -8 | 02M-T | Tunisia | Tameghza | 34.23 | 7.54 | AY487681 | AY487785 |
| 9 | *Natrix maura* -9 | ENAT12-SS | Spain | Tarifa. Cadiz | 36.08 | -5.42 | **KC570270** | **KC570228** |
| 10 | *Natrix maura* -10 | 03M-SS | Spain | Cadiz province | 36.50 | -6.20 | AY866530 | AY873708 |
| 11 | *Natrix maura* -11 | 05M-F | France | Herault department | 43.15 | 2.40 | AY487698 | AY487786 |
| 12 | *Natrix maura* -12 | ENAT7-ES | Spain | Sot de les Mines. Montseny | 41.77 | 2.35 | **KC570272** | **KC570232** |
| 13 | *Natrix maura* -13 | ENAT10-S | Spain | Camping Gredos. Avila | 40.34 | -5.16 | **KC570271** | **KC570229** |
| 14 | *Natrix maura* -14 | ENAT22-SS | Spain | Rota. Cadiz | 36.62 | -6.36 | **KC570273** | **KC570230** |
| 15 | *Natrix maura* -15 | 1110222SS | Spain | Mairena/Gelves. Sevilla | 37.34 | -6.03 | **KC570274** | **KC570231** |
| 16 | *Natrix tessellata* -1 | 07T-H | Iran | Kermanshah | 34.20 | 46.45 | AY487574 | AY487789 |
| 17 | *Natrix tessellata* -2 | 08T-E | Greece | Ioannina | 39.40 | 20.50 | AY487585 | AY487787 |
| 18 | *Natrix tessellata* -3 | 10T-J | Jordan | Jarash | 32.07 | 35.54 | AY487591 | AY873732 |
| 19 | *Natrix tessellata* -4 | 16T-K | Turkey | Yenicaga | 40.46 | 32.02 | AY487593 | AY873733 |
| 20 | *Natrix tessellata* -5 | 15T-Z | Kazakhstan | Ili river | 44.03 | 77.00 | AY487603 | AY487790 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 21 | *Natrix tessellata* -6 | 14T-U | Uzbekistan | Kungrad | 42.42 | 59.18 | AY487615 | AY487791 |
| 22 | *Natrix tessellata* -7 | 13T-G | Georgia | Agara | 42.03 | 43.49 | AY487624 | AY487788 |
| 23 | *Natrix tessellata* -8 | 17T-E | Greece | Crete | 35.11 | 25.42 | AY487631 | AY873728 |
| 24 | *Natrix tessellata* -9 | ENAT26 | Greece | Crete | 35.04 | 25.98 | **KC570309** | **KC570266** |
| 25 | *Natrix tessellata* -10 | 19T-N | Romania | Cluj-Napoca | 46.46 | 23.35 | AY866534 | AY873730 |
| 26 | *Natrix tessellata* -11 | 18T-V | Bulgaria | Sozopol | 42.25 | 27.41 | AY866533 | AY873729 |
| 27 | *Natrix tessellata* -12 | 20T-I | Italy | Grado | 42.42 | 13.28 | AY487669 | AY873726 |
| 28 | *Natrix natrix* -1 | E10118x1T | Tunisia | 10 km E of Tabarka | 36.96 | 8.94 | **KC570292** | **KC570248** |
| 29 | *Natrix natrix* -2 | E10118x2T | Tunisia | 9km S of Cabo Negro | 37.05 | 9.05 | **KC570290** | **KC570246** |
| 30 | *Natrix natrix* -3 | E10118x3T | Tunisia | Cabo Negro | 37.10 | 9.03 | **KC570291** | **KC570247** |
| 31 | *Natrix natrix* -4 | ENAT1M | Morocco | 30km NW of Chefchaouen | 35.27 | -5.49 | **KC570295** | **KC570251** |
| 32 | *Natrix natrix* -5 | E21023x8M | Morocco | 1km SW of Derdara. Rif | 35.10 | -5.30 | **KC570293** | **KC570249** |
| 33 | *Natrix natrix* -6 | e10127x1M | Morocco | 6km SE of Koudia El Mal | 35.37 | -5.57 | **KC570294** | **KC570250** |
| 34 | *Natrix natrix* -7 | 23N-ES | Spain | Osona | 41.51 | 2.12 | AY866536 | AY873713 |
| 35 | *Natrix natrix* -8 | e6116x9S | Spain | Trevinyo | 42.73 | -2.74 | **KC570288** | **KC570237** |
| 36 | *Natrix natrix* -9 | e6116x5S | Spain | Reparade. Ourense | 41.94 | -8.02 | **KC570287** | **KC570244** |
| 37 | *Natrix natrix* -10 | e6116x4S | Spain | Cilleruelo de Bezana. Burgos | 42.97 | -3.85 | **KC570289** | **KC570238** |
| n/p* | *Natrix natrix* -11 | ENAT3X | Spain | | - | - | **KC570310** | **KC570239** |
| 38 | *Natrix natrix* -12 | E21023x9P | Portugal | Santo Aleixo Restauracao | 38.06 | -7.15 | **KC570281** | **KC570235** |
| 39 | *Natrix natrix -13* | E21023x12S | Spain | Higuera de Vargas. Badajoz | 38.45 | -6.97 | **KC570282** | **KC570236** |
| 40 | *Natrix natrix -14* | E21023x10S | Spain | Aeropuerto de Sevilla | 37.42 | -5.89 | **KC570278** | **KC570233** |
| 41 | *Natrix natrix -15* | e6116x6S | Spain | Rio Guadalete | 36.76 | -5.36 | **KC570279** | **KC570234** |
| 42 | *Natrix natrix -16* | 22N-SS | Spain | Cadiz province | 36.00 | -6.00 | **AY866535** | **AY873714** |
| 43 | *Natrix natrix -17* | E21023x11S | Spain | Ayedo Fresnedilla. Jaen | 38.05 | -2.93 | **KC570284** | **KC570243** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 44 | *Natrix natrix -18* | E21023x13S | Spain | Rio Guadalmez. Cordoba | 38.53 | -4.65 | **KC570285** | **KC570245** |
| 45 | *Natrix natrix -19* | E11102x28S | Spain | Orijiva. Granada | 36.90 | -3.42 | **KC570283** | **KC570240** |
| 46 | *Natrix natrix -20* | e6116x8S | Spain | Tremp | 42.17 | 0.89 | **KC570286** | **KC570241** |
| 47 | *Natrix natrix -21* | e6116x7S | Spain | San Juan de la Peña | 42.52 | 0.70 | **KC570280** | **KC570242** |
| 48 | *Natrix natrix -22* | ENAT86 | Italy | Serro San Bruno. Calabria | 38.58 | 16.33 | **KC570305** | **KC570265** |
| 49 | *Natrix natrix -23* | e6116x2I | Italy | Near Castelo di Marescotto | 37.97 | 14.59 | **KC570304** | **KC570264** |
| 50 | *Natrix natrix -24* | ENAT85 | Italy | Sardinia | 39.28 | 9.42 | **KC570303** | **KC570258** |
| 51 | *Natrix natrix -25* | ENAT27 | Italy | Sardinia | 39.28 | 9.44 | **KC570302** | **KC570257** |
| 52 | *Natrix natrix -26* | ENAT84 | France | Corsica | 41.86 | 9.37 | **KC570301** | **KC570259** |
| n/p* | *Natrix natrix -27* | ENAT25 | France | Corsica | - | - | **KC570300** | **KC570256** |
| 53 | *Natrix natrix -28* | e6116x3I | Italy | 20Km NW of Asiago | 45.97 | 11.40 | **KC570299** | **KC570255** |
| 54 | *Natrix natrix -29* | 25N-I | Italy | Ticino | 46.00 | 8.48 | **AY487751** | **AY487795** |
| 55 | *Natrix natrix -30* | 24N-I | Italy | Torre San Gennaro | 40.32 | 18.05 | **AY487733** | **AY873715** |
| 56 | *Natrix natrix -31* | e6116x1I | Italy | 1.5Km E of Campagrina | 44.06 | 10.27 | **KC570298** | **KC570254** |
| 57 | *Natrix natrix -32* | e6116x11B | UK | Brandon. Norwich | 52.63 | 1.29 | **KC570297** | **KC570253** |

**Table S2**. List of samples used in the first analysis of the two-step approach for the estimation of divergence times (see Materials and Methods), with their corresponding GenBank accession codes.

| Taxon | Genbank Accession Codes | | Code Fig. S1 |
|---|---|---|---|
| | cytb | ND4 | |
| *Bitis arietans* | EU624304 | EU624213 | Bitigrie |
| *Cerberus rynchops* | AF471092 | U49327 | Cerbrync |
| *Contia tenuis* | AF471095 | AF402656 | Conttenu |
| *Coronella girondica* | AF471088 | AY487066 | Corogiro |
| *Diadophis punctatus* | AF471094 | DQ364667 | Diadpunc |
| *Dinodon semicarinatus* | AB008539 | AB008539 | Dinosemi |
| *Eirenis modestus* | AY486933 | AY487072 | Eiremode |
| *Elaphe quatorlineata* | AY486931 | AY487067 | Elapquat |
| *Farancia abacura* | U69832 | U49307 | Faraabac |
| *Hemorrhois algirus* | AY486911 | AY487037 | Hemoalgi |
| *Hemorrhois hippocrepis* | AY486916 | AY487045 | Hemohipp |
| *Hemorrhois nummifer* | AY376742 | AY487049 | Hemonumm |
| *Hypnale hypnale* | AY223561 | U41884 | Hypnhypn |
| *Lamprophis fuliginosus* | AF471060 | AF544664 | Lampfuli |
| *Macroprotodon cuculatus* | AF471087 | AY487064 | Macrcucu |
| *Malpolon insignitus* | AY188029 | FJ404320 | Malpmoin |
| *Malpolon moilensis* | DQ486333 | DQ486309 | Malpmoil |
| *Malpolon monspessulanus* | AY058965 | AY058989 | Maplmomo |
| *Mimophis mahfalensis* | AY188032 | AF544662 | Mimomahf |
| *Naja kaouthia* | EU624298 | EU624209 | Najakaou |
| *Naja naja* | EU624299 | AY713378 | Najanaja |
| *Naja nigricollis* | EU624300 | AY713377 | Najanigr |
| *Naja nivea* | AF217827 | AY058983 | Najanive |
| *Natrix maura* | KC570269 | KC570224 | ENAT17M |
| *Natrix natrix* | KC570295 | KC570251 | ENAT1M |
| *Natrix tessellata* | AY487585 | AY487787 | 08T-E |
| *Ophiophagus hannah* | AF217842 | AY058984 | Ophihann |
| *Ovophis chaseni* | AY352760 | AY352825 | Ovopchas |
| *Vipera ammodytes* | EU624314 | EU624232 | Vipeammo |

**Table S3**. The results of the SDMs for each time period, with the test and train AUC, null model results, standard deviation (SD) and ten percentile threshold (TPT) values.

| ***Backround A*** | **Test AUC** | **Train AUC** | **Null model 100: Train AUC median (95% CI)** | **SD** | **10 percentile** |
|---|---|---|---|---|---|
| Present | 0.8966 | 0.8717 | 0.682 (0.676-0.692) | 0.0512 | 0.2909 |
| Mid Holocene | 0.9006 | 0.8745 | 0.682 (0.676-0.692) | 0.0526 | 0.2893 |
| Last Glacial Maximum CCSM | 0.912 | 0.873 | 0.682 (0.676-0.692) | 0.0562 | 0.2406 |
| Last Glacial Maximum MIROC | 0.912 | 0.8747 | 0.682 (0.676-0.692) | 0.0446 | 0.243 |
| Last Glacial Maximum ENSEMBLE | 0.9128 | 0.8718 | 0.687 (0.677-0.694) | 0.0444 | 0.2495 |
| Last Inter Glacial | 0.9107 | 0.8756 | 0.687 (0.677-0.694) | 0.0526 | 0.3448 |
| ***Background B*** | **Test AUC** | **Train AUC** | | **SD** | **10 percentile** |
| Present | 0.9417 | 0.9208 | 0.680 (0.673-0.689) | 0.0407 | 0.2668 |
| Mid Holocene | 0.9431 | 0.911 | 0.680 (0.673-0.689) | 0.0354 | 0.2935 |
| Last Glacial Maximum CCSM | 0.9369 | 0.9004 | 0.680 (0.673-0.689) | 0.024 | 0.2525 |
| Last Glacial Maximum MIROC | 0.9378 | 0.9167 | 0.680 (0.673-0.689) | 0.0388 | 0.2709 |
| Last Glacial Maximum ENSEMBLE | 0.9403 | 0.9193 | 0.681 (0.670-0.687) | 0.0358 | 0.2836 |
| Last Inter Glacial | 0.9432 | 0.9097 | 0.681 (0.670-0.687) | 0.0372 | 0.2729 |

**Table S4**. Localities of *Natrix natrix* from North Africa used for species distribution modelling.

Species,Lat,Long
Natrix,35.712061001,-5.489186436
Natrix,35.351630052,-5.479031905
Natrix,35.261443466,-5.474125648
Natrix,35.267425503,-5.146914778
Natrix,35.164708722,-5.254492018
Natrix,35.015768141,-4.967811451
Natrix,35.066268177,-5.186849505
Natrix,34.911340125,-4.81094338
Natrix,35.002959607,-4.703504021
Natrix,35.002088504,-4.597770462
Natrix,34.915575106,-4.59095153
Natrix,35.007373654,-4.372952078
Natrix,33.715506158,-5.351041958
Natrix,33.533333125,-5.082486972
Natrix,33.416039168,-5.999034868
Natrix,32.388515635,-6.066604727
Natrix,32.479268489,-6.008965768
Natrix,32.497791652,-6.103434722
Natrix,32.115427721,-6.467860686
Natrix,36.604911668,2.990690994
Natrix,36.759685234,3.501696405
Natrix,36.921359732,7.683845095
Natrix,36.756747013,8.322800714
Natrix,36.721184048,8.694662025
Natrix,36.955817237,8.934312831
Natrix,37.048626226,9.051456088

# Chapter 4

## Review of the systematics, distribution, biogeography and natural history of Moroccan amphibians

Journal: *Zootaxa* 3661: 001-060

Authors: Wouter Beukema, Philip de Pous, David Donaire-Barroso, Sergé Bogaerts, Joan Garcia-Porta, Daniel Escoriza, Oscar J. Arribas, El Hassan El Mouden and Salvador Carranza

The amphibian fauna of the Kingdom of Morocco was traditionally regarded as poor and closely related to its European counterpart. However, an increase in research during the last decades revealed a considerable degree of endemism amongst Moroccan amphibians, as well as phenotypic and genotypic inter- and intraspecific divergence. Despite this in- crease in knowledge, a comprehensible overview is lacking while several systematic issues have remained unresolved. We herein present a contemporary overview of the distribution, taxonomy and biogeography of Moroccan amphibians. Fourteen fieldtrips were made by the authors and colleagues between 2000 and 2012, which produced a total of 292 new distribution records. Furthermore, based on the results of the present work, we (i) review the systematics of the genus *Salamandra* in Morocco, including the description of a new subspecies from the Rif- and Middle Atlas Mountains, *Salamandra algira splendens* ssp. nov.; (ii) present data on intraspecific morphological variability of *Pelobates varaldii* and *Pleurodeles waltl* in Morocco; (iii) attempt to resolve the phylogenetic position of *Bufo brongersmai* and erect a new genus for this species, *Barbarophryne* gen. nov.; (iv) summarize and assess the availability of tadpole-specific characteristics and bioacoustical data, and (v) summarize natural history data.

**Author contribution**: Second authorship reflects that I was the second main contributor to the paper. I have developed the concept together with Wouter Beukema and extensively contributed to the writing. I have also conducted the bioacoustic and morphological analyses.

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# ZOOTAXA

**3661**

## Review of the systematics, distribution, biogeography and natural history of Moroccan amphibians

WOUTER BEUKEMA[1,*], PHILIP DE POUS[2,3,4], DAVID DONAIRE-BARROSO[5], SERGÉ BOGAERTS[6], JOAN GARCIA-PORTA[3], DANIEL ESCORIZA[7], OSCAR J. ARRIBAS[8], EL HASSAN EL MOUDEN[9] & SALVADOR CARRANZA[3]

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## Table of contents



## Abstract


The amphibian fauna of the Kingdom of Morocco was traditionally regarded as poor and closely related to its European counterpart. However, an increase in research during the last decades revealed a considerable degree of endemism amongst Moroccan amphibians, as well as phenotypic and genotypic inter- and intraspecific divergence. Despite this increase in knowledge, a comprehensible overview is lacking while several systematic issues have remained unresolved. We herein present a contemporary overview of the distribution, taxonomy and biogeography of Moroccan amphibians.

Fourteen fieldtrips were made by the authors and colleagues between 2000 and 2012, which produced a total of 292 new distribution records. Furthermore, based on the results of the present work, we (i) review the systematics of the genus *Salamandra* in Morocco, including the description of a new subspecies from the Rif- and Middle Atlas Mountains, *Salamandra algira splendens* ssp. nov.; (ii) present data on intraspecific morphological variability of *Pelobates varaldii* and *Pleurodeles waltl* in Morocco; (iii) attempt to resolve the phylogenetic position of *Bufo brongersmai* and erect a new genus for this species, *Barbarophryne* gen. nov.; (iv) summarize and assess the availability of tadpole-specific characteristics and bioacoustical data, and (v) summarize natural history data.



**Key words:** North Africa, Maghreb, Amphibia, Anura, Urodela, tadpole, identification key

**Résumé**

La faune des amphibiens du royaume du Maroc a longtemps été considérée comme appauvrie et très proche de la faune européenne d'amphibiens. Cependant, une activité de recherche accrue au cours des dernières décennies a mis en évidence un fort niveau d'endémisme chez les amphibiens marocains, ainsi que de nombreux exemples de divergence phénotypique ou génétique entre espèces et au sein des espèces. Malgré cette accroissement de nos connaissances, on ne dispose toujours pas d'une synthèse exhaustive sur les amphibiens du Maroc et plusieurs incertitudes systématiques demeurent. Nous présentons ici une synthèse actualisée sur la distribution, la taxonomie et la biogéographie des amphibiens du Maroc.

Quatorze missions de terrains réalisées par les auteurs et des collègues entre 2000 et 2012 ont produit un total de 292 nouvelles données de distribution. De plus, nous (i) réévaluer la systématique du genre *Salamandra* au Maroc, inclus la description d'une nouvelle sous-espèce de *S. algira* du Rif et du Moyen-Atlas, *Salamandra algira splendens* ssp. nov.; (ii) présentons des données sur la variabilité morphologique intraspécifique des *Pelobates varaldii* et *Pleurodeles waltl* au Maroc; (iii) réévaluer la position phylogénétique de *Bufo brongersmai* et d'ériger un nouveau genre pour cette espèce, *Barbarophryne* gen. nov.; (iv) résumer et évaluer la disponibilité des caractéristiques spécifiques du têtards et des données bioacoustique par espèce, et (v) résument les données d'histoire naturelle en mettant l'accent sur ??les modèles d'activité.

## Introduction

The Kingdom of Morocco (henceforth Morocco) is one of the best studied countries on the African continent in terms of herpetology. Located at the north-western corner of the continent, Morocco is characterized by an exceptional heterogeneous landscape on a relatively small surface, encompassing temperate lowlands, humid mountain ranges, steppes and deserts (Bons & Geniez 1996). Preliminary inventories of Moroccan reptiles and amphibians started to appear during the mid to late 19th century, while earlier herpetological attention for the Maghreb (i.e. north-western Africa) had mostly been given to neighbouring Algeria (review in Boulenger 1891). These early contributions chiefly comprised compilations of collected specimens, presented species descriptions and provided minor notes on natural history (e.g. Boettger 1874, 1883; Camerano 1878; Boulenger 1889, 1891). However, as the amphibian fauna of Morocco was traditionally considered to be poor in diversity and closely related to its European counterpart (Boulenger 1891), this group benefited less of research efforts compared to the highly diverse reptilian fauna. The initial exploration and on-going description and analyses of Moroccan amphibian diversity can therefore be divided into two phases (see also Fig. 1.); (1) an early exploratory phase, approximately between the mid-19th century and the early 20th century; (2) a descriptive- and analytical period from the mid-20th up to now, in which phylogenetic, morphological, geospatial and distributional analyses are carried out in order to explore intra- and interspecific divergence and systematics. What follows is a summary of the history of amphibian studies in Morocco.

### Initial exploration (1870–1950)

As earlier work had mainly focussed on neighbouring Algeria, the first confirmed record of an amphibian species in Morocco was made by Boettger (1874), concerning '*Pleurodeles waltlii*' (=*Pleurodeles waltl* Michahelles, 1830). A few years later Camerano (1878) described *Discoglossus scovazzi* (which was however synonymized by Boulenger (1891) with *D. pictus*) in a work dedicated to Moroccan anurans, and recorded the presence of '*Rana esculenta*' (=*Pelophylax saharicus* (Boulenger 1913)), '*Bufo vulgaris*' (=*Bufo bufo* (Linnaeus 1758)), '*Bufo pantherinus*' (=*Amietophrynus mauritanicus* (Schlegel 1841), Fig. 2) and '*Hyla arborea*' (=*Hyla meridionalis* Boettger 1874). Boettger (1883) and Boulenger (1889) then added respectively '*Bufo viridis*' (=*Bufotes boulengeri* (Lataste 1879)) and '*Salamandra maculosa*' (=*Salamandra algira* Bedriaga 1883, Fig. 2) to the list of Moroccan amphibians, thereby compiling a total of eight amphibian species by the end of the 19th century (Boulenger 1891). During the early 20th century, Pellegrin (1925) presented evidence on the occurrence of the genus *Pelobates* Wagler 1830 in Morocco, while Galan (1931) noted the presence of the genus *Alytes* Wagler 1830 in the northern part of the country. Currently, these records are attributed to *Pelobates varaldii* Pasteur & Bons (1959) and *Alytes maurus* Pasteur & Bons (1962) which are considered to be cryptic species, hence their relatively late discovery. Apart from the North African endemic *A. mauritanicus*, all amphibians were at that time still considered to be conspecific with their European counterparts. The Moroccan herpetofauna was generally divided into geographical zones based on

biotic assemblages (e.g. Boulenger 1891; Werner 1929, 1931), without much attention for historical or biogeographical causes, which led to the observed pattern. Boettger (1883) was one of the few who proposed historical herpetofaunal exchange between Europe and North Africa during the Pliocene glaciations, by means of land bridges.

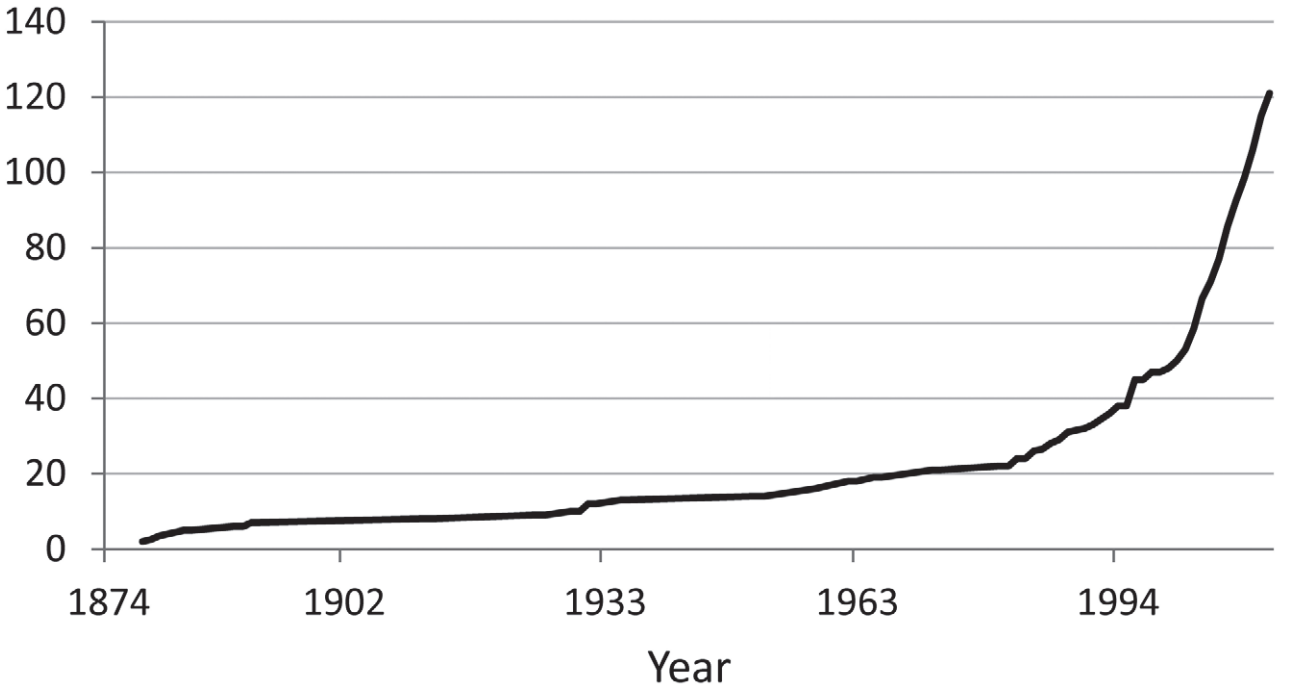


**FIGURE 1.** Cumulative number of studies (including *im partim*) involving Moroccan amphibians treated in the current manuscript.

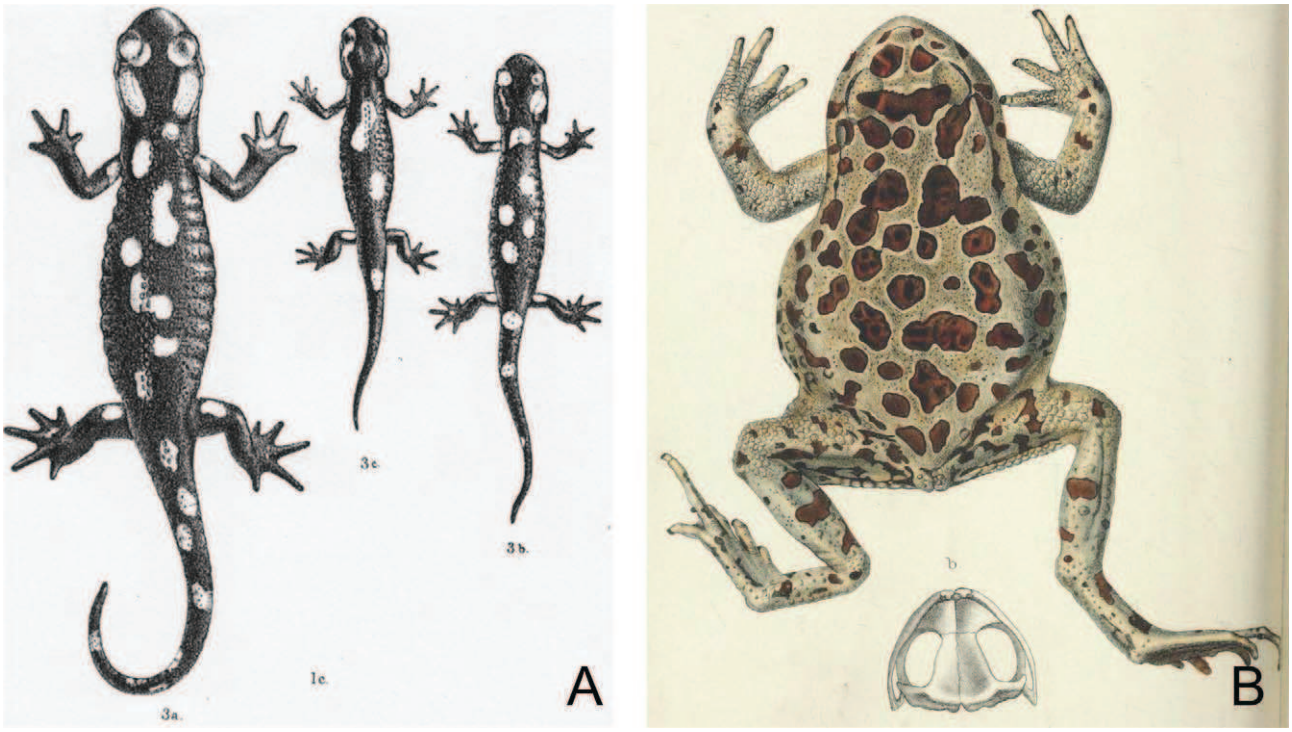


**FIGURE 2.** Historical drawings of Moroccan amphibians. A: *Salamandra algira tingitana* by Boulenger (1891); B: *Amietophrynus mauritanicus* by Boulenger (1881 "1880").

**Ongoing exploration, description and biogeographical analyses (1950–current)**

A period of revitalized interest in the Moroccan amphibian fauna was initiated by the monographic work of Pasteur and Bons (1959) which provided an elaborate overview of systematics, biology and ecology of all hitherto known species. Based on larval characteristics, these authors described *P. varaldii*, which represented the first endemic Moroccan amphibian species. By means of similar methodology, the second endemic taxon *A. maurus* was described by Pasteur and Bons (1962) soon thereafter. When Bons (1972) published his overview of the Moroccan herpetofauna, members of the genera *Alytes*, *Pelobates*, *Pelophylax* Fitzinger 1843 and *Salamandra* Garsault 1764 were either confirmed or suspected to represent endemic taxa, rather than being conspecific with their European relatives. Additionally, during the same year Hoogmoed (1972) surprised the herpetological community with the description of *Bufo brongersmai* Hoogmoed 1972. Despite early scepticism regarding the validity of this taxon due to its resemblance with *B. boulengeri*, the endemic Brongersma´s Toad has been shown to represent an ancient and well-diverged lineage within the Bufonidae (Grillitsch *et al*. 1989; Delfino *et al.* 2009; Van Bocxlaer *et al.* 2009; Pyron & Wiens 2011).

During the next decades an increase of species-specific research (e.g. Busack *et al.* 1985; Mellado & Dakki 1988; Destre *et al.* 1989; Melhaoui & Chavanon 1989; Benhachem & Benazzou 1992; De la Riva 1992; Mellado & Mateo 1992; Geniez & Soto 1994; Llorente *et al.* 1996; Vences & Glaw 1996) facilitated the creation of several comprehensive overviews. Consequently, Le Berre (1989) and Salvador (1996) provided information on amphibians of entire North Africa including notes on ecology, biology and distribution. Divulgations treating respectively the entire Moroccan and North African herpetofauna by Bons and Geniez (1996) and Schleich *et al*. (1996) appeared simultaneously, and provided a wealth of information on distribution and natural history of all taxa. In addition, inventories of remote regions continued, resulting in the discovery of *Amietophrynus xeros* (Tandy *et al.* 1976) and *Hoplobatrachus occipitalis* (Günther 1858) in the extreme southeastern Western Sahara (Geniez *et al.* 2004 and references therein). However, intraspecific variation both at genotypic and phenotypic level remained largely unexplored (but see e.g. Busack 1986), hence biogeographical scenarios for amphibian divergence in North Africa were proposed, but generally not elaborated upon.

Recent phylogeographical studies have shed light on historical diversification patterns of Moroccan amphibians. Especially the Gibraltar land bridge, its subsequent collapse during the Early Pliocene at the end of the Messinian Salinity Crisis and the climatic fluctuations during the Plio-Pleistocene have had a profound impact on diversification (e.g. Busack *et al.* 1986; Carranza & Wade 2004; Zangari *et al.* 2006; Beukema *et al*. 2010). Also, knowledge regarding the ecology, behaviour and phenology of Moroccan amphibians has been significantly increased as a result of detailed field studies (e.g. Gallix 2002; Donaire-Barroso & Bogaerts 2003b; El Hamoumi *et al.* 2007; Guarino *et al.* 2011). Lastly, intensified fieldwork during the last decennium has resulted in the discovery of a considerable number of new amphibian populations (e.g. Donaire-Barroso & Bogaerts 2003a,b; Harris *et al.* 2008; Harris *et al*. 2010; de Pous *et al.* 2012).

**Goals of this paper**

Despite a significant increase in knowledge on the biogeography, distribution and systematics of Moroccan amphibians, a contemporary synthesis is lacking, while a considerable amount of descriptive data has remained unpublished. At the most basic level, it is highly important to record and present distribution data which might facilitate conservation efforts to combat threats. To this aim, we compare personal distribution data with published information. Furthermore, we aim at:

(i) Providing a distribution review of the Moroccan amphibians;
(ii) Resolving the taxonomy of the genus *Salamandra*, including the description of a new subspecies and creation of a polytomous identification key;
(iii) Presenting data on intraspecific morphological variability of *P. varaldii* and *P. waltl* in Morocco, as previous studies (e.g. Pasteur & Bons 1959; Busack *et al.* 1985) have caused confusion regarding their (sub)specific status;
(iv) Resolving the systematic position of *B. brongersmai*, including the description of a new genus comprising this species;
(v) Summarizing and assessing the availability of tadpole-specific characteristics (including the creation of a dichotomous identification key) and bioacoustic data;
(vi) Summarizing natural history data with an emphasis on phenology, which might assist in future discovery and monitoring of amphibian populations.

## Materials and Methods

**Distribution assessment.** Existing distribution records for Morocco were compiled from various literature sources dating from Bons and Geniez (1996) to current publications (December 2012), depending on the species. While several additional amphibian species occur in the extreme south-eastern Western Sahara (Geniez *et al.* 2004) we did not visit this region, and conclusively do not include it into the current review. Accordingly, the study area is approximately located between N 36º00 to 28º00 and W 13º00 to 1º00 (Fig. 3). Although the work of Fahd *et al.* (2005) might contain new records for several species, these records were not georeferenced due to their geographic scale of 10x10 km. A total of fourteen fieldtrips were undertaken from 2000 to 2012 by the authors and colleagues. Records of amphibians taken by various GPS types during expeditions were compared to the existing distribution database, and transported into ArcGIS versions 9.2–10 using (and when relevant, transformed into) coordinate system GCS_WGS_1984. New records are presented here in relation to published records, while numerous reconfirmations were not taken into account.

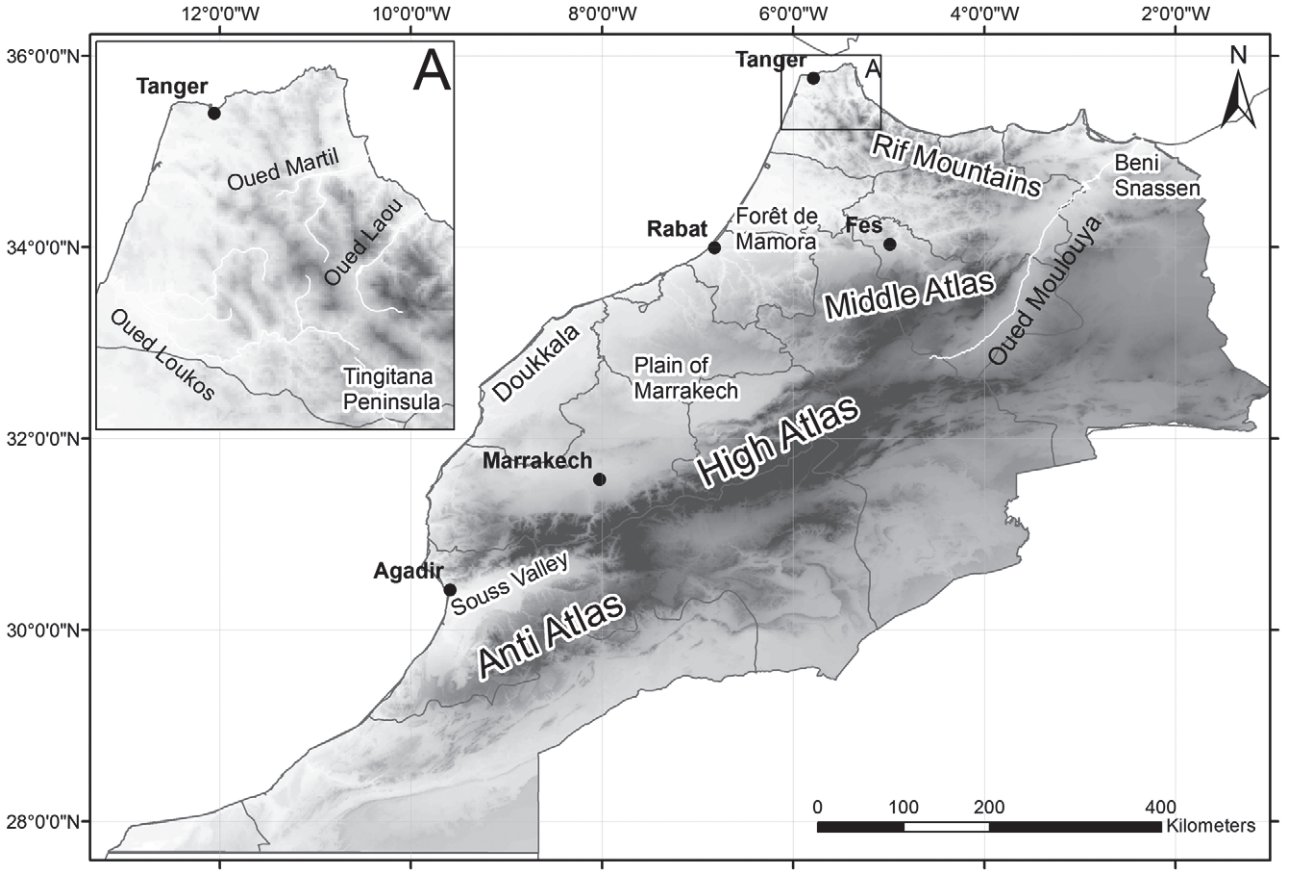


**FIGURE 3.** Topographical overview of the study area, indicating the most prominent geographical features and cities mentioned in the text. Darker colours indicate higher altitude. A: Inset of the Tingitana Peninsula.

**Species examination.** General tadpole morphology as presented in the text is shown in Figure 4.

Measurements were made to the nearest mm of (i) *S. algira* specimens from the Rif and Middle Atlas Mountains; (ii) of a collection of *P. waltl* from Fôret de Mamora (Rabat), and (iii) of a sample *P. varaldii* in order to assess intraspecific divergence.

The following measurements were taken of Moroccan *Salamandra algira* (see also Fig. 5) by D. Escoriza: SVL: snout-vent length, measured from tip of the snout to the anterior edge of the cloaca; LT: tail length measured from the anterior edge of cloaca to tip of tail; AL: anterior arm length, measured from the insertion from the body to the tip of the third finger; PL: posterior limb length, taken from the insertion to the tip of the third toe; AP: distance between the rear end of the forelimb and the anterior end of the hind limb. HL: head length from posterior edge of parotoid gland to tip of snout; HW: head width, measured at anterior edge of parotoids; PAL: length of the parotoid from the forward edge of the parotoid to the rear end of the parotoid; LJL: lower jaw length measured

from the oral commissure to the anterior of the jaw; SL: snout length, measured from the anterior end of the eye to the anterior end of snout; EN: distance between the eye and the nostril measured from the anterior end of eye to nostril; IN: distance between both nostrils. We additionally introduce the term 'discoloration' to define the variably present and occasionally difficult to observe red patches which appear through ontogeny.

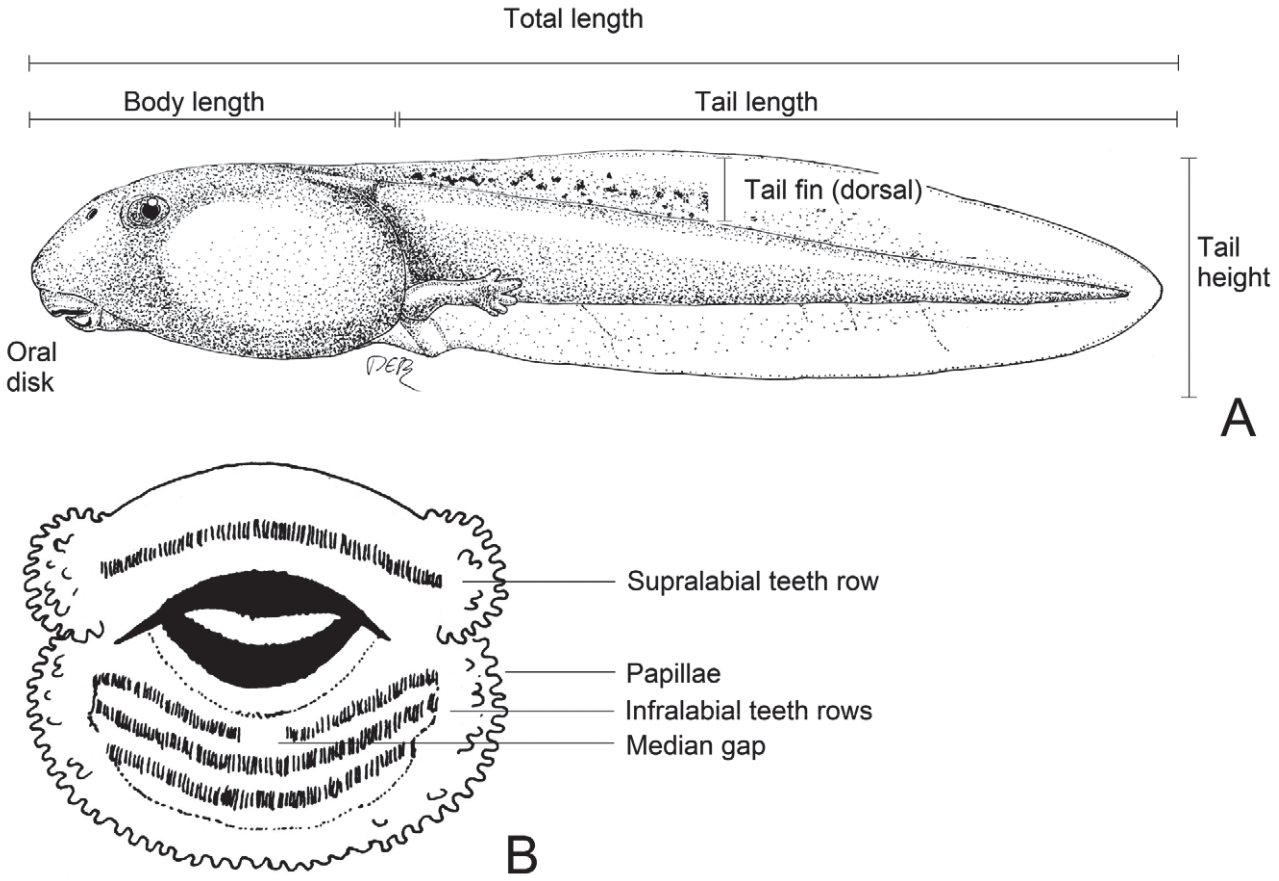


**FIGURE 4.** Tadpole morphology. Major features of A: Body morphology. B: Oral disc morphology. Drawing by DE.

A total of 65 *P. waltl* individuals were captured during January–April 2010 from temporary ponds in Forêt de Mamora, near Rabat. The following measurements were taken by a single person (P. de Pous) based on an adaptation of the measurement scheme presented by Carranza and Wade (2004): TL: total length, measured from the tip of the snout to the tip of the tail; SVL: snout-vent length, measured from tip of the snout to the anterior edge of the cloaca; LT: tail length measured from the anterior edge of cloaca to tip of tail; HL: head length from posterior edge of parotoid gland to tip of snout; HW: head width, measured at anterior edge of parotoids. Additionally, the sex of the measured individuals was noted. Descriptive statistics were produced and compared to published data (Pasteur 1958; González de la Vega 1988; Fontanet & Horta 1989).

Measurements of *P. varaldii* were based on an adaptation of the measurement scheme presented by Ugurtas *et al.* (2002). Of a total of 60 individuals of *P. varaldii* (30 males and 30 females) from Forêt de Mamora near Rabat, the following characteristics were measured by P. de Pous: L: total body length, measured from the snout to the centre of the cloaca; F: femur length, measured from the centre of the cloaca to the distal end of the femur measured in the bent hind limb; P: pes length, measured from the metarsal heel to the apex of the longest ($4^{th}$) toe; Lpa: forelimb length, measured as the length of the humerus + length from the distal articulation of the humerus to the apex of the longest ($3^{th}$) finger; Lpp: hind limb length, measured as the length of the femur + the length of the tibia + the foot length from the heel to the tip of the longest ($4^{th}$) toe; DpPp: length of the hind limb, measured from the distal end of the inner metarsal tubercle to the tip of the toe; Cint: inner metarsal tubercle length; Spi: minimum inter-narial space; Lo: eye length, measured from the anterior to the posterior eyelid commissure; Dno: nostril to eye distance, measured from the nostril to the anterior eyelid commissure; HW: head width, measured between the corners of the mouth; Weight (to the nearest gram). Presence or absence of red dots on the eyelids (Eye), forelimbs

(FL), hindlimbs (HL), dorsum (D) and head (H) was recorded for each individual. Additionally, the sex of the measured individuals was noted; males were distinguished from females by the presence of swollen humeral glands, which are typically developed during the breeding period. Descriptive statistics were produced and compared to published data (Lizana *et al*. 1994; Marangoni & Tejedo 2008) of the sister species *Pelobates cultripes* (Cuvier 1829) from southwestern Europe.

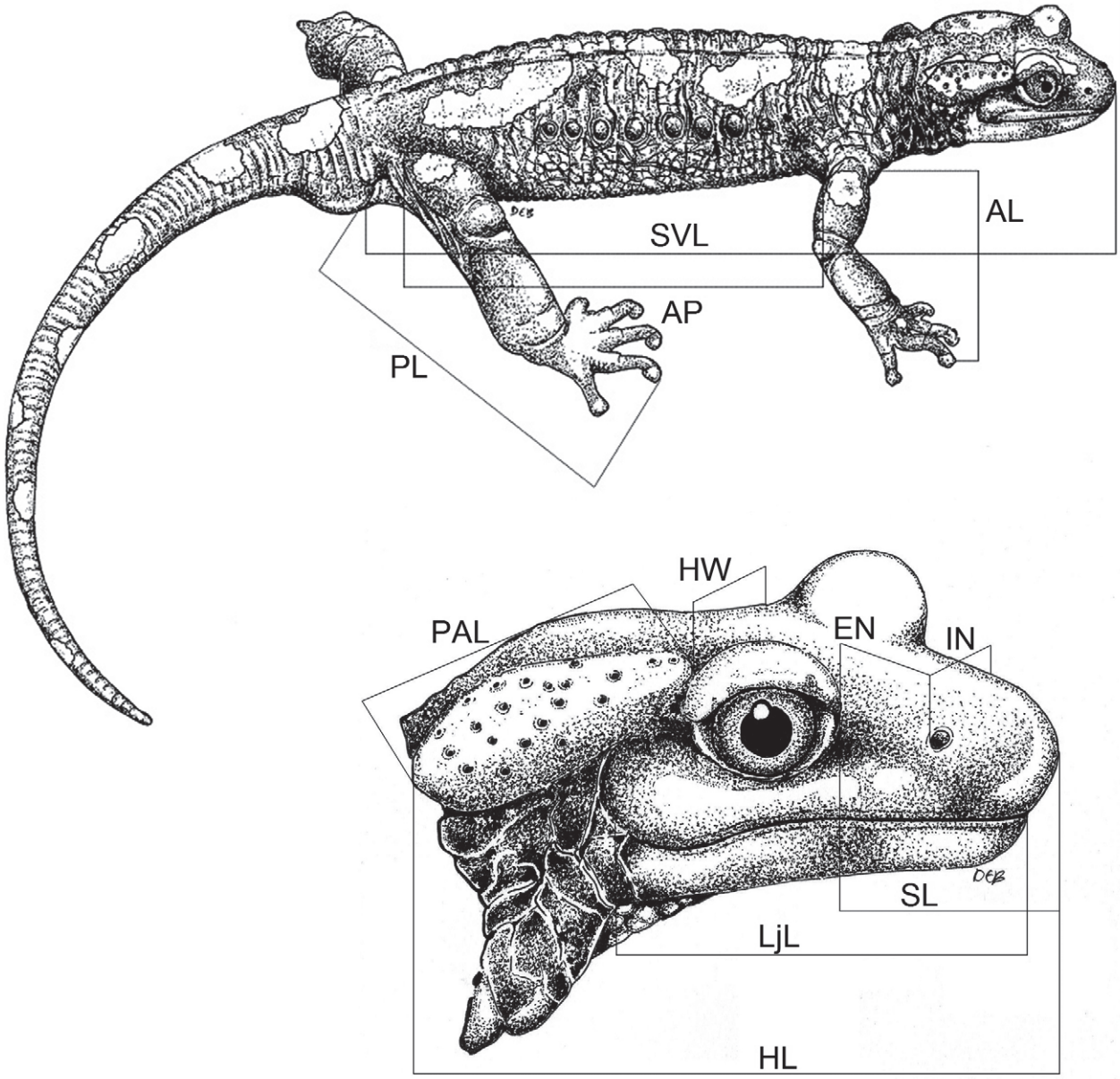


**FIGURE 5.** Measurement scheme implemented for Moroccan *Salamandra algira*. Drawing by DE.

Collections which were used to examine or deposit material are as follows; Doñana Biological Station (EBD), Sevilla, Spain; Museu de Ciències Naturals, Zoologia (MCNC), Barcelona, Spain; Naturhistorisches Museum Wien (NHMW), Vienna, Austria; Netherlands Centre for Biodiversity Naturalis (RMNH), Leiden, the Netherlands; Peabody Museum of Natural History—Yale University (PMNH); Society for Preservation of Herpetological Diversity (SPHD, personal collection of P. de Pous); Zoölogisch Museum Amsterdam (ZMA), Amsterdam, the Netherlands and Zoologisches Forschungsmuseum Alexander Koenig (ZMFK), Bonn, Germany.

**Phylogenetic position of *B. brongersmai*.** In order to recover the phylogenetic position of *B. brongersmai* we constructed a time-calibrated tree of 174 species of the Bufonidae family. This tree either presents more markers, a denser sampling of bufonid members or improved inpretability when compared to previous analyses (e.g. Pramuk *et al.* 2008; Van Bocxlaer *et al.* 2009; Van Bocxlaer *et al.* 2010; Pyron & Wiens 2011). In addition, we calculated

alternative phylogenetic positions of *B. brongersmai* and their frequencies (see below). The molecular dataset was based on three mitochondrial fragments (tRNAval, 16S and ND2) and two nuclear fragments (CXCR4 and NCX1) obtained from GenBank, determined by Van Bocxlaer *et al.* (2009) and Van Bocxlaer *et al.* (2010) (available upon request). Fragments were either partial (ND2, CXCR4, NCX1) or complete (tRNAval, 16S) for *B. brongersmai*. The unalignable regions of the tRNAval and 16S markers were removed by means of Gblocks (Castresana 2000), eliminating the misaligned regions and the positions with more than 50% missing data. Analysis was performed by means of a Bayesian relaxed molecular clock approach (BRMC) as implemented by the package BEAST v. 1.5.2 (Drummond & Rambaut 2007). A Yule branching process with a uniform prior and an uncorrelated branch rate variation was modelled by means of a resampling from a lognormal distribution. The model of evolution was set to GTRGAMMAI. The clock model and the evolutionary models were applied independently to four partitions: (i) mitochondrial protein-coding (ND2); (ii) mitochondrial RNA-coding (tRNAval and 16S); (iii) nuclear CXCR4; and (iv) nuclear NCX1.

The four calibration points described in Garcia-Porta *et al.* (2012) were used to obtain the ultrametric tree in time units. All these calibration points were already employed in previous studies to calibrate timescales encompassing the whole Bufonidae family (Pramuk *et al.* 2008; Van Bocxlaer *et al.* 2009, 2010, see references therein). Five independent Markov chain Monte Carlo (MCMC) analyses were performed; each chain ran for 25,000,000 generations, sampling parameters and trees every 1000 generations. These five independent runs converged on very similar posterior estimates and were combined using LogCombiner version 1.4 after excluding the first 5,000,000 generations in each one (Rambaut & Drummond 2007). Tracer 1.2 (Rambaut & Drummond 2007) was used to confirm convergence and good mixing of the five combined MCMC chains. Finally, we generated the maximum clade credibility tree (Fig. 6) with median node heights using the TreeAnnotator program (also included in BEAST package), setting the posterior probability limit to 0.5. Given that the phylogenetic position of *B. brongersmai* was poorly resolved (see results) we calculated its alternative phylogenetic positions, and their frequency, across a sample of 1,500 trees of the BEAST posterior by means of the branch attachment frequency (BAF) algorithm implemented in the package Phyutility 2.2.4 (Smith & Dunn 2008). This permitted us to integrate this phylogenetic uncertainty into the genus description, allowing us to compare *B. brongersmai* with all the potentially related genera.

**Niche modeling.** Niche models were generated by the presence/background algorithm Maxent, version 3.3.3k (Philips *et al.* 2006) based on selected climatic parameters and occurrence records. The climatic dataset of de Pous *et al.* (2011) was used in combination with the complete distribution dataset presented in the current paper, comprising 2057 records. Maximum entropy is achieved by the constraint that the expected value of each variable must equal the mean value at the presence points (the empirical average) (Phillips *et al.* 2006). In other words, this means that the model minimizes the relative entropy between two probability densities (one from the presence data and one from the landscape) defined in covariate space (Elith *et al.* 2010). The model output displays the relative occurrence probability of a species within the grid cells of the study area. Maxent was used with default settings, while performing ten crossvalidate replicates for each species in order to gain an average suitability prediction. Suitability in the resulting predictive distribution maps ranges from blue (unsuitable) to red (highly suitable).

**Bioacoustic analyses.** Calls were recorded by a single person (P. de Pous) using a Olympus LS-10 Digital Linear PCM Recorder with an internal microphone at a sampling rate of 44.1 kHz at 16 bit and stored as .wav files on a SD Card. Depending on the species, the following bioacoustic variables were analysed: dominant frequency (frequency at which peak amplitude occurs), note length (length in seconds of a note, where a note is a discrete bundle of pulses; a note also refers to "pulse group" sensu e.g. Schneider 2001), pulses per note (where each pulse is defined as a burst of energy, several of which together comprise a note), pulse rate (calculated as pulse count for a note divided by note length) and interpulse (the amount of time from the end of one pulse to the beginning of the following pulse). Measurements, spectograms, oscillograms and power spectrums were made using SoundRuler ver. 0.9.6.0 (Gridi-Papp 2007), whereas Audacity ver. 2.0.1. (Audacity Team 2012) was used to obtain frequency information through Fast Fourier Transformation (FFT length 512 points) at Hanning window function. Air temperature and humidity were measured (CM-DT321) immediately after each sound recording to the nearest 1°C and 3.5% relative humidity.

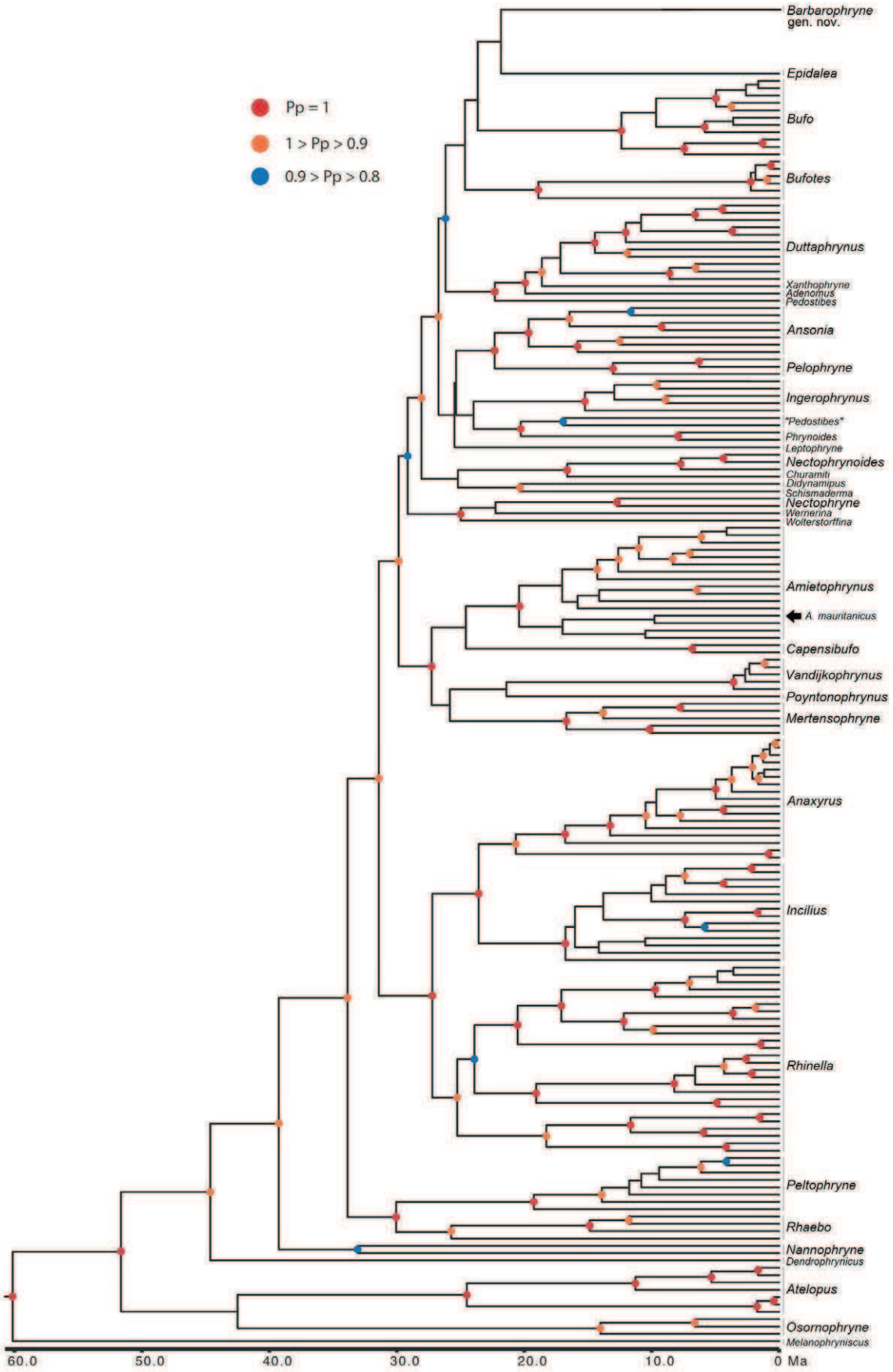


**FIGURE 6.** Time-calibrated tree showing the radiation of the family Bufonidae and the most likely phylogenetic position of *Barbarophryne* gen. nov. The dots represent nodes with posterior probabilities (pp) greater than 0.8. The colors, from blue to red, order the nodes from lower to high support.

**TABLE 1.** New distribution records for Moroccan amphibian species.

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| *Salamandra algira* | South of Jbel Tazekka | 33.94 | –4.23 |
| | Jbel Tazekka | 34.05 | –4.22 |
| | South of Taza | 34.06 | –4.05 |
| | North of Bab Bou-Idir | 34.09 | –4.10 |
| | Jbel Tazekka | 34.09 | –4.19 |
| | Bouadel | 34.53 | –4.51 |
| | Northeast of Rhafsai | 34.63 | –4.86 |
| | South of Tleta-Ketama | 34.79 | –4.63 |
| | South of Tleta-Ketama | 34.81 | –4.67 |
| | West of Ketama | 34.94 | –4.63 |
| | North of Mokrisset | 34.95 | –5.36 |
| | Southwest of Bab Berret | 34.96 | –4.96 |
| | Between Bab Berret and Ketama | 34.96 | –4.68 |
| | East of Bab Berret | 34.99 | –4.82 |
| | Northwest of Bab Berret | 35.02 | –5.02 |
| | Southwest of Chefchaouen | 35.09 | –5.40 |
| | Southwest of Chefchaouen | 35.13 | –5.37 |
| | Ahl Serif | 35.14 | –5.77 |
| | Southwest of Souk-el-Arba-des-Beni-Hassan | 35.26 | –5.49 |
| | Southwest of Souk-el-Arba-des-Beni-Hassan | 35.27 | –5.44 |
| | North of Chefchaouen | 35.29 | –5.32 |
| | West of Souk-el-Arba-des-Beni-Hassan | 35.32 | –5.50 |
| *Pleurodeles waltl* | North of Safi | 32.35 | –9.28 |
| | Southeast of Had-Harrara | 32.39 | –9.05 |
| | Southeast of Had-Harrara | 32.40 | –9.05 |
| | Southeast of Oualidia | 32.66 | –8.94 |
| | Between El Jadida and Oualidia | 32.92 | –8.72 |
| | Northeast of Souk-Jemaa-des-Oulad-Abbou | 33.14 | –7.91 |
| | Northeast of Souk-Jemaa-des-Oulad-Abbou | 33.22 | –7.83 |
| | North of Timahdite | 33.26 | –5.08 |
| | Northeast of Souk-Jemaa-des-Oulad-Abbou | 33.26 | –7.76 |
| | Northeast of Ben-Slimane | 33.62 | –7.09 |
| | Northeast of Ben-Slimane | 33.65 | –7.04 |
| | Beteen Bouznika and Ben-Slimane | 33.65 | –7.14 |
| | Northwest of Ben-Slimane | 33.68 | –7.27 |
| | South of Sidi Yahya-des-Zaër | 33.74 | –6.94 |
| | South of Skhirat | 33.76 | –7.01 |
| | Southeast of Rabat | 33.82 | –6.53 |
| | East of Tiflet | 33.86 | –6.22 |

*..... continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | Southeast of Rabat | 33.87 | –6.60 |
| | East of Rabat (Western Mamora) | 34.01 | –6.70 |
| | East of Rabat (Western Mamora) | 34.02 | –6.50 |
| | East of Rabat (Western Mamora) | 34.03 | –6.69 |
| | East of Rabat (Western Mamora) | 34.05 | –6.55 |
| | Central Mamora | 34.08 | –6.20 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.08 | –6.67 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.08 | –6.65 |
| | Central Mamora | 34.10 | –6.19 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.14 | –6.67 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.16 | –6.60 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.16 | –6.67 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.16 | –6.66 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.17 | –6.63 |
| | Between Rabat and Kénitra (Western Mamora forest) | 34.18 | –6.63 |
| | North of Fes | 34.18 | –5.03 |
| | South of Sidi-Yahya-du-Rharb (Central Mamora) | 34.20 | –6.34 |
| | South of Sidi-Yahya-du-Rharb (Central Mamora) | 34.21 | –6.31 |
| | South of Sidi-Yahya-du-Rharb (Central Mamora) | 34.25 | –6.24 |
| | Northeast of Kénitra | 34.27 | –6.50 |
| | Northeast of Kénitra | 34.28 | –6.47 |
| | West of Esskhourra | 34.49 | –5.10 |
| | South of Ouazzane | 34.62 | –5.54 |
| | Midway between Larache and Kenitra | 34.65 | –6.40 |
| | South of Ouazzane | 34.74 | –5.58 |
| | Southeast of Larache | 35.02 | –5.99 |
| | Ahl Serif | 35.14 | –5.76 |
| | South of of Maïsra | 35.31 | –5.73 |
| | North of Maïsra | 35.33 | –5.71 |
| | Southeast of Asilah | 35.34 | –5.99 |
| | South of Tanger airport | 35.68 | –5.96 |
| *Alytes maurus* | South of Bab Bou-Idir | 34.05 | –4.15 |
| | Southwest of Bab Bou-Idir | 34.05 | –4.17 |
| | Jbel Tazekka | 34.11 | –4.16 |
| | South of Ketama | 34.87 | –4.56 |
| | North of Mokrisset | 34.97 | –5.37 |
| | West of Bab Berret | 35.02 | –5.02 |
| | East of Chefchaouen | 35.17 | –5.17 |
| | West of Souk-el-Arba-des-Beni-Hassan | 35.27 | –5.49 |

*..... continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | West of Souk-el-Arba-des-Beni-Hassan | 35.32 | –5.51 |
| | West of Es-Sebt-de-Saïd | 35.39 | –5.24 |
| | South of Tetouan | 35.52 | –5.34 |
| *Discoglossus pictus* | Beni Snassen | 34.82 | –2.39 |
| | Eastern Beni Snassen | 34.83 | –2.13 |
| | Beni Snassen | 34.87 | –2.13 |
| | West of Saïdia | 35.08 | –2.35 |
| | Saïdia (village) | 35.10 | –2.26 |
| *Discogloccus scovazzi* | Tizi-n-Test | 30.93 | –8.27 |
| | Imilchil | 32.20 | –5.59 |
| | Kasba-Tadla | 32.50 | –6.04 |
| | Southeast of Oualidia | 32.61 | –8.93 |
| | Southeast of Oualidia | 32.68 | –8.87 |
| | Oualidia | 32.71 | –9.00 |
| | Between El Jadida and Oualidia | 32.92 | –8.72 |
| | Northeast of Ben-Slimane | 33.62 | –7.08 |
| | North of Ben Slimane | 33.64 | –7.13 |
| | Northwest of Ben-Slimane | 33.68 | –7.26 |
| | Southwest of Tiflet | 33.86 | –6.22 |
| | Rabat | 34.03 | –6.71 |
| | Jbel Tazekka | 34.05 | –4.17 |
| | Central Mamora | 34.05 | –6.38 |
| | South of Taza | 34.07 | –4.04 |
| | South of Taza | 34.15 | –4.01 |
| | North of Moulay-Idriss | 34.28 | –5.46 |
| | South of Oujda | 34.56 | –2.06 |
| | Northeast of Rhafsai | 34.65 | –4.84 |
| | North of Tahar-Souk | 34.75 | –4.26 |
| | Northeast of Melloussa | 34.77 | –5.60 |
| | East of Moulay Bousselham | 34.85 | –6.22 |
| | Between Tleta Ketama and Zerkat | 34.86 | –4.46 |
| | South of Ketama | 34.87 | –4.55 |
| | South of Targuist | 34.89 | –4.32 |
| | West of Ketama | 34.94 | –4.63 |
| | Bab-Berret | 34.99 | –4.82 |
| | West of Bab Berret | 35.00 | –4.94 |
| | Ksar-el-Kebir | 35.01 | –5.89 |
| | Northwest of Ksar-el-Kebir | 35.02 | –5.99 |
| | East of Ksar el-Kebir | 35.02 | –5.76 |

*..... continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | West of Bab Berret | 35.03 | −5.01 |
| | West of Draa-el-Asef | 35.04 | −5.50 |
| | Northwest of Ksar-el-Kebir | 35.06 | −6.07 |
| | East of Ksar el-Kebir | 35.08 | −5.55 |
| | West of Derdara | 35.12 | −5.36 |
| | Ahl Serif | 35.15 | −5.76 |
| | West of Chefchaouen | 35.17 | −5.31 |
| | Talembote | 35.24 | −5.18 |
| | Between Chefchaouen and Souk-Khemis-des-Beni-Arouss | 35.27 | −5.43 |
| | West of Souk-el-Arba-des-Beni-Hassan | 35.32 | −5.50 |
| | Northeast of Souk-el-Arba | 35.37 | −5.55 |
| | Ichouchen | 35.40 | −5.26 |
| | Asilah | 35.45 | −6.04 |
| | South of Tetouan | 35.54 | −5.39 |
| | West of Daidatz | 35.68 | −5.93 |
| | Northwest of Melloussa | 35.79 | −5.68 |
| | Jbel Musa | 35.89 | −5.40 |
| *Amietophrynus mauritanicus* | Southwest of Abouda | 28.97 | −10.23 |
| | South of Amerzgane | 30.91 | −7.23 |
| | Tizi-n-Test | 30.93 | −8.27 |
| | North of Safi | 32.35 | −9.28 |
| | Southwest of Oualidia | 32.67 | −8.93 |
| | Jbel Tazekka | 34.06 | −4.27 |
| | North of Taza | 34.45 | −3.92 |
| | Southeast of Fes-el-Bali | 34.48 | −5.02 |
| | South of Oujda | 34.53 | −2.06 |
| | Northwest of Taounate | 34.56 | −4.73 |
| | North of Taounate | 34.59 | −4.61 |
| | South of Kassita | 34.79 | −3.75 |
| | Taforalt | 34.82 | −2.39 |
| | South of Kassita | 34.85 | −3.72 |
| | West of Mont-Areul | 34.99 | −3.07 |
| | South of Bni-Boufrah | 35.01 | −4.31 |
| | Ksar-el-Kebir | 35.01 | −5.89 |
| | Southwest of Chefchaouen | 35.02 | −5.39 |
| | West of Draa-el-Asef | 35.04 | −5.57 |
| | South of Bni-Boufrah | 35.05 | −4.29 |
| | Saïdia | 35.09 | −2.25 |
| | West of Draa-el-Asef | 35.09 | −5.53 |

..... *continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | West of Saïdia | 35.11 | –2.32 |
| | West of Draa-el-Asef | 35.11 | –5.46 |
| | Ahl Serif | 35.13 | –5.76 |
| | Torres-de-Alcala | 35.14 | –4.32 |
| | Ahl Serif | 35.20 | –5.75 |
| | Talembote | 35.24 | –5.19 |
| | South of Souk-Khemis-des-Beni-Arouss | 35.24 | –5.65 |
| | Northwest of Chefchaouen | 35.27 | –5.40 |
| | South of Souk-el-Arba-des-Beni-Hassan | 35.30 | –5.38 |
| | Souk-Khemis-des-Beni-Arouss | 35.32 | –5.63 |
| | Southwest of Zinat | 35.37 | –5.55 |
| | Southwest of Zinat | 35.43 | –5.44 |
| | North of Briex | 35.63 | –5.98 |
| | Northeast of Melloussa | 35.80 | –5.59 |
| | West of Tanger | 35.82 | –5.84 |
| *Barbarophryne brongersmai* | Southwest of Agz | 30.61 | –6.64 |
| | Tasla | 30.55 | –6.82 |
| | North of Mechra-Benâbbou | 32.66 | –7.79 |
| | Tamri | 30.73 | –9.83 |
| | South of Tnine Ameliou | 29.20 | –10.11 |
| *Bufo bufo* | Azrou | 33.40 | –5.23 |
| | North of Ifrane | 33.65 | –5.04 |
| | Southwest of Taza | 34.05 | –4.19 |
| | Southwest of Taza | 34.08 | –4.15 |
| | Southwest of Taza | 34.09 | –4.11 |
| | South of Taza | 34.15 | –4.01 |
| | South of Bab Taza | 35.02 | 5.21 |
| | Talansetoune, Bab Taza | 35.07 | –5.17 |
| | Ahl Serif | 35.12 | –5.77 |
| | Southeast of Souk-el-Arba | 35.28 | –5.54 |
| | Southwest of Larbaa Beni Hassen | 35.28 | –5.40 |
| | Northwest of Chefchaouen | 35.28 | 5.47 |
| | Northwest of Moulay Abdeslam | 35.33 | –5.53 |
| | North of Moulay Abdeslam | 35.37 | –5.54 |
| | South of Tetouan | 35.54 | –5.38 |
| *Bufotes boulengeri* | Tasla | 30.55 | –6.82 |
| | Agdz | 30.64 | –6.58 |
| | Merzouga | 31.21 | –3.99 |
| | North of Essouria | 31.57 | –9.53 |

..... *continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | Imilchil | 32.20 | –5.59 |
| | Southeast of Oualidia | 32.64 | –8.92 |
| | North of Aguelmam | 33.07 | –5.03 |
| | Moyen Atlas | 33.13 | –5.35 |
| | South of Azrou | 33.33 | –5.23 |
| | Azrou | 33.40 | –5.23 |
| | Debdou south | 33.87 | –3.17 |
| | Debdou south | 33.96 | –3.04 |
| | East of Rabat | 34.05 | –6.63 |
| | Southwest of Taza | 34.09 | –4.11 |
| | Debdou north | 34.12 | –3.01 |
| | Beni Snassen | 34.83 | –2.20 |
| *Hyla meridionalis* | Northeast of Tafraoute | 29.75 | –8.83 |
| | South of Amerzgane | 30.91 | –7.23 |
| | Tizi-n-Test | 30.93 | –8.27 |
| | North of Safi | 32.35 | –9.28 |
| | South of Oualidia | 32.60 | –9.06 |
| | South of Oualidia | 32.67 | –8.93 |
| | Southeast of Oualidia | 32.68 | –8.87 |
| | South of Sidi Moussa | 32.89 | –8.73 |
| | Northeast of Ben-Slimane | 33.62 | –7.09 |
| | Northeast of Ben-Slimane | 33.65 | –7.04 |
| | Beteen Bouznika and Ben-Slimane | 33.66 | –7.15 |
| | Northwest of Ben-Slimane | 33.68 | –7.27 |
| | South of Sidi Yahya-des-Zaër | 33.74 | –6.94 |
| | South of Skhirat | 33.76 | –7.01 |
| | Debdou | 33.89 | –3.04 |
| | Fes | 33.98 | –5.05 |
| | East of Rabat (southern Mamora) | 34.01 | –6.47 |
| | East of Rabat | 34.04 | –6.73 |
| | Between Rabat and Kénitra (Western Mamora) | 34.08 | –6.64 |
| | Central Mamora | 34.10 | –6.20 |
| | South of Sidi-Kacem | 34.14 | –5.71 |
| | Between Rabat and Kénitra (Western Mamora) | 34.18 | –6.66 |
| | East of Kénitra (northern Mamora) | 34.21 | –6.31 |
| | East of Kénitra (northern Mamora) | 34.25 | –6.24 |
| | East of Sidi Slimane | 34.26 | –5.86 |
| | East of Kénitra (northern Mamora) | 34.30 | –6.38 |
| | Northwest of Fes | 34.31 | –5.47 |

*..... continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | Ksar-el_Kebir | 35.02 | –5.93 |
| | East of Ksar-el-Kebir | 35.02 | –5.86 |
| | West of Ksar el-Kebir | 35.02 | –5.99 |
| | West of Ksar el-Kebir | 35.04 | –6.05 |
| | Ahl Serif | 35.07 | –5.83 |
| | Derdara | 35.10 | –5.32 |
| | West of Draa-el-Asef | 35.10 | –5.48 |
| | Ahl Serif | 35.11 | –5.78 |
| | West of Saïdia | 35.12 | –2.34 |
| | Northwest of Derdara | 35.13 | –5.36 |
| | Ahl Serif | 35.14 | –5.76 |
| | Northwest of Chefchaouen | 35.18 | –5.31 |
| | Ahl Serif | 35.21 | –5.73 |
| | Northwest of Chefchaouen | 35.26 | –5.42 |
| | Northwest of Chefchaouen | 35.27 | –5.49 |
| | Southeast of Asilah | 35.34 | –5.99 |
| | Beni Ider | 35.46 | –5.42 |
| | South of Tetouan | 35.58 | –5.33 |
| | West of Daidatz | 35.68 | –5.93 |
| | Melloussa | 35.74 | –5.61 |
| *Pelobates varaldii* | - | - | - |
| *Pelophylax saharicus* | SW of Abouda | 28.97 | –10.23 |
| | SW of Imi n'Kern | 30.75 | –6.08 |
| | SW of Tazzarine | 30.79 | –5.57 |
| | Northwest of Ouerzazate | 30.90 | –7.24 |
| | Northwest of Ouerzazate | 30.94 | –7.21 |
| | Southeast of Risani | 31.27 | –4.16 |
| | South of Agoudim | 32.38 | –5.17 |
| | South of Ait Labbes | 32.45 | –4.49 |
| | Kasba-Tadla | 32.50 | –6.04 |
| | Southwest of Oualidia | 32.60 | –9.06 |
| | South of Oualidia | 32.64 | –8.96 |
| | Southeast of Oualidia | 32.68 | –8.87 |
| | Southwest of Sidi Moussa | 32.89 | –8.73 |
| | Bab-Bou-Idir | 34.08 | –4.14 |
| | Central Mamora | 34.10 | –6.20 |
| | East of Sidi-Yayha-du-Rharb | 34.27 | –6.24 |
| | Northeast of Sidi-Kacem | 34.28 | –5.46 |
| | Northwest of Taounate | 34.56 | –4.73 |

*..... continued on the next page*

**TABLE 1.** (Continued)

| Species | Locality | Coordinates | |
|---|---|---|---|
| | | Lat | Long |
| | Souk-el-Arba-du-Rharb | 34.69 | –6.00 |
| | Eastern Beni Snassen | 34.82 | –2.14 |
| | North of Mokrisset | 34.95 | –5.36 |
| | West of Bab Berret | 35.01 | –4.94 |
| | Northwest of Ksar-el-Kebir | 35.04 | –6.03 |
| | Northwest of Ksar-el-Kebir | 35.06 | –6.07 |
| | West of Derdara | 35.12 | –5.36 |
| | Ahl Serif | 35.13 | –5.75 |
| | Ahl Serif | 35.16 | –5.77 |
| | Between Taourirt and El Aïoun | 35.17 | –5.31 |
| | Talembote | 35.24 | –5.18 |
| | Northwest of Chefchaouen | 35.26 | –5.42 |
| | Southwest of Souk-el-Arba-des-Beni-Hassan | 35.27 | –5.45 |
| | South of Souk-el-Arba-des-Beni-Hassan | 35.29 | –5.32 |
| | Southwest of Tleta-des-Beni-Yder-Cherki | 35.36 | –5.57 |
| | West of Daidatz | 35.68 | –5.93 |
| | North of Melloussa | 35.78 | –5.61 |
| | R'milet | 35.79 | –5.86 |

## Results

**Distribution assessment.** A total of 292 new distribution records divided over all Moroccan amphibians except *P. varaldii* (treated in de Pous *et al.* 2012) were identified and are presented in Table 1, as well as per species.

**Species examination.** Measurement data from all currently recognized Moroccan *Salamandra* spp. are summarized in Table 2.

Descriptive statistics derived from the measurements of the Moroccan *P. varaldii* are summarized in Tables 4–5, and compared to available published morphological data of the sister species *P. cultripes*.

**Phylogenetic position of *B. brongersmai*.** The maximum clade credibility tree obtained (Fig. 6) yielded most of the clades compatible with previously published phylogenies of the Bufonidae (Van Bocxlaer *et al.* 2009, 2010; Pyron & Wiens 2011). In accordance with Pyron and Wiens (2011) the genus *Pedostibes* Günther 1876 "1875" was revealed to be polyphyletic. The crown age of the Bufonidae was estimated at approximately 60 Ma (95% HDP = 47.9–75.8 Mya), compatible with previous estimates (Roelants *et al.* 2007; Van Bocxlaer *et al.* 2009, 2010) and with the age of the oldest attributed fossil (55 Ma old) (Báez & Nicoli 2004). The phylogenetic position of *B. brongersmai* remained poorly resolved, with the summary tree showing it is sister to *Epidalea calamita* (Laurenti 1768) with 0.70 of posterior probability. However, the BAF analysis showed (not shown, available upon request) that *B. brongersmai* is probably related to the clade of Eurasian toads, as in the 90% of the trees *B. brongersmai* branches with this group. *Bufo brongersmai* falls in clades other than this one in just 9% of the trees.

**Bioacoustics.** Call recordings of anuran species, which were previously only reported in 'grey' literature or were either incomplete or unknown are presented in Figure 7. Calls of *B. brongersmai* and *B. boulengeri* were recorded on March 15th, 2009 southwest of Agdz (N 30.54, W -6.81 and N 30.6, W -6.57 respectively). Calls of *A. mauritanicus* and *H. meridionalis* were recorded on April 20th, 2009 southeast of Oualidia (Doukkala region) at N 32.68, W -8.87. Calls of *P. varaldii* were recorded in an aquarium in Rabat on January 15th, 2008. Descriptions of the calls are presented in the specific accounts below.

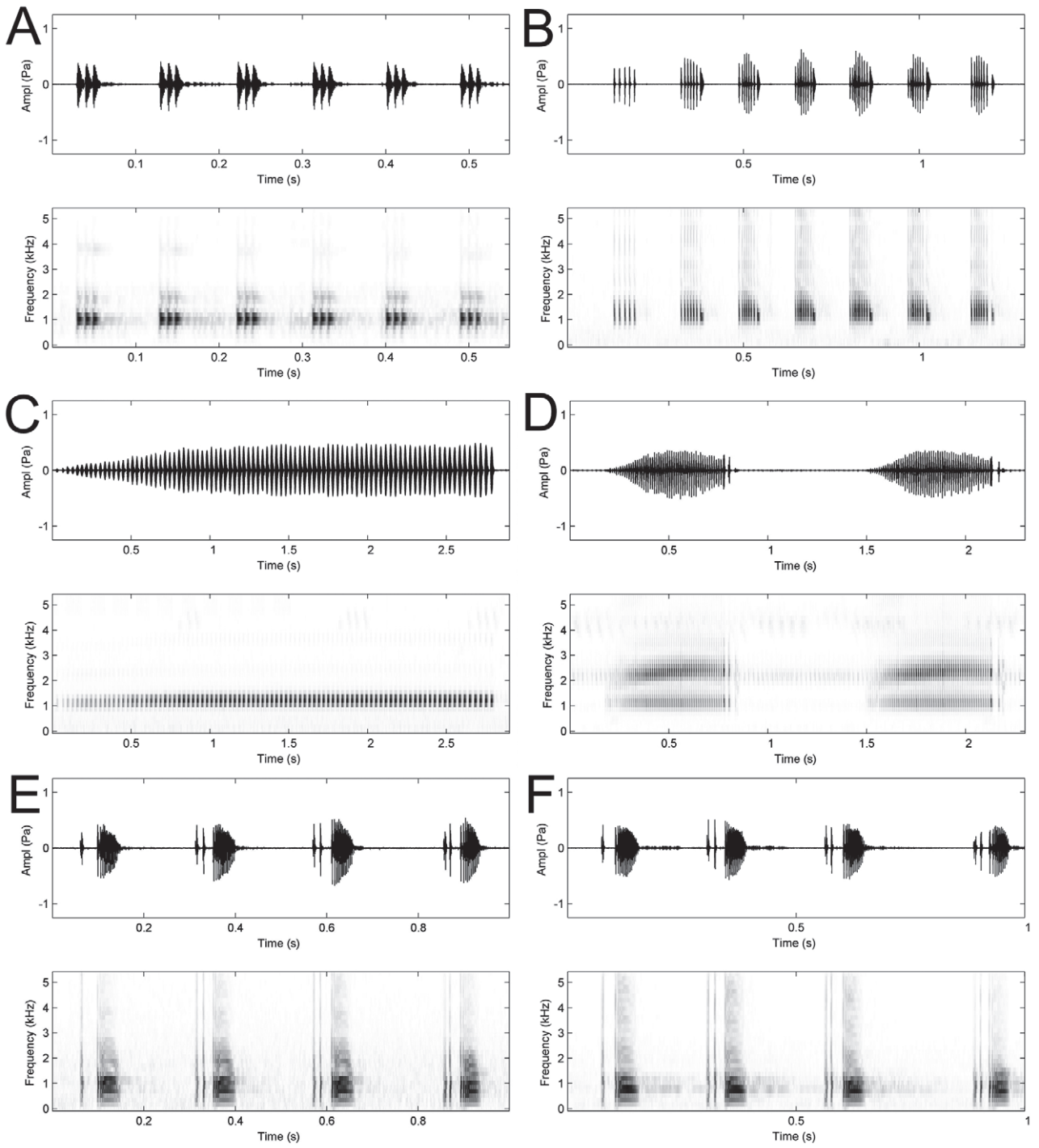


**FIGURE 7.** The amplitude (upper) and frequency (lower) of the advertisement call of respectively A: *Amietophrynus mauritanicus*. B: *Barbarophryne brongersmai*. C: *Bufotes boulengeri*. D: *Hyla meridionalis*. E-F show the amplexus calls of respectively a *Pelobates varaldii* male and female.

**Dichotomous identification key to Moroccan tadpoles**

The key presented here uses basic characters independent of tadpole size to identify species excluding those which occur in the extreme southeast of the Western Sahara. Two additional anuran species, *A. xeros* and *H. occipitalis* occur in the latter region, while *Tomopterna* sp. (see Zimkus & Larson 2011) might occur there (Geniez *et al.* 2004). The tadpole of *A. xeros* remains undescribed, whereas the latter two species likely represent species complexes (e.g. Zimkus & Larson 2011). Available tadpole descriptions are based on material from the Afrotropical Realm (Grillitsch *et al.* 1988; Grosjean *et al.* 2004), and cannot be unambiguously assigned to North African populations. We have therefore not included these species in the identification key.

As tadpole descriptions for African *B. bufo* and *D. scovazzi* are lacking, information on these taxa was added by means of synapomorphic characters in order to correct for missing data. Information on tooth rows is summarized using the Labial Tooth Row Formula (LTRF) following Altig and McDiarmid (1999). Accordingly, fractional notation displays the number of tooth rows on the upper (numerator) and lower (denominator) labia and the number of rows with medial gaps. Supralabial rows are numbered from the lip to the mouth, while infralabial rows are numbered from the mouth to the lip. Numbers in parentheses indicate rows with medial gaps, while numbers in brackets indicate variation in the presence of a median gap.

Tadpoles of bufonid species native to Morocco resemble each other closely in terms of colour and general morphology. Specific ratios which were previously used for identification such as the interorbital space in relation to the internarial space (Hoogmoed 1972) have been revealed to show considerable overlap between species (Grillitisch *et al.* 1989). Furthermore, syntopic occurrence of species (apart from *B. brongersmai* and *B. bufo*) is no exception. Comparison of multiple characteristics and measurements is therefore advised to positively identify bufonid tadpoles in Morocco.

1a. Eyes position dorsally . . . 3
1b. Eyes positioned laterally . . . 2
2a. Light, uniform colour . . . . *Pelobates varaldii* [Tail fins high, dorsal fin extending to anterior of body; metatarsal tubercle present; LTRF 4-5(2-4-5)/5(1-4)]
2b. Colour olive-green with golden specks . . . . . *Hyla meridionalis* [Tail fins high, dorsal fin extending to midbody; LTRF 2/3(3)]
3a. Spiracle sinistral . . . 6
3b. Spiracle midventral . . . 4
4a. Distribution west of the Moulouya Basin . . . 5
4b. Distribution east of the Moulouya Basin . . . . *Discoglossus pictus* [Tail fins with fine pattern of brown polygonal meshes; LTRF 2/3(1)]
5a. Tail fins with fine pattern of brown polygonal meshes . . . . *Discoglossus scovazzi* [Other details lacking]
5b Tail fins transparent with large dark spots. . . . *Alytes maurus* [Large dark spots also on tail; LTRF 2/3(1); distribution in Rif Mountains]
6a. Tail twice body length or less . . . 7
6b. Tail more than twice body length . . . . . *Pelophylax saharicus* [Elongated body; dark stripe running from anterior body through 2/3 of central tail; LTRF 1/3[1]]
7a Dark uniform colour . . . 8
7b Dark colour with abundant golden specks . . . . . *Amietophrynus mauritanicus* [Dark grey underside and tail; LTRF 2(2)/3]
8a Dorsal tail fin ending posterior to base of the tail . . . 9
8b Dorsal tail fin ending at the base of the tail. . . . *Bufo bufo* [Distribution in humid Rif and Atlas Mountains; other details lacking]
9a Tail fins unspotted . . . . *Barbarophryne brongersmai* [Distribution in (semi) desert; LTRF 2(2)/3]
9b Tail fins with brown spots . . . *Bufotes boulengeri* [Dorsal tail fin higher than ventral fin; dark dorsal and lateral patches visible in large individuals; LTRF 2(2)/3]

**Polytomous identification key to Moroccan members of the genus *Salamandra***

1a. Red discoloration present . . . 2
1b. Red discoloration absent . . . 2
2a. Underside of body and limbs dark . . . 3
2b. Underside of body and limbs dark with small white specks or rosettes . . . 3
2c. Underside of body and limbs dark with red discoloration and occasionally few tiny white specks . . . 4a
3a. Gular coloration dark . . . 4
3b. Gular coloration dark in combination with small white specks . . . 4
3c. Gular coloration dark with red discoloration, occasionally in combination with small white specks . . . 4a
4a. Distribution in Rif Mountains east of Chefchaouen or Middle Atlas Mountains . . . . *Salamandra algira splendens* ssp. nov. [red discoloration often highly extended in the Central Rif]
4b. Distribution on the Tingitana Peninsula north of Chefchaouen . . . *Salamandra algira tingitana*
4c. Distribution in the Beni Snassen Massif . . . *Salamandra algira spelaea*

## Systematics

New nomenclatural acts implemented in this study are pointed out below in bold characters. The National Red List Status displayed in each species account was provided by Pleguezuelos *et al*. (2010).

**Order Urodela Duméril**

**Family Salamandridae Goldfuss**

**Subfamily Salamandrinae Goldfuss**

**Genus *Salamandra* Garsault 1764**

Dubois and Raffaëlli (2009) recently revised the taxonomy of North African *Salamandra* and recognized three species, one of which undescribed, and placed these in the subgenus *Algiandra* Dubois and Raffaëlli 2009. Their rationale for these changes depended on prior demonstrated mitochondrial divergence (Donaire-Barroso & Bogaerts 2003a; Escoriza & Comas 2007), and descriptive data on several morphological characters. However, as the supporting data for recognition of these taxa at species level is incomplete (morphology) or inconsistent (mitochondrial relations, reproductive behaviour) with current knowledge (e.g. Beukema *et al.* 2010, see below for details), a new rearrangement of the North African *Salamandra* taxa is proposed here. Additionally, due to the fact that an extraordinary degree of adaptive, intraspecific divergence is present within the genus *Salamandra*, morphological synapomorphic characters of proposed subgenera by Dubois and Raffaëlli (2009) are ambiguous and vary significantly even within recognized taxa. This is at least true for the proposed characters size, colour(s) and associated pattern, cranial morphology and altitudinal distribution (Eiselt 1958). As recognition of subgenera within *Salamandra* could unnecessarily complicate the already confusing systematics of the genus, we do not follow this proposal.

**North African Fire Salamander, *Salamandra algira* Bedriaga 1883**
Fig. 8A–H

The genus *Salamandra* most likely colonized the African continent during the Messinian Salinity Crisis via the Gibraltar land bridge (Escoriza *et al.* 2006; Beukema *et al*. 2010). Subsequent divergence was initiated approximately 3.6 mya on both sides of the Moulouya River in eastern Morocco, due to a period of cyclic fluctuations in climate and consequently vegetation in north-western Africa (Beukema *et al.* 2010). Climatic oscillations during the Upper Pliocene and Pleistocene have driven at least mitochondrial divergence in the hitherto recognized taxa (Beukema *et al.* 2010).

The first report of the genus *Salamandra* in Morocco was made by Boulenger (1889), based on material collected by M. Henry Vaucher from the 'Benider Hills near Tanger'. Subsequent collectors could not verify this claim, which led to doubts on the actual presence of *Salamandra* in Morocco (e.g. Hediger 1935 and references therein). By the mid-twentieth century, however, a considerable number of records had been published (Pasteur & Bons 1959), although general knowledge remained particularly limited. Recent publications have provided detailed accounts on distribution, reproductive behaviour and consequently updated subspecific taxonomy (Donaire-Barroso & Bogaerts 2001; Donaire-Barroso *et al.* 2001; Bogaerts & Donaire-Barroso 2003; Donaire-Barroso & Bogaerts 2003a; Bogaerts *et al.* 2007). *Salamandra algira* sensu lato was divided into two subspecies by Donaire-Barroso and Bogaerts (2003a), by separation of *S. a. tingitana* Donaire-Barroso and Bogaerts 2003 from the nominate *S. a. algira* (NHMW 9251 designated neotype from the Edough Massif near Annaba, Algeria). Subsequently, Escoriza *et al.* (2006) rediscovered a population of *S. algira* in the Moroccan Beni Snassen Massif, upon which Escoriza and Comas (2007) described the hitherto endemic *S. a. spelaea* Escoriza and Comas 2007. Based on prior published evidence, Dubois and Raffaëlli (2009) elevated *S. a. tingitana* to species status based on ´very different morphology, its viviparous mode of reproduction and its different ethology, as shown by its special requirements in captivity´, and suggested that the populations from the Rif- and Atlas Mountains represented an undescribed species. These former statements however remained undefined, while the taxonomic value of 'special requirements in captivity' is at least doubtful. Moreover, viviparity is not characteristic for *S. a. tingitana* as a whole (Beukema *et al.* 2010). At this moment, only mtDNA data are available to demonstrate the divergence between populations on each side of the Moulouya Basin. Available morphological data have revealed minor size differences and chromatic features between described taxa, such as the absence/presence of red discoloration and

gular colouration. However, these characteristics vary widely within and among populations, as well as between subspecies (Bogaerts & Donaire-Barroso 2003; this paper). Local adaptive divergence of populations throughout the North African range therefore seems to be responsible for the majority of the observed differentiation (Bogaerts & Donaire-Barroso 2003; Beukema *et al.* 2010). Distribution data therefore remains critical in determination of individuals. As a result, we do not consider the current evidence sufficient to recognize multiple species within North Africa, and regard all previously described *Salamandra* taxa from this region as subspecies. However, as shown by Bogaerts and Donaire-Barroso (2003), Steinfartz *et al.* (2000) and Beukema *et al.* (2010), populations from the Rif- and Middle Atlas Mountains are distinct in terms of colour pattern and mtDNA, in respect to those located on the Tingitana Peninsula north of the Oued Laou and east of the Moulouya Basin. The description of *S. a. spelaea* by Escoriza and Comas (2007) and preservation of *S. a. algira* for Moroccan populations excluding *S. a. tingitana* (e.g. Beukema *et al.* 2010) moreover makes *S. algira algira* paraphyletic. Therefore, populations from the Rif- and Middle Atlas Mountains are herein recognized as a new subspecies. We provide detailed accounts on all Moroccan subspecies of *S. algira* below, while tentatively restricting *S. a. algira* to all Algerian populations.

### *Salamandra algira spelaea* Escoriza and Comas 2007

Fig. 8A.

**Background information.** While Moroccan presence of *S. algira* remained restricted to areas characterized by high rainfall west of the Moulouya Basin (Pasteur & Bons 1959), Melhaoui and Chavanon (1989) reported on a finding of a single individual on Jbel Ouartass, south of Berkane in the Beni Snassen Massif. Due to the karstic nature of this massif and presence of *S. algira* across the Algerian border near Oran, Melhaoui and Chavanon (1989) presumed populations of Beni Snassen to have gone unnoticed due to their nocturnal behaviour, while expecting them to be more widely dispersed across the massif. Subsequently, no additional sightings were recorded for nearly two decades, after which Escoriza *et al.* (2006) rediscovered the presence of *S. algira* in the Beni Snassen Massif. A year later, Escoriza and Comas (2007) described this isolated population as *S. a. spelaea* (holotype: MNCN 2005-05550; type locality: "Ouartass, Beni Snassen massif, Northeast-Morocco (Locality 5, at approximately 1300 m above sea level)"). The presence of minor red discoloration on several parts of the head and body appears to be congruent with descriptions of individuals from Rhar el Maden located near Remchi, north-western Algeria (Doumergue 1901), which is situated less than 100 km eastwards of the Beni Snassen Massif.

**Natural history.** Within the Beni Snassen Massif, *S. a. spelaea* can be found from 600–1300 m, occurring in the direct vicinity of water bodies which are used for reproduction (Escoriza & Comas 2007). Generally, populations are located in mixed forests consisting of *Quercus*, *Pinus* and *Olea* trees, characterized by an abundance of karstic limestone crevices and fissures (Escoriza *et al.* 2006). Due to the nature of the soil most water bodies used for reproduction are man-made, such as cattle watering troughs or springs, although small puddles and temporary streams are also used to deposit larvae. The onset of the activity period is initiated by the late autumn rains, and continues throughout the winter. Active individuals have been observed in November during humid or rainy weather, both in the late afternoon and night. During these observations, air temperature varied between 5.8 and 7 ºC while humidity ranges between 75–85% (Escoriza & Comas 2007). Larvae have been found in November and January, while fully metamorphosed juveniles were observed in mid-February (Escoriza & Comas 2007).

**Distribution.** The distribution map (Fig. 9A) is composed of the single record from Bons and Geniez (1996). The range of *S. a. spelaea* was thought to be restricted to an area of less than 45 km2 (Escoriza & Comas 2007), but is found largely continuous within (D. Escoriza pers. obs.). See also Fig. 10.

**National Red List Status.** Vulnerable (as part of *S. algira*). *Salamandra algira spelaea* was proposed to qualify as Endangered by Escoriza and Comas (2007). The entire range of *S. a. spelaea* is subject to intensive human impact by means of logging, construction of buildings, canalization of natural springs and construction of fountains which prevent access to the limited water bodies by pregnant females. These actions have led to considerable desertification, especially on the southern slopes of the massif (Escoriza & Comas 2007).


**FIGURE 8.** Members of the Salamandridae family native to Morocco. A: *Salamandra algira spelaea* Beni Snassen (DE). B-D: *Salamandra algira tingitana*; B: Jbel Musa (PdP); C: Tagramt (SB); D: Cudia Adru (DD). E-H: *Salamandra algira splendens* ssp. nov.; E: Bou Iblane (DD); F: Chefchaouen (DD) (holotype specimen); G: Bab Berret (DE); H: Ketama (DD). I-K: *Pleurodeles waltl*; I: Forêt de Mamora (WB); J: Casablanca (Gabriel Martínez del Mármol); K: Moulay Abdeslam (SB).

***Salamandra algira tingitana*** **Donaire-Barroso and Bogaerts 2003**
Figs. 8B–E.

**Background information.** Information about *S. algira* remained scarce for more than a century after the initial description of Bedriaga (1883) which was based on individuals from north-eastern Algeria. Both Bedriaga (1883)

and Pasteur and Bons (1959) reported *S. algira* to be larvae-bearing. Hence, observations of populations which deposit fully-developed juveniles among the limestone outcrops in extreme north-western Morocco (Donaire-Barroso & Bogaerts 2001; Donaire-Barroso *et al.* 2001) were unexpected. Subsequent research showed that populations from northwestern Morocco consistently lack red discoloration while often showing white specks or rosettes on the underside (Fig. 10A&B). The northernmost populations were shown to possess a high tendency towards hypoluteism and melanism (Bogaerts & Donaire-Barroso 2003). As a result of the aforementioned characteristics, Donaire-Barroso and Bogaerts (2003a) described *S. a. tingitana* (holotype: MNCN 41037; type locality: "500 m altitude on Jabal Muse (= Jabal Mousa) north Morocco"). Recent studies have shown *S. a. tingitana* to possess a more extended distribution than initially thought, while mtDNA studies showed the taxon to consist of three clades which diverged during the Pleistocene (Beukema *et al.* 2010). Only the northernmost clade deposits fully-developed juveniles, while populations located in the triangle formed by Tetouan, Ksar el-Kébir and Chefchaouen deposit larvae. The boundary between these is approximately located near the Oued Martil, with larvae occasionally having been found west of Tetouan (D. Donaire-Barroso pers. obs.), the southernmost population bearing fully-developed juveniles according to Beukema *et al.* (2010).

**Natural history.** Across the largely Mediterranean-influenced region located between the Strait of Gibraltar and Tetouan, *S. a. tingitana* inhabits chiefly limestone outcrops from sea level up to 390 m. Both barren eroded mountain summits (e.g. the type locality, Jbel Musa) as well as hilly terrain covered by maquis, *Pinus* and *Quercus* trees have been recorded as habitat (Donaire-Barroso & Bogaerts 2003a). Populations recorded in this area deposit fully-developed juveniles, even though temporary surface water is available at multiple sites (Donaire-Barroso & Bogaerts 2001; Donaire-Barroso *et al.* 2001; Donaire-Barroso & Bogaerts 2003a). On rare occasions, either a single or small number of larvae can be deposited in these water bodies during periods of abundant rainfall (Martínez-Medina 2001; Donaire-Barroso & Bogaerts 2003a). The activity period ranges from October to March, throughout which individuals are exclusively nocturnal, and only emerge from their shelters during humid or rainy weather (Martínez-Medina 2001; Donaire-Barroso & Bogaerts 2003a). Low temperatures (< 10º C) and presence of considerable wind does not have a negative influence on activity in general, in contrast to most European *Salamandra* populations (Thiesmeier & Günther 1996; Thiesmeier 2004; pers. obs. D. Donaire-Barroso & S. Bogaerts). During the day, individuals hide under superficial shelters such as rocks in humid weather throughout the activity period, while they retreat deep into karstic crevices from April to September (Donaire-Barroso & Bogaerts 2003a). Up to 17 fully developed juveniles can be deposited between October to March (Donaire-Barroso *et al.* 2001).

Southwards, the occurrence is limited to mountainous or hilly terrain between Tetouan, Ksar el-Kébir and Chefchaouen up to at least 1274 m (Fôret de Bouachem), where populations generally occur close to springs or brooks in (half-open) forests, agricultural terraces and limestone outcrops (e.g. Donaire-Barroso & Bogaerts 2003b). General activity of these larvae-bearing populations is likely similar to that of the populations which deposit fully-developed juveniles, although the former might benefit from a more humid, Atlantic climate among the western mountain ranges of the Tingitana Peninsula (Beukema *et al.* 2010). Larvae have been encountered in streams, springs and occasionally in temporary ponds. Donaire-Barroso and Bogaerts (2003b) encountered larvae at 700 m a.s.l. approximately 30 km south-west of Tetouan at the end of February, while D. Donaire-Barroso and W. Beukema (pers. obs.) observed recently metamorphosed juveniles with gill remnants at 400 m a.s.l. north-east of Ksar el-Kebir during the same period.

**Distribution.** The distribution map (Fig. 9A) is composed of records from Bons and Geniez (1996), Donaire-Barroso and Bogaerts (2003a) and Beukema *et al.* (2010). While most records fill in gaps within the continuous mountain ranges, several are located at the periphery of the distribution area, suggesting a wider range during the Pleistocene. An earlier suggested, isolated occurrence in the Ahl Serif northeast of Ksar-el-Kebir based on predictive species distribution modelling by Beukema *et al.* (2010) has been confirmed. As the niche model on Fig. 9A is composed of climatic data only (as opposed to that shown in Beukema *et al.* 2010), it is likely overpredicting the distribution of *S. algira* in Morocco. See Fig. 10 for the distribution of *S. a. tingitana* in relation to other Moroccan *Salamandra algira* ssp.

**National Red List Status.** Vulnerable (as part of *S. algira*).

**Taxonomic comment.** The original description of *S. a. tingitana* by Donaire-Barroso and Bogaerts (2003a) appeared in Pod@rcis, an online bilingual herpetological journal which has irregularly appeared since 2000. As such, this publication appeared well before a recent amendment of the International Code of Zoological

Nomenclature (ICZN) which permits electronic publication of new scientific names and nomenclatural acts (ICZN 2012). However, as detailed in the colophon of each Pod@rcis issue, copies of published papers were deposited on paper and CD-ROM at the PNMH, RMNH, Koninklijke Bibliotheek (The Hague, Netherlands), ZMA and ZMFK. Pod@rcis therefore meets the requirements of Article 8.6 of the ICZN, representing a published work.

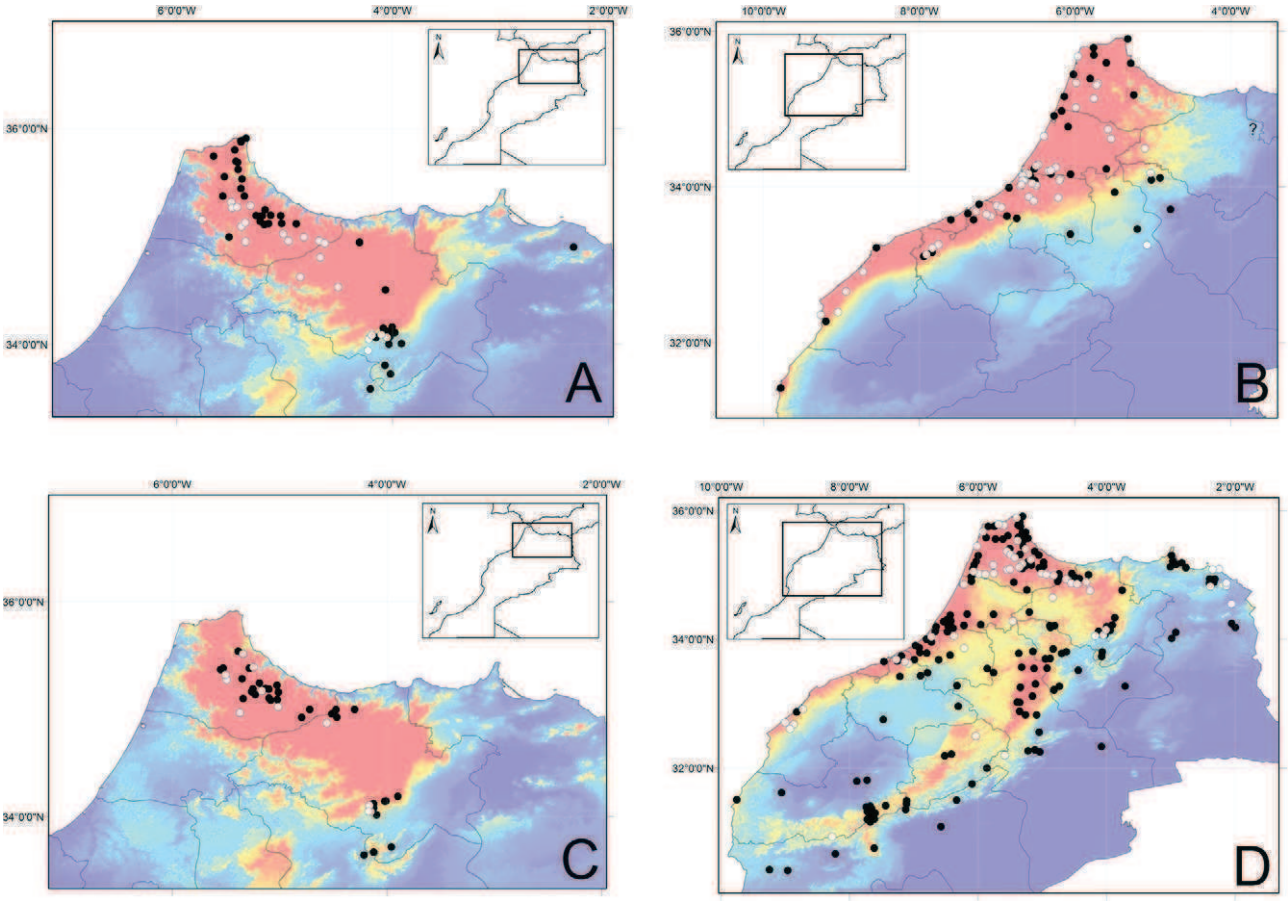


**FIGURE 9.** Niche models and distribution records of *Salamandra algira* (A); *Pleurodeles waltl* (B); *Alytes maurus* (C); and *Discoglossus* spp. (D) in Morocco. Warmer colours indicate higher climate suitability. Dark spots indicate records derived from literature, white spots show new records.

### *Salamandra algira splendens* Beukema, de Pous, Donaire-Barroso, Bogaerts, Garcia-Porta, Escoriza, Arribas, El Mouden & Carranza, ssp. nov.

Figs. 8F–H

*Salamandra algira* ssp.—Steinfartz *et al.* 2000: p. 410.
*Salamandra algira algira*—Donaire-Barroso and Bogaerts 2003a: p. 97.
*Salamandra* sp.—Dubois and Raffaëlli 2009: p. 35.

**Holotype.** RMNH 40173, an adult male collected at Aïn Tissimilan, Jebel el Kelaâ, Chefchaouen, western Rif Mountains, Morocco (N 35°10.5, W 5°14.6, 700 m a.s.l.) in November 1996 by David Donaire-Barroso and César Barrio (Fig. 8F).

**Paratypes.** EBD 29787, an adult female, collected at N 35°10.5, W 5°14.6, Aïn Tissimilan, Jebel el Kelaâ, Chefchaouen, western Rif Mountains in November 1996 by David Donaire-Barroso and César Barrio. MCNC 2010-0136, a sub adult female collected at N 35º02.1, W 5º01.5, 1445 m a.s.l., Bab Berret, western Rif Mountains, Morocco in December 2009 by Daniel Escoriza and Félix Amat (Fig. 8G). MCNC 2010-0128, 2010-0129, two adult males collected at N 34º85.7, W 2°21.0 and 1450 m a.s.l., Jebel Tazekka, Middle Atlas Mountains, Morocco in December 2009 by Daniel Escoriza and Félix Amat.

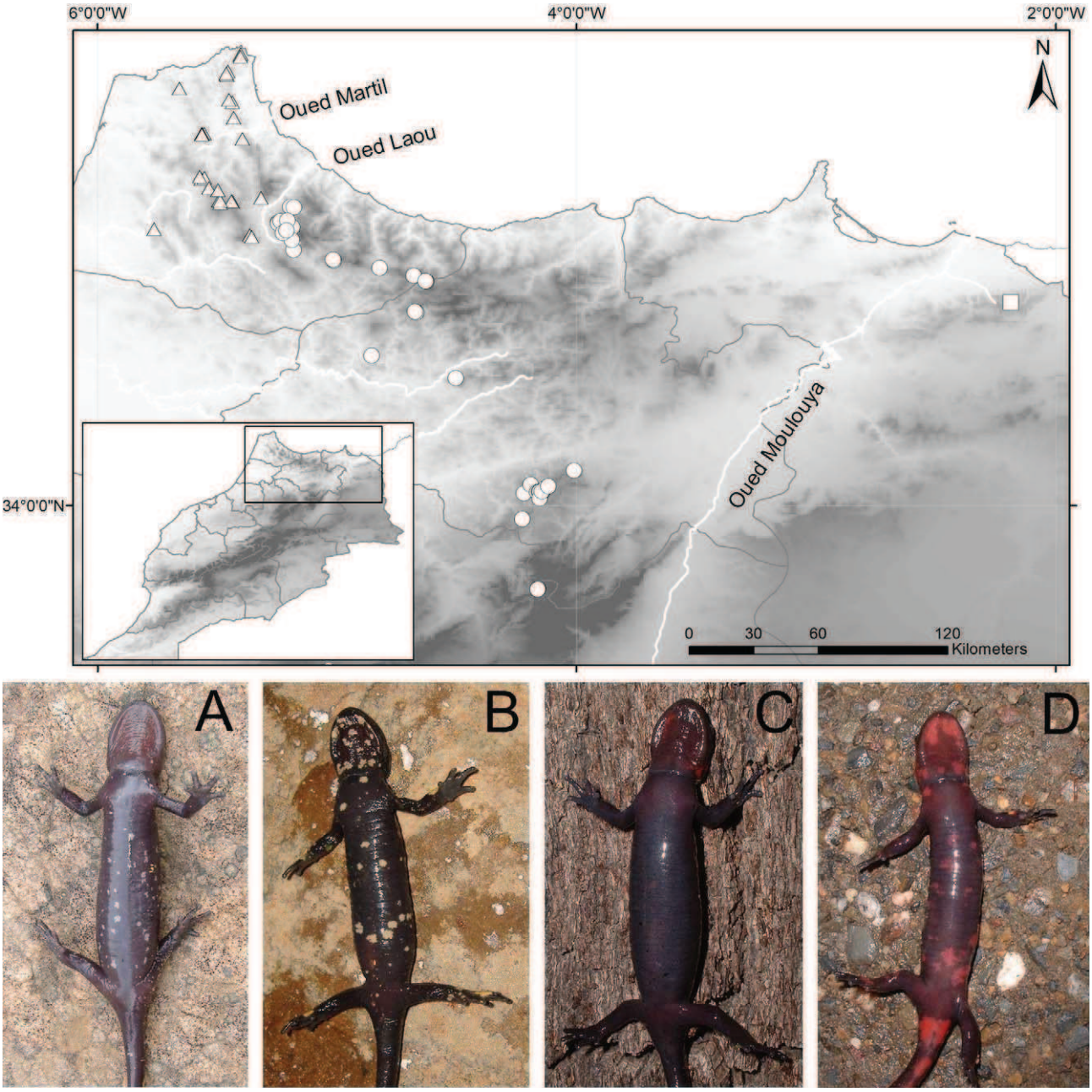


**FIGURE 10.** Upper panel: distribution of *Salamandra algira* ssp. in Morocco, indicating identified geographical barriers. Lower panel: variation in ventral pattern and coloration among Moroccan *Salamandra algira* ssp. A: *Salamandra algira tingitana*, west of Tetouan; B: *Salamandra algira tingitana*, Moulay Abdeslam; C: *Salamandra algira splendens* ssp. nov., Bou Iblane; D: *Salamandra algira splendens* ssp. nov., Ketama.

**Etymology.** The present participle *splendens* (Latin, from splendere = to shine) refers to the aesthetically pleasing appearance of this taxon, which is manifested in its pattern of bright yellow patches and red discolorations on a black background.

**Diagnosis.** A slender, large-sized (215 mm total length) North African member of the genus *Salamandra* having the combination of an elongated flat head, 1½ times longer than wide; long well separated limbs, fingers and tail; black coloration with rounded or elongated yellow markings and red discoloration; larvae-depositing. *Salamandra algira splendens* ssp. nov. can be distinguished from all other North African *Salamandra* taxa by a combination of colour pattern and distribution. *Salamandra algira algira* never possesses red discoloration and is restricted to the eastern Algerian Tell Atlas, comprising the Kabylies and the Annaba Peninsula (Algeria). *Salamandra algira tingitana* similarly never possesses red discoloration and is found exclusively north of the Oued

Laou. Northern populations of *S. a. tingitana* are characterized by hypoluteism (Fig. 8B, C) and bear fully-developed young, whereas larvae-bearing is the rule in *S. a. splendens* ssp. nov. *Salamandra algira spelaea* possesses only few and small red specks distributed across the head and body which do not extend to the degree observed in *S. a. splendens* ssp. nov. when present in the latter. Additionally, the former taxon is found exclusively in the Beni Snassen Massif east of the Moulouya Basin.

**Description of the holotype.** Habitus slender. Head flat, longer than wide. Snout short, slightly truncate in dorsal view, rounded in profile while exceeding beyond lower jaw. Parotoids large and elongated, with glandular pores, pointing slightly backwards. Double dorsolateral gland row from the posterior of the head to the basis of the hind legs. Skin smooth. Gular fold present. Limbs long and clearly separated from the body; fingers and toes long. Four fingers (fingers relative length 3>2>4>1) and five toes (toes relative length 4>3>2>5>1), all without webbing. Tail long, laterally slightly compressed, decreasing in width towards the tip. Measurements (in mm): SVL 94.0, LT 86.0, AL 31.0, PL 33.6, HL 25.0, HW 15.3 and PAL 13.2.

**Colour of holotype in life.** Body black with six elongated bright yellow patches on the dorsum. Parotoids and eyes covered by yellow patches, bordered by red discoloration. Yellow patches with red discolouration present at the base of each limb. Additional small red discolorations are present on the borders of the mouth, limbs, tail and gular region.

**Colour of holotype in preservative.** Body black. Yellow patches appear whitish. Red discoloration faded to dark brown, with smaller patches being difficult to distinguish.

**Variation.** Measurements of the type series are summarized in Table 2. All paratypes show black body coloration. Yellow patches are without exception present on the eyes, parotoids and at the basis of each limb. Yellow patches on the dorsum and tail show random, variable patterns but are always rounded, occasionally elongated. Of the four paratypes, three possess red discoloration on the head, tail and gular region. In addition to the paratype series, a total of 12 adult individuals (8 males and 4 females) from National Park Tazekka were measured in the field during mid-December 2012 by S. Bogaerts and W. Beukema and subsequently released in order to document morphological variation. All of these individuals showed red discolorations on the head, surrounding the paratoids. Eleven individuals showed red discoloration on the gular region (see also Fig. 10C), nine on the tails, eight on the legs and one on the ventral side (see also Fig. 10D). Eight individuals show few (< 10) tiny, irregularly-shaped whitish specks on the gular region; in two of these, the specks are present in a higher number while few are also present on the venter. Two males show glandular skin protuberances.

**TABLE 2.** Morphological measurements (mm) of Moroccan *Salamandra algira* ssp. Abbreviations defined in the text. Numbers displays range; average ± SD.

| Measurement | ***Salamandra algira splendens* ssp. nov. type series (*n* = 5).** | ***Salamandra algira splendens* ssp. nov. Tazekka (*n* = 12).** | ***Salamandra algira tingitana* (*n* = 8) Donaire-Barroso & Bogaerts 2003a** | ***Salamandra algira spelaea* (*n* = 8) Escoriza & Comas 2007** |
|---|---|---|---|---|
| SVL | 85.4–103.4; 93.3 ± 7.6 | 101.1–115.8; 110.5 ± 4.7 | 26–106.4; 73,1 ± 26.9 | 36.4–127.7; 97.3 ± 37.0 |
| LT | 76.6–101.2; 85.8 ± 11.4 | 75.3–99.7; 91.5 ± 7.3 | 15.0–81.0; 51.2 ± 22.2 | 24.9–108.2; 68.7 ± 27.6 |
| AL | 26.8–31.2; 29.2 ± 2.2 | 28.4–37.7; 34.8 ± 2.5 | 7.5–34.2; 21.7 ± 8.5 | 10.2–51.6; 33.7 ± 15.2 |
| PL | 30.1–36.1; 32.6 ± 2.8 | 32.6–40.8; 37.3 ± 2.7 | 7.2–37.2; 23.2 ± 9.8 | 11.5–50.8; 34.9 ± 14.9 |
| AP | 46.8–55.9; 49.0 ± 4.6 | 57.0–64.2; 60.2 ± 2.3 | - | - |
| HL | 19.5–27.9; 23.9 ± 3.5 | 25.0–28.7; 27.0 ± 1.3 | 8.0–25.5; 18.4 ± 6.0 | 9.6–29.2; 22.0 ± 7.7 |
| HW | 12.8–17.6; 15.3 ± 2.0 | 16.9–19.5; 18.0 ± 1.0 | 6.0–18.1; 13.2 ± 4.2 | 7.4–21.0; 15.4 ± 5.1 |
| PAL | 9.3–14.6; 12.2 ± 2.3 | - | - | 4.9–16.4; 12.3 ± 4.6 |
| LJL | 13.5–17.8; 15.5 ± 1.8 | - | 5.1–15.4; 11.3 ± 3.9 | 6.2–19.2; 14.5 ± 5.2 |
| SL | 6.1–7.0; 7.6 ± 1.3 | - | 2.5–7.6; 5.7 ± 1.8 | 2.6–8.9; 6.4 ± 2.3 |
| EN | 3.6–4.7; 4.1 ± 0.5 | - | - | - |
| IN | 6.3–7.3; 6.7 ± 0.5 | - | - | - |

**Natural history.** *Salamandra algira splendens* ssp. nov. has been found between 600–2000 m a.s.l. in Middle Atlas Mountains, and between 280–1700 m a.s.l. in the western Rif Mountains. Within this range, the distribution is largely limited to forests characterized by *Abies maroccana*, *Cedrus atlantica*, *Pinus* sp. and *Quercus* sp. or open karstic limestone formations (Donaire-Barroso & Bogaerts 2003b; Martínez-Medina 2007). At lower elevations populations can occur in shrub land chiefly composed of *Pistacia* sp. usually in the vicinity of brooks or springs. Individuals have incidentally been encountered in caves (Aellen 1951). The terra typica consists of a rocky limestone mountain slope, where only Mediterranean maquis and garrigue is present due to human activities such as logging and the grazing of livestock. However, the combination of sufficient altitude, high annual rainfall and porous limestone outcrops characterized by many crevices seem to allow survival of *S. a. splendens* ssp. nov. at this location.

The activity pattern of *S. a. splendens* ssp. nov. ranges from late autumn to early spring, which coincides with periods of high rainfall. The earliest observation of an active individual was made on 9 October 2001, when four adult males were found along a stream south of Ras el Mâ (Taza). South of the latter locality in National Park Tazekka 12 active adult individuals were observed along a humid limestone cliff in a single night in December 2012, foraging and mating during temperatures around 5ºC. During a rainy night on the 15–16th of November 1996 near Chefchaouen, mating behaviour and deposit of larvae was observed amongst approximately 100 adult individuals. At higher altitudes the activity period is shifted towards late spring and early summer, as periods of frost and snow limit activity in the winter. Larvae are generally deposited from October to May in small water bodies, which vary from sources, streams, temporary rain-filled ponds to small man-made concrete water reservoirs and irrigation channels. Dorda (1984) observed larvae and recently metamorphosed juveniles near Chefchaouen in late February. We encountered different sized larvae on December 9th, 1998 near Chefchaouen, ranging from recently deposited individuals of 39 mm to larvae close to metamorphosis measuring 58 mm. On January 4th, 2012 we observed similar size differences (ranging from 40 mm up to 75 mm) among larvae on Jebel Tazekka, south of Ras el Ma (Middle Atlas). Well-developed larvae were additionally observed during the end of April near Bab Berret (central Rif). Larvae of *S. a. splendens* ssp. nov. can be found together with larvae and adults of *A. maurus*, *D. scovazzi*, *H. meridionalis* and *P. saharicus*. Cannibalism in combination with a prolonged aquatic period and large growth (TL = 74 mm) has been observed (Escoriza *et al.* 2006). Metamorphosed juveniles can be found from beginning of January until the end of April, rarely up to summer or even past summer.

**Distribution.** The occurrence of *S. a. splendens* ssp. nov. is restricted to the western- and central Rif Mountains (from Chefchaouen to Imassinen and Jebel Aâloul, Jebel Rhelem) and the north-eastern Middle Atlas Mountains (from Tazekka to the Bou Iblane Massif) as previously described by Bons and Geniez (1996), Bogaerts and Donaire-Barroso (2003), Fahd *et al.* (2005), Escoriza *et al.* (2006) and Bogaerts *et al.* (2007). Populations on the Rif- and Middle Atlas Mountains are separated by a large stretch of non-suitable lowland and have been separated from each other for approximately 0.7 my (Beukema *et al.* 2010). The distribution map (Fig. 9A) is composed of records from Bons and Geniez (1996), Donaire-Barroso and Bogaerts (2003a), Bogaerts *et al.* (2007), Fahd and Mediani (2007) and Fahd *et al.* (2007). While most records fill in gaps within the continuous mountain ranges, several are located at the periphery of the distribution area, suggesting a wider range during the Pleistocene. As the niche model on Fig. 9A is composed of climatic data only (as opposed to that shown in Beukema *et al.* 2010), it is likely overpredicting the distribution of *S. algira* in Morocco. See Fig. 10 for the distribution of *S. a. splendens* ssp. nov. in relation to the other Moroccan *Salamandra algira* ssp.

**National Red List Status.** Vulnerable (as part of *S. algira*).

### Subfamiliy Pleurodelinae Tschudi

### Genus *Pleurodeles*

### Sharp-Ribbed Newt, *Pleurodeles waltl* Michahelles 1830

Figs. 8I–K.

**Background information.** Initial reports of *Pleurodeles* sp. in Morocco were attributed to *Pleurodeles poireti* (Gervais 1835), which added considerable confusion to the already ambiguous systematic situation of the genus

*Pleurodeles* in North Africa (Pasteur 1958; Carranza & Arnold 2004). Furthermore, Alluaud (1923) suggested that *P. poireti* and *P. waltl* occurred in sympatry around Rabat, which was finally refuted by Pasteur and Bons (1959) and Pasteur (1968), who unambiguously attributed Moroccan populations to *P. waltl*. Recent research has shown that populations of Sharp-Ribbed Newts in Morocco are closely related to those from south, south-eastern and eastern Spain, suggesting that they diverged during the Holocene. This relation has been attributed to a very recent either natural (Batista *et al.* 2003) or anthropogenic (Carranza & Arnold 2004) colonization of the African continent from the Iberian Peninsula. However, Stoetzel *et al.* (2010a) and Bailon *et al.* (2011) reported on fossilized Pleistocene *Pleurodeles* remains from several northwestern Moroccan sites, possibly attributable to an earlier than expected presence of *P. waltl* or the prior existence of a currently extinct lineage. The latter hypothesis is especially interesting due to a suggested Upper Miocene colonization of the African continent by *Pleurodeles* sp. via the Gibraltar land bridge, which subsequently diverged into the current *Pleurodeles nebulosus* (Guichenot 1850) and *P. poireti* in the eastern Maghreb, but disappeared from Morocco (Carranza & Arnold 2004; Veith *et al.* 2004).

Moroccan *P. waltl* have been described as morphologically different from their Iberian counterparts due to their smaller total length, lower dorsal tail crest, rapid development of the crest during the reproductive period and the rare display of the distress call (Pasteur 1958). This has been interpreted as the possible occurrence of an undescribed taxon in Morocco (e.g. Pasteur & Bons 1959; Schleich *et al.* 1996). The measurements presented by Pasteur (1958) were based on an undisclosed number of samples from uncertain origin, in which only a distinction was made between 'Morocco' and 'Iberia'. According to these data, Moroccan individuals are characterized by an average SVL of 58.8 mm (max TL 218 mm), while Iberian individuals show an average SVL of 100.5 mm (max TL 280 mm, in contrast to the erroneous report of 300 mm by Schleich *et al.* 1996). The current measurements based on 65 individuals (50 females, 16 males) originating from Forêt de Mamora, near Rabat show an average SVL and TL of respectively 79.0 and 173.2 (max 270.0) mm for females, and 62.3 and 131.4 mm (max 145.3) for males (Table 3). The recovered sexual dimorphism might be partially related to the relative small amount of males measured. These data reveal larger average sizes for Moroccan *P. waltl* when compared to those presented by Pasteur (1958), of which at least the females are of comparable length to those from the Iberian Peninsula. However, it has to be noted that there is considerable geographical variation regarding size of *P. waltl* on the Iberian Peninsula, ranging from a maximum TL of 312 and 286 mm for respectively males and females in Huelva (González de la Vega 1988) to a maximum TL of 259 and 251 mm (average 176.84 and 175.63 mm) for respectively males and females in Catalunya (Fontanet & Horta 1989). In conclusion, the presence of considerable interpopulation variation in terms of at least size differences in combination with the very recent African colonization by *P. waltl* (Batista *et al.* 2003; Carranza & Arnold 2004) make it seem unlikely that a distinct subspecies inhabits Morocco.

**TABLE 3.** Morphological measurements (mm) of *Pleurodeles waltl* from Fôret de Mamora, Rabat, Morocco. Numbers display average ± SD (range).

| | **Male** | **Female** |
|---|---|---|
| *N* | 16 | 50 |
| TL | 13.14 ± 0.82 (11.93 – 14.53) | 17.32 ± 3.12 (11.75 – 27.00) |
| SVL | 6.23 ± 0.48 (5.76 – 7.09) | 7.9 ± 1.53 (5.30 – 12.90) |
| LT | 6.91 ± 0.46 (6.17 – 7.48) | 9.42 ± 1.80 (6.45 – 14.10) |
| HL | 1.73 ± 0.13 (1.54 – 2.01) | 2.13 ± 0.32 (1.70 – 3.05) |
| HW | 1.24 ± 0.08 (1.10 – 1.41) | 1.71 ± 0.30 (1.15 – 2.50) |

**Natural history.** The main distribution of *P. waltl* comprises the Atlantic lowlands ranging from Tanger southwards to Essaouira, where the species generally occupies temporary ponds and flooded fields (Pasteur & Bons 1959). Several populations are found in the western Rif- and Middle Atlas Mountains, where they can be found in (temporary) ponds and slow flowing streams (D. Donaire-Barroso & W. Beukema, pers. obs.) or lakes (e.g. Dayets, Stemmler 1965). Reproductive activity is dependent of altitude, starting with the onset of the winter (lowland) or spring (mountains) rains, during which males develop extended dorsal and ventral tail fins. Along the

Atlantic Coast and at lower altitudes inland, larvae can be found from January onwards (Dorda 1984; Lapeña *et al.* 2011; pers. obs. P. de Pous). Juveniles are mostly found on land, also during the winter, only becoming aquatic once they reach sexual maturity (Pasteur & Bons 1959). *Pleurodeles waltl* is a highly aquatic newt which may stay aquatic outside of the reproductive period. However, as most of these water bodies dry out during summer, aestivation takes place on land under stones, logs or in fissures in clay soils (Pasteur & Bons 1959). The natural history of populations in the western Rif Mountains and Middle Atlas Mountains is not well known.

**Distribution.** Within Morocco, *P. waltl* mainly occurs in the north-western part of the country, following the Atlantic coast to the south as far as Essaouira (Bons & Geniez 1996) where its presence was recently confirmed by Harris *et al.* (2008). While the species is especially common in lowland areas (e.g. Pasteur & Bons 1959; El Hamoumi 1988; El Hamoumi & Himmi 2010), *P. waltl* also occurs in mountainous terrain of the western Rif (Fahd & Mediani 2007). A single record from the eastern Rif Mountains (Talamagaït, indicated in Fig. 9B by a question mark, Mellado & Mateo 1992) is in need of confirmation. Additionally, *P. waltl* occurs sporadically in the Middle Atlas in the vicinity of Dayets (Stemmler 1965; Bons & Geniez 1996). The distribution map (Fig. 9B) is composed of records from Bons and Geniez (1996), Carretero *et al.* (2004) and Harris *et al.* (2010). New distribution records fill in prior large gaps in the north-western range of *P. waltl,* showing the species to be commonly present in the lowlands and mountainous areas of the Tingitana Peninsula. A second significant population cluster is located around Rabat, in the cork oak forests of Mamora, Temara and Ben-Slimane. Additionally, the species appears to be widespread on the Doukkala plain, between the occurrences near El Jadida and Safi (Bons & Geniez 1996; Carretero *et al.* 2004).

**National Red List Status.** Near Threatened.

## Order Anura

## Family Alytidae Fitzinger

### Moroccan Midwife Toad, *Alytes (Baleaphryne) maurus* Pasteur and Bons 1962

Figs. 11A–C.

**Background information.** A few decades after the initial discovery of the genus *Alytes* in Morocco by Galan (1931), Pasteur and Bons (1962) attributed the African populations to *Alytes obstetricans maurus* based on divergent tadpole morphology. In contrast, Arntzen and Szymura (1984) suggested Moroccan populations to be of anthropogenic origin and identical with *Alytes obstetricans* (Laurenti 1768), based on minimal recovered electrophoretic divergence in respect to Iberian and Western European populations. The position of *A. maurus* within *Alytes* would remain obscure for some time. Concurrently however, the discovery of extant *Alytes muletensis* (Sanchíz & Adrover 1979 "1977") on the Balearic island of Mallorca (Mayol *et al.* 1980) led to a systematic rearrangement of the genus (Dubois 1987 "1986"). While the subgenus *Baleaphryne* was initially proposed solely for *A. muletensis*, Donaire-Barroso and Bogaerts (2003b) elevated *A. maurus* to species level and placed the taxon in *Baleaphryne* Sanchíz & Adrover, 1979 "1977". The validity of the subgenus, and confirmation of the correct attribution of its members, was subsequently provided by means of osteological, mitochondrial and nuclear evidence (Martínez-Solano *et al.* 2004; Gonçalves *et al.* 2007), albeit not explicitly. According to the most recent phylogenetic analyses (Gonçalves *et al.* 2007), *A. maurus* forms a trichotomy with the Iberian *Alytes dickhilleni* Arntzen and García-París 1995 and *A. muletensis*. A more recent study including several nuclear genes places *A. maurus* with high support as sister to *A. dickhilleni*, while *A. mulentensis* is shown to be sister to the *A. maurus-A. dickhilleni* clade (M. Vences pers. comm.). The collapse of the Gibraltar land bridge at the end of the Miocene has most likely led to the rapid diversification of the *Baleaphryne* members, separating the ancestor of *A. maurus* on the African continent (Martínez-Solano *et al.* 2004). The current, fragmented distribution range of *A. maurus* is likely a result of increasing temperatures throughout the recent Quarternary, as a fossil record of a *Baleaphryne* sp. from the Upper Pleistocene in the arid Jbilets northwest of Marrakech (Hossini 2001) suggest a much wider historical distribution.

**Bioacoustics.** Márquez *et al.* (2011) recently described the advertisement call of male individuals recorded in the Middle Atlas Mountains. Males were encountered calling at dusk and during rainy nights, located beneath

cover objects or out in the open. Pulse duration ranges between 77–217 ms (mean 97.3 ms), while emitted at intervals of several seconds. To the ear, the call resembles a short tonal burst of sound with a very brief rise time and a longer fall time. While the advertisement call of *A. maurus* is significantly shorter in duration than that of *A. dickhilleni*, it falls within intraspecific variation of *A. obstetricans.*

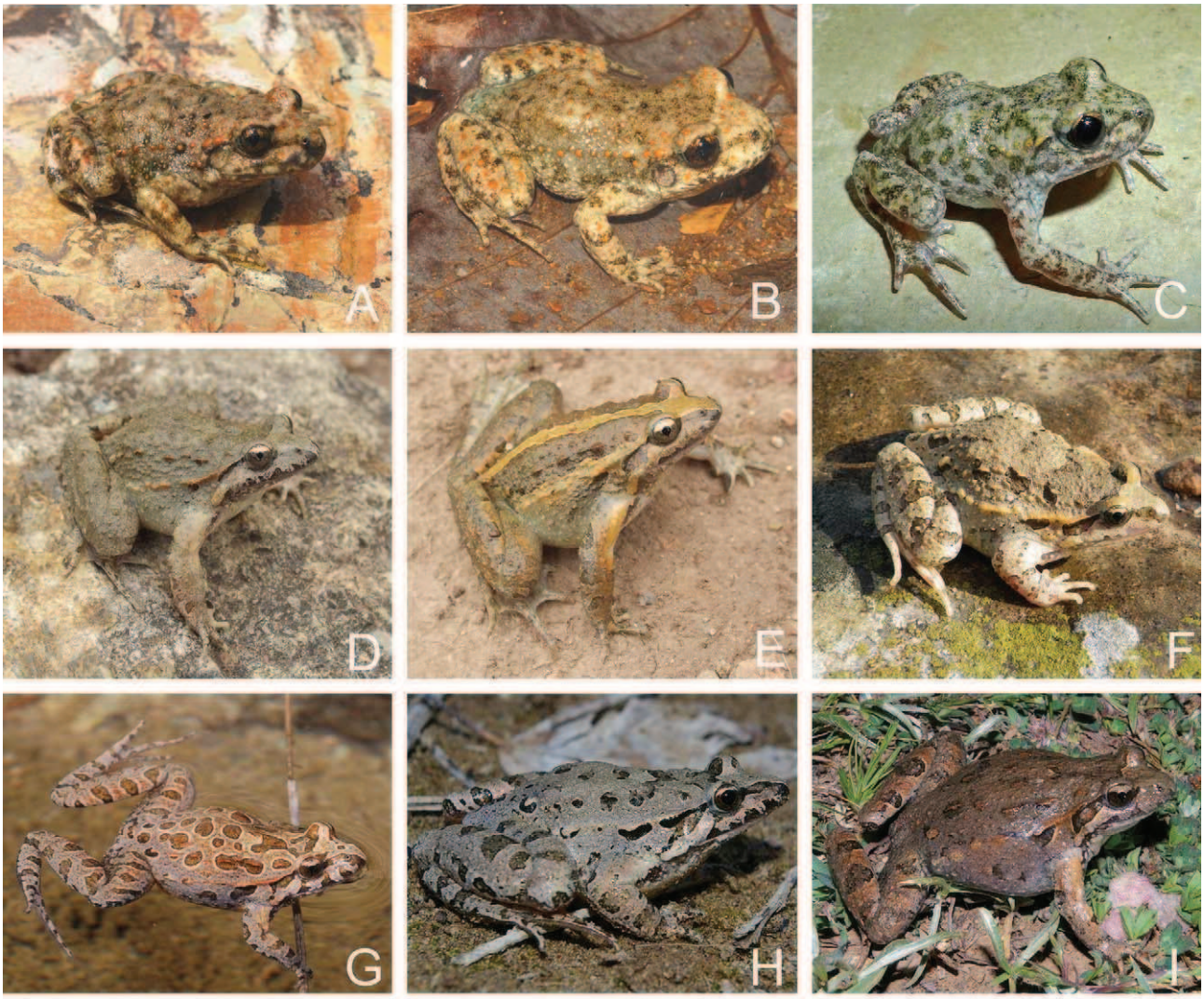


**FIGURE 11.** Members of the Alytidae and Discoglossidae families native to Morocco. A-C: *Alytes maurus*; A–B: Tazekka (Gabriel Martínez del Mármol); C: Chefchaouen (SB). D-G: *Discoglossus scovazzi*; D–E: Jbel Musa (WB); F: Oualidia (WB). I-J: *Discoglossus pictus*; G: Beni Snassen (DE); H: Saidia (SB); I: Beni Snassen (SB).

**Tadpole.** Pasteur and Bons (1962) gave a comprehensive description of the tadpole of *A. maurus* (Fig. 12A). The following account is a summary of the original description. Fully developed tadpoles are characterized by their large size (up to 90 mm), midventral spiracle and spotted tail. Eyes positioned dorsally on head. Interorbital space at least twice as wide as the internarial space. Oral disc ventral, significantly wider than interorbital space. Two tooth rows on the upper labium and three on the lower. Upper supralabial row composed of two series, lower supralabial row and all infralabial rows composed of at least three series. Upper infralabial row with very narrow median gap. Spiracle midventral. Colour grey, brown or olive with conspicuous pattern of smaller and larger golden and dark patches and spots.

**Natural history.** Available natural history information on this species has been presented and summarized by Donaire-Barroso and Bogaerts (2003b) and Donaire-Barroso *et al.* (2006). *Alytes maurus* is commonly found in open forests, agricultural terraces bordered by stone walls and open, rocky valleys in the vicinity of streams or water sources. Dominant vegetation consists mainly of *Quercus* sp., *Juniperus* sp. and *Olea europaea*. In in the Middle Atlas populations are additionally encountered in *Cedrus atlantica* forest (Donaire-Barroso *et al.* 2006).

Occurrence in high-altitude mountain meadows has occasionally been noted (Harris *et al.* 2008). Habitats are often dominated by an undergrowth of maquis and high abundance of rocks and (karstic limestone) rocky outcrops. Near Chefchaouen in the western Rif Mountains advertisement calls have been heard from February until April, while larvae close to metamorphosis were observed in August (Donaire-Barroso & Bogaerts 2003b). In the Middle Atlas (Taza), advanced-staged larvae were encountered in August and September (D. Donaire-Barroso pers. obs.). It is not uncommon for larvae to grow to large sizes and hibernate in permanent streams. Indeed, larvae have also been observed during January and February (Donaire-Barroso & Bogaerts 2003b). Populations have been encountered from 200 up to 2142 m a.s.l. (Donaire-Barroso *et al.* 2006), although most occur at intermediate altitudes (Donaire-Barroso & Bogaerts 2003b). Sympatric occurrence with larvae-bearing populations of *S. a. tingitana* and *S. a. splendens* ssp. nov. is common.

**Distribution.** Donaire-Barroso and Bogaerts (2003b) and Donaire-Barroso *et al.* (2006) reviewed the distribution of this species in the Rif Mountains and Middle Atlas Mountains, respectively. *Alytes maurus* is commonly found in brook valleys of the western- and central Rif Mountains and several small peripheral ranges. Presence in the Middle Atlas Mountains seems to be limited to the Tazekka and Bou Iblane Massifs. Despite prior mentions of *A. maurus* north of the Oued Martil (e.g. in Ceuta, Bons & Geniez 1996), such records have not been confirmed by subsequent inventories of the area (e.g. Martínez-Medina 2001; Donaire-Barroso & Bogaerts 2003b) and are consequently not displayed on the map. The distribution map (Fig. 9C) includes records from Bons and Geniez (1996), Donaire-Barroso and Bogaerts (2003b), Donaire-Barroso *et al.* (2006), Fahd and Mediani (2007), Fahd *et al.* (2007) and Harris *et al.* (2008). Newly discovered localities largely fill in gaps within the known distribution range. The niche model (Fig. 9C) shows potential suitable areas in the Middle Atlas and east of the Moulouya Basin, which are unoccupied due to major geographical barriers separating these from the realized distribution.

**National Red List Status.** Near Threatened.

## Family Discoglossidae Günther

Divergence between *Alytes* and *Discoglossus* Otth 1837, hitherto grouped in the Alytidae, took place according to most recent estimations during the Late Jurassic (Bossuyt & Roelants 2009) or Cretaceous (Blackburn *et al.* 2010). Conclusively, Bossuyt and Roelants (2009) suggested reinstatement of the Discoglossidae in recognition of the long separate evolutionary history of the latter genus. We herein follow this suggestion, which was also adopted by Pyron and Wiens (2011).

### Moroccan Painted Frog, *Discoglossus scovazzi* Camerano 1878

Figs. 11D–F.

**Background information.** *Discoglossus scovazzi* was initially separated from *D. pictus* based on the presence of a distinct tympanum, which would be absent in the latter. As this character was proven to occur randomly in species of the genus *Discoglossus*, Boulenger (1891) placed *D. scovazzi* in the synonymy of *D. pictus*. This situation would not change for nearly a century, after which Lanza *et al.* (1986), Fromhage *et al.* (2004), Zangari *et al.* (2006) and most recently Pabijan *et al.* (2012) proposed several biogeographical scenarios for the genus based upon mtDNA and nDNA data, revealing the Moroccan populations to be significantly diverged. As this situation has not been settled, two potential scenarios for diversification of *Discoglossus* spp. are currently considered. The older scenario involves vicariance due to the breakup and subsequent rifting of the Hercynian orogeny after the opening of the western Mediterranean Basin in the late Oligocene (Pabijan *et al.* 2012). This process resulted in present day Corsica, Sardinia, Calabria, the Balearic Islands, parts of the Algerian Atlas (Kabylies), the Peloritan Massif of Sicily and parts of the Betic–Rif mountain belt of Spain and Morocco, nearly all of which are currently occupied by *Discoglossus* spp. Alternatively, it has been proposed that the genus *Discoglossus* colonized Africa twice at the end of the Messinian Salinity Crisis, via both the Gibraltar land bridge in the west, and a second connection in the eastern Maghreb (Zangari *et al.* 2006). The reflooding of the Mediterranean acted as a vicariant event, isolating *Discoglossus* at both sides of the Maghreb.

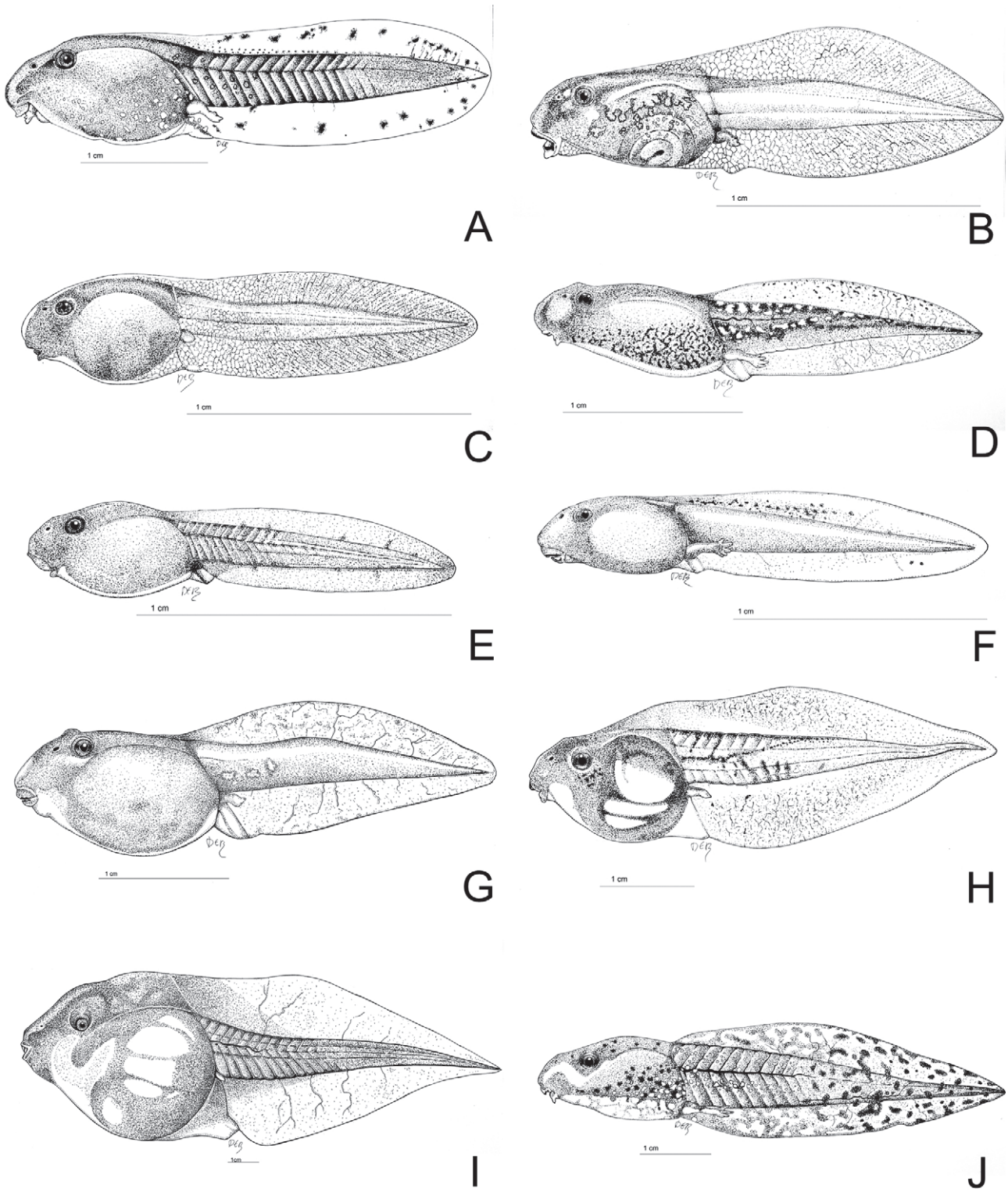


**FIGURE 12.** Tadpoles of Moroccan anurans. A: *Alytes maurus*, Ketama. B: *Discoglossus scovazzi*, Safi. C: *Discoglossus pictus*, Tafuyalt (Algeria). D: *Amietophrynus mauritanicus*, Mohammedia. E: *Barbarophryne brongersmai*, Smimou. F: *Bufo bufo*, Tazekka. G: *Bufotes boulengeri*, Dayet Aoua. H: *Hyla meridionalis*, Mohammedia. I: *Pelobates varaldii*, Kenitra. J: *Pelophylax saharicus*, Sidi Ifni. All drawings made by DE.

Fromhage *et al*. (2004) showed Moroccan painted frogs to represent a highly divergent mtDNA (12S and16S rRNA) lineage compared to *D. pictus*, based on which these authors elevated *D. scovazzi* to species level. Additionally, Fromhage *et al*. (2004) revealed *D. scovazzi* to display a closer relationship with the Iberian *Discoglossus* than with *D. pictus*. Although Zangari *et al.* (2006) found similar results as Fromhage *et al*. (2004) at

the molecular level (albeit showing low bootstrap support), *D. scovazzi* and *D. pictus* were revealed to group together based on nDNA. According to the former authors, the nDNA results suggest historical contact between *D. scovazzi* and *D. pictus* up to 1.8 mya, when the general climate turned colder and drier. However, Zangari *et al.* (2006) also suggested that *D. pictus* expanded its range during the Pleistocene, thus reaching Morocco. While the Moulouya Basin likely acted as a barrier between these two species (Zangari *et al.* 2006) it is not clear whether they currently remain separated. Additional sampling of *Discoglossus* populations in north-eastern Morocco is therefore necessary in order to clarify the distribution and potential contact areas of both taxa (see also Pabijan *et al.* 2012).

**Tadpole.** Tadpole illustrations adapted to resemble Moroccan *Discoglossus* sp. were provided by Pasteur and Bons (1959), based on the description of Algerian material by Boulenger (1897). As these adaptations have not been specified or designated both in terms of morphological characteristics and origin, we regard the tadpole of *D. scovazzi* as currently undescribed. A preliminary drawing of the body morphology is displayed in Fig. 12B.

**Bioacoustics.** The advertisement call of *D. scovazzi* was studied in individuals originating from Ceuta (Sebta) by Vences and Glaw (1996). With a duration of approximately 200-210 ms the advertisement call is shorter than that of *D. pictus* (Glaw & Vences 1991). Comparable with the latter species, *D. scovazzi* also expresses its call in a series generally without intervals. Vences and Glaw (1996) commented on bioacoustic differentiation between *Discoglossus* species and noted that the call of *D. scovazzi* bears more similarity to that of *D. pictus* than to *Discoglossus galganoi* Capula, Nascetti, Lanza, Bullini and Crespo 1985.

**Natural history.** Adult *D. scovazzi* can either be uniform coloured, striped or spotted (Fig. 11D-F). The occurrence and abundance of these phenotypes differs per population (Pasteur & Bons 1959). North African *Discoglossus* spp. are in general characterized by their cryptic behaviour outside of the breeding season (Doumergue 1901; Le Berre 1989). Adult individuals are usually found near small water bodies such as temporary ponds, slow-flowing streams and water sources, but also submerged tracks of vehicles or roadside ditches. Diurnal activity is restricted. *Discoglossus scovazzi* is one of the first amphibian species to reproduce with the onset of winter rains. W. Beukema (pers. obs.) observed small tadpoles during the end of October in the northernmost Tingitana Peninsula. Martínez-Medina (2001) encountered recently deposited larvae in November and December within the same area. Dorda (1984) encountered larvae close to metamorphosis at the end of February near Tetouan, and reproductive activity near Kenitra. Metamorphosis was also observed in March near Rabat (P. de Pous pers. obs.). At higher altitudes, Galan (1931) found juveniles in the central Rif Mountains during June. Werner (1931) found larvae and juveniles near Tadlest (2250 m a.s.l.), but only juveniles near Asni (1600 m a.s.l.) during June.

**Distribution.** The Moroccan Painted Frog is an endemic species, which occurs in the north-western lowlands and mountains, southwards to the Souss Valley. *Discoglossus scovazzi* is known from a large number of aquatic biotopes throughout Morocco from sea level up to the Atlas Mountains, excluding the Saharan areas. The distribution map (Fig. 9D) is composed of records from Bons and Geniez (1996), Fahd and Mediani (2007), Fahd *et al.* (2007) and Harris *et al.* (2008). New records reveal the distribution of *D. scovazzi* to be almost continuous from the Rif Mountains southwards to Casablanca. Several new records on the Doukkala plain confirm presence of this species in the area.

**National Red List Status.** Least Concern.

### Painted Frog, *Discoglossus pictus* Otth 1837

Figs. 11G–I.

**Background information.** See *D. scovazzi*.

**Tadpole.** Lataste (1879) provided a comprehensive account on the tadpole of *D. pictus* and its development which was however based on a combination of individuals originating from Algeria and 'Spain', as *D. galganoi* was not yet recognized as a separate entity. Individuals from the latter area were provided by Eduardo Boscá, who at that time operated from Ciudad Real. While the exact origin of these individuals cannot be retraced with certainty, it is highly likely that the tadpole descriptions are based on multiple taxa. However, Boulenger (1897) gave a description of *D. pictus* tadpoles originating from Algeria, which can be unambiguously attributed to *D. pictus* sensu stricto. The following is a summary of the description by Boulenger (1897), supplemented by details

from Doumergue (1901). Fully developed tadpoles are characterized by a midventral spiracle and pattern of fine, brown polygonal meshes on the tail fins. Eyes positioned dorsally on head. Interorbital space 1½ times as wide as the internarial space. Body relatively elongated. Spiracle midventral. Tail length three to four times tail height, broadly rounded at the end. Dorsal tail fin slightly elevated anteriorly, does not extend onto the dorsum. Mouth elliptical, bordered by single line of papillae, narrowly interrupted in the middle of the upper lip. Two tooth rows on the upper and three on the lower labium. First supralabial and first infralabial row consist of one or two series, others always of two. Upper infralabial row with very narrow median gap. Dorsal and lateral colour brown or olive, venter lighter than dorsum. Tail fins with network of fine brown polygonal meshes, although this is very difficult to observe in young larvae. Development of pattern (i.e. dorsal stripes or spots) is usually visible before metamorphosis. A drawing of the tadpole is displayed in Fig. 12C.

**Bioacoustics.** The advertisement call of *D. pictus* has been described by Glaw and Vences (1991), based on individuals originating from Sicily and the introduced Franco-Iberian metapopulation (the latter being part of the former NE Maghrebian taxon *D. p. auritus* Herón-Royer 1888). No significant differences were found between call characteristics from both regions. The advertisement call of *D. pictus* lasts for approximately 250 ms but is often expressed in a series, mostly characterized by a lack of intervals. The mean duration of the expiratory and inspiratory pulse groups are approximately half the length of the call (Glaw & Vences 1991).

**Natural history.** Only incidental reports on the natural history of *D. pictus* in Morocco have been made. The diet of *D. pictus* consists of insects (Doumergue 1901). Fahd *et al.* (2005) encountered larvae and recently metamorphosed juveniles in the Beni Snassen Massif at the end of May, while Escoriza and Comas (2007) commented on the presence of larvae in the same mountains during November. Larvae were also observed in Beni Snassen during early March, while reproductive activity was observed in December (P. de Pous & W. Beukema pers. obs.). Doumergue (1901) noted that the activity period in Algeria starts in autumn, while large numbers of aquatic individuals were observed in December. Additional observations were made up to April, but mostly consisted of small numbers of individuals. Reproductive activity at higher altitudes can either take place in autumn or early spring (Doumergue 1901).

**Distribution.** The presence of *D. pictus* has only been confirmed from eastern Morocco (Zangari *et al.* 2006). *Discoglossus pictus* appears to be widespread in the north-eastern part of the country, from the Mediterranean lowlands southwards to semi-desert areas. The exact distribution border of *D. pictus* is not known. Whether the distribution of this species extends westwards beyond the Moulouya Basin, and if secondary contact with *D. scovazzi* occurs remain interesting topics for future research (Pabijan *et al.* 2012).

## Family Bufonidae Gray

Despite the accumulating support for recognition of the 'Green Toad group' as a separate evolutionary unit (Stöck *et al.* 2006; Van Bocxlaer *et al.* 2009), several authors have advocated retaining *Bufo* for all species from the Western Palaearctic and Central Asia (Dubois & Bour 2010; Speybroeck *et al.* 2010). Additional confusion concerning attribution of either *Pseudepidalea* Frost, Grant, Faivovich, Bain, Haas, Haddad, de Sá, Channing, Wilkinson, Donnellan, Raxworthy, Campbell, Blotto, Moler, Drewes, Nussbaum, Lynch, Green and Wheeler 2006 or *Bufotes* Rafinesque 1815 as the correct generic epithet for the 'Green Toad group' (i.e. Frost *et al.* 2006; Dubois & Bour 2010) hampered taxonomic action. Dubois and Bour (2010) provided a detailed nomenclatural assessment, revealing *Pseudepidalea* to represent a junior objective synonym of *Bufotes*, thereby giving priority to the latter. However, Dubois and Bour (2010) listed these nomina as subgenera rather than genera, based on the presence of otherwise intergeneric hybridization and the restricted sampling size of prior studies focussing on the Bufonidae. According to the results presented herein which comprise a dense sampling of bufonid members (Fig. 6), most genera currently recognized by Frost (2013) represent well-supported monophyletic units (see also Van Bocxlaer *et al.* 2010). We herein follow this taxonomy, while as a result treat the main bufonid clades inhabiting the Western Palaearctic and Central Asia as genera. Additionally, we note that recognition of these genera is supported by all priority Taxon Naming Criteria (TNC; Vences *et al.* 2013), i.e. Monophyly, Clade Stability and Phenotypic Diagnosability, see e.g. Boulenger (1881 "1880", 1897); Gallix (2002); Guillon *et al.* (2004); Frost *et al.* (2006); Delfino *et al.* (2009); Van Bocxlaer *et al.* (2009, 2010); Pyron and Wiens (2011); current study.

**Berber Toad, *Amietophrynus mauritanicus* (Schlegel 1841)**
Figs. 13A–C.

**Background information.** While traditionally assumed to be a Maghrebian endemic, Werner (1931) already suggested that *A. mauritanicus* was related to sub-Saharan Bufonidae. Harris and Perera (2009) showed that the former "*Bufo*" *mauritanicus* is indeed part of a sub-Saharan clade of toads recently assigned to the genus *Amietophrynus* (Frost *et al.* 2006). These results were confirmed by Van Bocxlaer *et al.* (2009) who suggested transferring the species from "*Bufo*" to *Amietophrynus*. Recent mitochondrial and nuclear analyses have recovered a basal position for *A. mauritanicus* with respect to the other members of the *Amietophrynus* genus (Pyron & Wiens 2011), which is in contrast with the results presented herein (Fig. 6). Despite its large distribution throughout the Maghreb, intraspecific genetic variation of *A. mauritanicus* appears to be minimal due to post-glacial expansion into its current range, possibly originating from southern populations (Harris & Perera 2009).

**Tadpole.** Pasteur and Bons (1959) gave a description of the tadpole of *A. mauritanicus* (Fig. 12D). Fully developed tadpoles are mainly characterized by colour pattern. Body shape ovoid. Eyes positioned dorsally on head. Spiracle sinistral. Oral disc ventral. Two tooth rows on the upper and three on the lower labium. Lower supralabial row with median gap. Lowest infralabial row about same length as both other infralabial rows. Lower horny beak V-shaped with dark, keratinized part only occupying 1/3 of height. Tail more than four times as long as deep. Dorsal tail fin ending at the muscular base of the tail. Dorsal and lateral colour black, venter and tail dark grey. Body spotted with abundant tiny golden dots. In general, tadpoles remain small (up to 30 mm) while metamorphosing at about 10 mm (Doumergue 1901; Hoogmoed 1972). The tadpoles of *A. mauritanicus* are herbivorous (Doumergue 1901; Pasteur and Bons 1959).

**Bioacoustics.** Roussel and Amar (1985) described the advertisement call of a Saharan and a Mediterranean population of *A. mauritanicus*, both located in Algeria. Call duration of the Mediterranean population averaged 0.57 ± 0.01 seconds, consisting of 12.3 ± 1.5 pulses with an average length of 20.0 ± 1.5 ms (interval 37.0 ± 2.2 ms). Conversely, call duration of the Saharan population averaged 0.63 ± 0.08 seconds, consisting of 13.5 ± 0.6 pulses with an average length of 25.3 ± 3.1 (interval 31.0 ± 2.5 ms). Calls are usually emitted in short series. To the ear, the call sounds short and low. The advertisement call presented in Fig. 7A was recorded at 14.8°C (85.80% humidity).

**Natural history.** A certain degree of sexual dimorphism is present, in which the dorsal patches of the male are usually less defined when compared to those of the female, while females are conspicuously larger. Uniform-coloured individuals are relatively common at the southern margin of the distribution (Pasteur & Bons 1959, Fig. 13C), which has led to occasional confusion with *B. bufo* in the past when natural history information on the latter species was scarce (Hordies & Van Hecke 1982). *Amietophrynus mauritanicus* can be commonly encountered throughout Morocco, usually in the vicinity of permanent or temporary water bodies. Based on a study of several reproduction sites in western and central Morocco, El Hamoumi *et al.* (2007) listed *A. mauritanicus* as a temporary pond breeder, highly dependent on seasonal rain patterns (see also Doumergue 1901). Reproduction in the Mediterranean part of the distribution takes place between April and June, while Saharan populations reproduce between February and April, comparatively late in comparison to the occasionally sympatric *B. brongersmai* and *B. boulengeri* (Doumergue 1901; Roussel & Amar 1989; Guillon *et al.* 2004; P. de Pous pers. obs.). Consequently, water temperatures recorded during calling activity (18–24 ºC) are considerably higher when compared to other Moroccan Bufonids (Roussel & Amar 1989). The larval period of *A. mauritanicus* is relatively long. Metamorphosis occurs in July (Hediger 1935; Stemmler 1972; Schweiger 1992). The diet of a population located in the Middle Atlas Mountains mainly consisted of Coleoptera, Hymenoptera, Formicidae and Dermaptera (Chillasse *et al.* 2002). Diet diversity increased when general prey availability was low.

**Distribution.** *Amietophrynus mauritanicus* is common throughout Morocco both in the lowland and mountains, while it is limited to the vicinity of oasis and oueds in the Saharan part of the country. The distribution map (Fig. 14A) is composed of records from Bons and Geniez (1996), Brito (2003), Herrmann and Herrmann (2003), Fahd *et al.* (2007), Harris *et al.* (2008), Ramos and Díaz-Portero (2008), Harris *et al.* (2010), Stoetzel *et al.* (2010b), Barnestein *et al.* (2010) and Barata *et al.* (2011). New records fill in gaps within the known distribution area throughout Morocco.

**National Red List Status.** Least Concern.


**FIGURE 13.** Members of the Bufonidae family native to Morocco. A-C: *Amietophrynus mauritanicus*; A-B: Akkechour (WB); C: Ouarzazate (PdP). D-F: *Barbarophryne brongersmai*; D: Tamri (WB); E: Sidi Ifni (Gabriel Martínez del Mármol); F: Mechra-Benâbbou (WB). G-I: *Bufo bufo*; G-H: Oukaimeden (PdP); I: Tetouan (WB). J-L: *Bufotes boulengeri*; J: Oualidia (WB); K: Sidi Ifni (Gabriel Martínez del Mármol); L: Saidia (PdP).

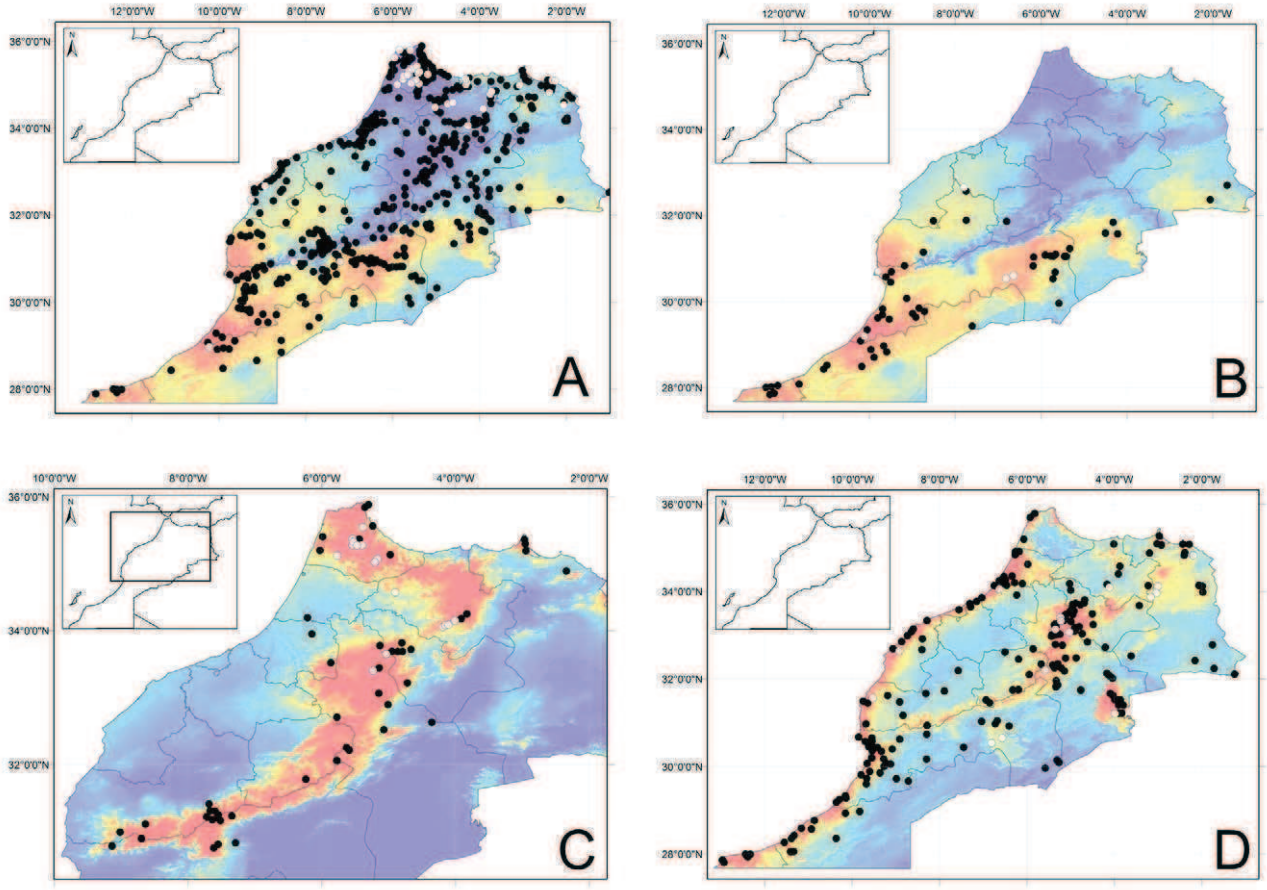


**FIGURE 14.** Niche models and distribution records of Bufonidae members in Morocco. A: *Amietophrynus mauritanicus*. B: *Barbarophryne brongersmai*. C: *Bufo bufo*. D: *Bufotes boulengeri*. Warmer colours indicate higher climate suitability. Dark spots indicate records derived from literature, white spots show new records.

### *Barbarophryne* Beukema, de Pous, Donaire-Barroso, Bogaerts, Garcia-Porta, Escoriza, Arribas, El Mouden & Carranza, gen. nov.

*Bufo*—Hoogmoed 1972. Zoologische Mededelingen 47 (5): 50.
*Pseudepidalea*—Frost *et al.* 2006. Bulletin American Museum of Natural History, 297: 365.
*Bufo (Epidalea)*—Doglio *et al.* 2008. Atti VIII Congresso Nazionale Societas Herpetologica Italica: 183.
"*Pseudepidalea*"—Van Bocxlaer *et al.* 2009. BMC Evolutionary Biology 9: 131.
*Pseudoepidalea*—García-Muñoz *et al.* 2009. Herpetology Notes 2: 231. Incorrect subsequent spelling.

**Type species.** *Bufo brongersmai* Hoogmoed 1972, by present designation.

**Definition and diagnosis.** *Barbarophryne* gen. nov. can be distinguished from other bufonid genera by the combination of the following characters: (i) small-sized adults (up to 51 mm in females); (ii) absence of warts on the dorsal surface of the head; (iii) almost circular parotoid glands (near as wide as long – at most 1½ times as long as wide –); (iv) nearly round tympanum; (v) absence of a gland in the tibia; (vi) paired distal subarticular tubercle on the fourth toe. *Barbarophryne* gen. nov. exhibits 2n = 22 biarmed chromosomes, with a noticeable decrease in size between larger elements (pairs 1–6) and the remaining five pairs. Pairs 1–6 and 11 are metacentric, whereas pairs 7–10 are submetacentric (Herrero *et al.* 1993). Although the basic karyotypic characteristics of *B. brongersmai* are similar to the members of the former *Bufo* species, it differs from *Amietophrynus* which generally show 20 chromosomes. Herrero *et al.* (1993) compared the C-band pattern of *B. brongersmai* to the patterns studied by Schmid (1978, 1980) for *B. bufo*, *E. calamita* and *B. viridis*, and recovered two main differences: (i) a low number of telomeric C-bands (only pairs 6 and 8); and (ii) a pronounced pericentromeric band in the short arm of pair 4.

**Etymology (derivatio nominis).** *Barbaro* (Latin, Barbaris, relative to Barbary, NW African region north of the Sahara) and *phryne* (from Phrynos (m) / Phryne (f), Greek for toad). The generic name is feminine.

**Distribution.** South-western Maghreb region of North Africa. Currently endemic to Morocco and the northernmost Western Sahara, but expected to occur in Algeria.

**Species included.** *Barbarophryne brongersmai* (Hoogmoed 1972) **comb. nov.** Monotypic.

**Taxonomic comment.** Hoogmoed (1972) described Brongersma's toad from the vicinity of Tiznit near the Moroccan Atlantic coast, which he tentatively placed within the "*B. viridis* - *B. calamita* group" based on morphological similarities. Subsequent research in the context of larval morphology, karyology, osteology and bioacoustics however revealed *B. brongersmai* to be well-diverged from all other bufonid taxa in the Western Palearctic (Grillitsch *et al.* 1989; Herrero *et al.* 1993; Delfino *et al*. 2009; Doglio *et al*. 2009). The majority of authors dealing with *B. brongersmai* during the last decades assumed a sister relationship with *B. boulengeri*, which eventually led to the attribution of *B. brongersmai* to the newly erected 'Green Toad' genus *Pseudepidalea* by Frost *et al.* (2006). In turn, Dubois and Bour (2010) revealed *Pseudepidalea* to represent a junior objective synonym of *Bufotes*, thereby giving priority to the latter as nomen for the Green Toad group. Subsequent mtDNA and nDNA analyses by Stöck *et al.* (2006), Van Bocxlaer *et al.* (2009), Pyron and Wiens (2011) and Garcia-Porta *et al.* (2012) recovered a polyphyletic pattern amongst the 'Green Toads' due to the inclusion of *B. brongersmai*, which was confirmed by the analyses presented herein (Fig. 6). The phylogenetic position of the present study reveals that *B. brongersmai* is most likely related to the clade of Eurasian toads (see Results section). In summary, we base our decision to place *B. brongersmai* in the newly erected genus *Barbarophryne* gen. nov. on the following priority TNC (sensu Vences *et al.* 2013); (i) Clade Stability (in favour of higher clade stability for *Bufotes* by exclusion of *B. brongersmai*) and (ii) Phenotypic Diagnosability (tadpole morphology: Grillitsch *et al.* 1989; bioacoustics: Doglio *et al*. 2009; current paper; osteology: Delfino *et al*. 2009), in addition to the secondary TNC Adaptive Zone (apparent from unique osteological adaptations to life in arid environments; Delfino *et al*. 2009).

### *Barbarophryne brongersmai* (Hoogmoed 1972)

Figs. 13D–F.

**Background information.** While the validity of *B. brongersmai* was shortly doubted after its description (Benhachem *et al.* 1989), subsequent data on larval morphology, karyology, osteology and bioacoustic data confirmed its specific status (Grillitsch *et al.* 1989; Herrero *et al.* 1993; Delfino *et al.* 2009; Doglio *et al.* 2009). The origin and interfamily relationships of *B. brongersmai* in regard to other bufonid species remain unresolved (see Results section of the present paper). The current calibration suggests that separation from other Eurasian Bufonidae would have occurred during the Oligocene, between 20 and 30 mya. The mountain ranges of the Western Palearctic were formed throughout that period as a result of the Alpine orogeny (Rosenbaum *et al.* 2002). Being one of the main vicariant events during the Late Mesozoic and Cenozoic, the Alpine orogeny likely had profound impact on divergence within the Bufonidae (see also Van Bocxlaer *et al.* 2009, 2010). Indeed, most contemporary bufonid genera diverged throughout that period (Van Bocxlaer *et al*. 2009). Initial separation of *Barbarophryne* gen. nov. during the Oligocene therefore seems a likely scenario, although comprehensive evidence is still lacking.

**Tadpole.** The following diagnosis is a summary of the elaborate descriptions given by Hoogmoed (1972) and Grillitsch *et al.* (1989). A drawing of the tadpole is shown in Fig. 12E. Fully developed tadpoles are characterized by their flattened body shape and generally small interorbital space. Eyes positioned dorsally on head. Interorbital space varying from equal to nearly 1½ times as wide as the internarial space (Grillitsch *et al.* 1989). Oral disk approximately 1½ times as wide as the interorbital space. Spiracle sinistral, pointing straight backwards or slightly upwards. Body 1¼ times as wide as deep, $1^{2}/_{3}$ times as long as wide. Tail less than four times as long as deep. Dorsal tail fin occasionally higher than ventral tail fin, ending posterior of the muscular base of the tail. Tail tip rounded. Oral disc ventral, about one and a half times as wide as interorbital space. Clusters of papillae at the corners of the mouth, upper and lower lip free of papillae. Two tooth rows on the upper and three on the lower labium. Lower supralabial row with median gap. Colour uniform dark brown to black, ventral side turning slightly lighter during ontogeny. Tail fins unspotted.

Hoogmoed (1972) described the interorbital space to be equal to the distance between the nostrils, and

considered this as one of the most important characters to separate *B. brongersmai* from *B. boulengeri*. However, as shown by Grillitsch *et al.* (1989) this distance varies considerably, nearly approaching the distance observed in *B. boulengeri* (interorbital space 1½ times the distance between the nostrils). As the dorsal tail fin can be higher than the ventral tail fin in both species while they present similar colours, identification in the field is troublesome. The interorbital space in relation to both the internarial space and the oral disk, as well as presence/absence of spots on the tail are the most informative characters for identification.

**Bioacoustics.** Hoogmoed (1972) described that a male *B. brongersmai* emitted several 'short squeaks' from beneath a large stone lying at the edge of a pond, but did not assume that this represented an advertisement call as it was highly different from the call of other Bufonidae species that he was familiar with. Bogaerts (2001) described the advertisement call, based on captive individuals, as consisting of several short pulses lasting for approximately 1–2 seconds, thus confirming the observation of Hoogmoed (1972). Bioacoustics of *B. brongersmai* have been analysed first by Gallix (2002) and then by Doglio *et al*. (2009). The call is variable and composed of a train of 3 to 8 pulses, with a fundamental frequency ranging from 1217 to 1890 Hz. The call length varies between 0.53 to 3.11 seconds. Pulse length ranges from 0.03 to 3.11 seconds. Interpulse length varies from 0.09 to 0.14 seconds, while the pulse rate ranges from 6.39 to 6.73 seconds. Intervals between calls are irregular. The frequency of release calls is higher, while the length of this call and number of pulses are highly variable. The advertisement call analysed herein (Fig. 7B) was recorded at 22.7ºC (34% humidity) and consisted of 5 notes composed of a series of 5 to 7 pulses (median = 7), with a dominant frequency ranging from 1217 to 1276 Hz. Note length varied between 0.77 to 1.10 seconds (average 0.98 ± 0.145). Pulse length ranged from 0.055 to 3.11 seconds (average 0.064 ± 0.004, n = 32). Interpulse length varied from 0.09 to 0.14 seconds (average 0.106 ± 0.011, n = 27,), while the pulse rate ranged from 6.39 to 6.73 seconds (average 6.52 ± 0.126).

**Natural history.** *Barbarophryne brongersmai* is an explosive breeder dependent on incidental periods of rain during spring, which uses all kinds of small, temporary water bodies such as rain puddles, temporary streams and ponds and occasionally man-made water reservoirs (Gallix 2002; García-Muñoz *et al.* 2009) for reproduction. Site fidelity is relatively strong. Reproduction has been observed between mid-March and the beginning of April (Hoogmoed 1972; Guillon *et al.* 2004). Eggs are deposited in small clutches, and attached onto stones or vegetation underwater (Bogaerts 2001). Despite the general aridity, which characterizes the distribution of *B. brongersmai*, the larval period is rather long (Gallix 2002). Recently metamorphosed juveniles have been observed in May (García-Muñoz *et al.* 2009).

**Distribution.** Initially, *B. brongersmai* was only known from the southern Moroccan Atlantic coast near Tiznit, and the adjacent Western Sahara (Hoogmoed 1972). Destre *et al.* (1989) provided considerable range extensions along the entire sub-Atlas region of Morocco, and northwards onto the arid plain of Marrakech. Additional records have been published by Guillon *et al.* (2004) and Doglio *et al.* (2009). The distribution map (Fig. 14B) is composed of records from Bons and Geniez (1996), Brito (2003), Herrmann and Herrmann (2003), Guillon *et al.* (2004), Harris *et al.* (2008), Ramos and Díaz-Portero (2008) and Doglio *et al.* (2009). New records fill in gaps within the sub-Atlas distribution of the species, which seems to be (nearly) continuous. The new record (see also Fig. 13F) north of Mechra-Benâbbou is close to that of De la Riva (1992), but significant due to its location north of the Oued Rbiaa.

**National Red List Status.** Near Threatened.

### Common Toad, *Bufo bufo* (Linnaeus 1758)

Figs. 13G–I

**Background information.** Bons (1972) presumed that *Bufo bufo spinosus* Daudin 1803 likely consisted of several 'geographic races' due to the large morphological variability within the distribution of this subspecies. However, likely due to a lack of supporting evidence proving otherwise, Bons (1972) attributed North African populations nonetheless to *B. b. spinosus*. The main reason for this decision was a closer resemblance of African populations to *B. b. spinosus* (large size, numerous warts) in comparison to *B. b. bufo* (see also Pasteur & Bons 1959; Salvador 1996). As a result of recent phylogeographical analyses of the *B. bufo* complex (Litvinchuk *et al.* 2008; Garcia-Porta *et al.* 2012; Recuero *et al.* 2012) the attribution of African populations to *B. b. spinosus* has to be revaluated. A long separate evolutionary history of African and Iberian populations was recently revealed by means of mtDNA

and nDNA (Garcia-Porta *et al.* 2012; Recuero *et al.* 2012). Dating estimates place the split in the Lower Pliocene at approximately 3 mya, which would imply transmarine migration (Garcia-Porta *et al.* 2012), although Recuero *et al.* (2012) suggested migration through the Gibraltar land bridge despite incongruent timing of events. Furthermore, also Moroccan and Tunisian populations seem well-differentiated based on allozyme and mtDNA data (Litvinchuk *et al.* 2008; Garcia-Porta *et al.* 2012; Recuero *et al.* 2012).

**Tadpole.** Not described. The tadpole description by Pasteur and Bons (1959) was based on that of Boulenger (1897), which was in turn based on European material. A preliminary drawing of the body morphology is shown in Fig. 12F.

**Bioacoustics.** See Pasteur and Bons (1959) for an interpretation of the advertisement call as described by Lataste (1876). A comparison with the calls of European populations has not been made.

**Natural history.** While *B. bufo* can be commonly encountered at high altitudes in the High Atlas (e.g. Bons & Geniez 1996; Harris *et al.* 2008) observations in north Morocco and the Middle Atlas are much less common (Mellado & Mateo 1992; Martínez Medina 2001; Fahd *et al.* 2005). El Hamoumi *et al.* (2007) characterized breeding sites in the Middle Atlas as permanent, stagnant, relatively large water bodies with significant aquatic vegetation. Reproductive activity has been observed in spring at high altitudes >2500 in the High Atlas (Salvador 1996). The diet of a population located in the Middle Atlas Mountains mainly consisted of Coleoptera, Hymenoptera Formicidae and Dermaptera (Chillasse *et al.* 2002). Diet diversity increased when prey availability was low. Ontogenetic pattern change has been reported to occur (see Werner 1931 regarding the High Atlas Mountains; pers. obs. authors in the western Rif Mountains). Juveniles are characterized by fragmented bright yellow to red colour on the dorsal, lateral and partially ventral sides, while the same colour is usually present in bands on the extremities (Fig. 13I). In contrast, adult individuals are coloured uniform brown to olive, without any discernible pattern (Fig. 13G&H). Additionally, webbing on the hind feet is highly extended in comparison to European populations (Fig. 13G&H).

**Distribution.** Within Morocco, *B. bufo* mainly occurs in the Rif and Atlas Mountains, while several isolated occurrences have been reported from the Gourougou- and Beni Snassen Massifs (Mellado & Mateo 1992; Bons & Geniez 1996). Presence of *B. bufo* at the latter is however doubtful and in need of confirmation, as no individuals were encountered during prospection for the generally syntopic *S. algira* (Escoriza *et al.* 2006; Escoriza & Comas 2007), while the single known record near Tafoughalt stems from a particularly low (< 900 m), Mediterranean influenced section of the massif. Occurrence of *B. bufo* in the north-western lowlands near the Atlantic coast has been confirmed based on fossil remains from the Upper Pleistocene (e.g. Ould Sabar & Michel 1996; Bailon & Aouraghe 2002; Stoetzel *et al.* 2010a). Additionally, several historical records from the same geographical area are known (e.g. Camerano 1878; Pellegrin 1912). Current persistence is however doubtful due to a lack of sightings. The record from Hordies and Van Hecke (1982) near Ouarzazate is most likely based on an uniform coloured *A. mauritanicus* (Fig. 13C). While the occurrence of *B. bufo* in northern Morocco seems fragmented (Bons & Geniez 1996; Martínez-Medina 2001; Fahd *et al.* 2005), the species seems relatively common at higher altitudes of the Atlas Mountains (Bons & Geniez 1996; but see Fahd *et al.* 2007). The distribution map (Fig. 14C) is composed of records from Bons and Geniez (1996), Harris *et al.* (2008), Harris *et al.* (2010) and Stoetzel *et al.* (2010b). While a significant new number of records from the Rif Mountains and the Middle Atlas mountains are herein presented, the distribution of *B. bufo* in Morocco remains fragmented.

**National Red List Status.** Near Threatened.

**Taxonomic comment.** Due to the long independent evolutionary history of the African and Iberian populations, but the lack of morphological and acoustic data of the different clades within *B. bufo*, we follow the suggestion of Garcia-Porta *et al.* (2012) in temporary recognition of *B. b.* ssp. for the African populations. This is in contrast to Recuero *et al.* (2012) who grouped populations of Iberia, south-western France and Africa as *B. spinosus*, which to our opinion cannot be corroborated based on available evidence.

### African Green Toad, *Bufotes boulengeri* (Lataste 1879)

Figs. 13J–L.

**Background information.** African green toads were denominated by Lataste (1879) as *B. boulengeri* based on the presence of a gland on the tibia and a conspicuous dorsal stripe. However, Lataste (1879) noted that the presence of

latter character was not consistent, while it also occurred in populations outside the African continent. Boulenger (1880, 1891) considered these characters to fall within intraspecific variation of *B. viridis* sensu lato, as he encountered the former morphological character in several Asian and European specimens, while he confirmed the dorsal stripe not to be consistently present (see also Fig. 13K&L). Subsequently, after more than a century Stöck *et al.* (2006) showed the African green toads to be highly divergent at the mtDNA level as a result of post Miocene divergence. Intraspecific differentiation is however particularly low (Batista *et al.* 2006; Stöck *et al.* 2008a) likely due to rapid post-glacial expansion into the current range.

**Tadpole.** Pasteur and Bons (1959) provided a description of the tadpole of *B. boulengeri* based on that of Boulenger (1897), which was in turn based on European material. However, Pasteur and Bons (1959) modified their description based on tadpoles originating from Morocco. A drawing of the tadpole is shown in Fig. 12G. Fully developed tadpoles are mainly characterized by their dorsal tail fin, which is generally higher than the ventral tail fin. Body shape ovoid. Eyes positioned dorsally on head. Interorbital space 1½ as wide as the internarial space. Tail less than four times as long as deep. Dorsal tail fin higher than ventral fin, ending posterior of the muscular base of the tail. Spiracle sinistral. Oral disc ventral. Oral disk as wide as the interorbital space. Two tooth rows on the upper and three on the lower labium. Lower supralabial tooth row with median gap of variable width. Pasteur and Bons (1959) described tadpoles of *B. boulengeri* to be uniform dark brown or dull olive-grey coloured, often in combination with darker dorsal and lateral patches; venter greyish-white; tail fins greyish or bluish often including brown spots or dots. This description is nearly identical to that of Boulenger (1897) regarding *B. viridis*. In contrast, Doumergue (1901) describes tadpoles of *B. boulengeri* to be uniform black, while this author does not mention the presence of patches. In our experience, tadpoles are either uniform black, greyish or dark brown coloured. As is the case with *B. viridis* tadpoles, *B. boulengeri* likely develops dorsal and lateral patches through ontogeny, explaining the variable presence of this character. Tadpoles of *B. boulengeri* have been reported to be larger than their European counterparts (up to 62 mm), and are carnivorous (Doumergue 1901; Pasteur & Bons 1959; Hoogmoed 1972). See the account of *B. brongersmai* regarding comments on larval identification of these two highly similar species.

**Bioacoustics.** Roussel and Amar (1985) described the advertisement call of a Saharan population of *B. boulengeri* from Algeria, recorded at a water temperature of 18º C. Note duration averaged 5.60 ± 0.5 seconds, consisting of 126.0 ± 13.0 pulses with an average length of 24.6 ± 2.2 ms (interval 21.2 ± 1.3 ms). The currently analysed notes (n = 7) of two males recorded at 21ºC were composed of a train of 90 to 136 pulses (median = 109), with a dominant frequency ranging from 1205 to 1240 Hz. The note length varied between 2.71 to 4.17 seconds (averagely 3.31 ± 0.509), while the pulse rate ranged from 32.19 to 33.45 seconds (averagely 32.94 ± 0.457). To the ear, the call sounds like a long, high vibrating noise, not unlike that of an insect. The number of pulses of the advertisement call presented here is similar to those recorded by Roussel and Amar (1985, Fig. 7C). Call duration is considerably shorter than that presented by the latter authors, despite being recorded at a higher temperature. It has to be noted that the amount of pulses in the advertisement call of the recently described *Bufotes siculus* Stöck, Sicilia, Belfiore, Buckley, Lo-Brutto, Lo-Valvo and Arcuelo 2008 (up to 75 at 16ºC) is considerably lower than that of *B. boulengeri*. However, a statistical comparison based on call data recorded at a full range of temperatures is lacking. Therefore, a definitive conclusion on call divergence between these species stands out.

**Natural history.** *Bufotes boulengeri* is a characteristic inhabitant of relatively arid, open landscapes. In this aspect, *B. boulengeri* is less generalistic than *A. mauritanicus*, but can nevertheless be found from coastal habitats up to mountain plateaus in the High Atlas (Bons & Geniez 1996). The diet has been described to mainly consist of insects, especially beetles (Doumergue 1901). This species is a temporary pond breeder, which is highly dependent on seasonal rains (Le Berre 1989). Reproduction can take place from January to the beginning of May (Pasteur & Bons 1959; El Hamoumi *et al.* 2007; pers. obs. P. de Pous, W. Beukema). In contrast to other Moroccan bufonids which variably reproduce throughout the season, *B. boulengeri* shows only one or two peaks in breeding activity (Gallix 2002). Bons (1959) observed reproductive activity in the first week of April in the Doukkala, while P. de Pous and W. Beukema noted activity in the same area in mid-March during heavy rains. Reproduction was observed in the plain of Marrakesh between mid-March and the beginning of April (Guillon *et al.* 2004). Pasteur and Bons (1959) described metamorphosis to occur in August or September. Populations located at higher altitudes likely reproduce from spring onwards. While individuals occasionally display a dorsal stripe as described by Lataste (1879), the dorsal pattern in general is highly variable (Schleich *et al.* 1996; El Oualidi & Jaziri 2001).

**Distribution.** The distribution map (Fig. 14D) is composed of records from Bons and Geniez (1996), Brito

(2003), Herrmann and Herrmann (2003), Mateo *et al.* (2003), Fahd *et al.* (2007), Donaire *et al.* (2011), Barnestein *et al.* (2010) and Barata *et al.* (2011). Donaire *et al.* (2011) commented on the distribution in northern Morocco and the apparent enigmatic absence of *B. boulengeri* in the northwestern Rif Mountains. The latter is herein supported by the high climatic unsuitability shown by the niche model in Fig. 14D. The majority of the new records are located in the eastern and southern part of the species distribution in Morocco.

**National Red List Status.** Least Concern.

**Taxonomic comment.** Herein we accept the attribution of the adjective *boulengeri* at species level for the North African green toad populations based on the high molecular differentiation and allopatric occurrence with respect to other green toads (Stöck *et al.* 2006, 2008).

## Family Hylidae Rafinesque

## Subfamily Hylinae Rafinesque

### Mediterranean Tree Frog, *Hyla meridionalis* Boettger 1883

Figs. 15A–C.

**Background information.** The presence of *Hyla* in Morocco has been reported from as early as the Upper Pliocene based on fossil remains (Bailon 2000). In a recent phylogeographical study, Recuero *et al*. (2007) recovered three subgroups divided into two major clades by means of mtDNA analyses, between which the High Atlas Mountains seem to act as a geographical barrier. Surprisingly, the southern clade is closely related to southern Iberian populations, while the northern clade shows affinity to circum-Pyrenean populations. As a result Recuero *et al.* (2007) presumed *H. meridionalis* to be of African ancestry, with European populations originating from relatively recent arrival (either by means of natural colonization or anthropogenic introduction). Stöck *et al.* (2008b) and Stöck *et al.* (2012), based on both mtDNA and nDNA analyses could not confirm the results of Recuero *et al.* (2007), although the former studies were limited by small sample sizes. Conclusively, while an African origin for *H. meridionalis* seems credible based on fossil and mtDNA data, further research is needed to clarify intraspecific variation of this species across its entire distribution range (Stöck *et al.* 2008b; Stöck *et al.* 2012).

**Tadpole.** The tadpole of *H. meridionalis* was described by Héron-Royer (1884) based on material from "Midi de France", and by Boulenger (1897) by means of specimens of uncertain origin. A drawing of the tadpole is shown in Fig. 12H. Fully developed tadpoles are characterized by their olive-greenish dorsal colour and lateral positioned eyes. Body shape ovoid. Eyes positioned laterally on head. Interorbital space one 1 ½ times as wide as the internarial space. Spiracle sinistral, directed upwards. Tail twice the length of the body. Muscular part of the tail discontinuously interspersed, and bordered above and below by a black line. Dorsal tail fin high and rounded, extending onto the dorsum near the position of the eyes. Ventral tail fin equally developed, extending on the venter beyond the anus. Lips bordered with papillae, absent in the middle of the upper border, usually forming two rows on the lower lip. Two tooth rows on the upper (unlike one, as displayed by Pasteur & Bons 1959) and three on the lower labium. Lower supralabial row with large median gap. Colour olive or green with a golden gloss, lateral parts interspersed with golden spots. Venter white with golden or yellowish spots. Tail and tailfins inconspicuously grey or back spotted.

**Bioacoustics.** Schneider (1968) described the advertisement call of *H. meridionalis* originating from southern France. The call is characterized by a higher number of pulses (paired with longer call duration) compared to other European *Hyla* spp., which however decrease as temperature decreases (averagely 41.95 pulses at 10ºC, 36.85 at 20ºC). Call interval is relatively long, ranging from nearly 2.5 seconds at 6 ºC to 823.20 ms at 19 ºC. The recordings of the advertisement call (n= 13) of two males presented herein (Fig. 7D) recorded at 14.8°C with a humidity of 85.80% were composed of 35 to 57 pulses (median = 41), with a dominant frequency ranging from 2194 to 2359 Hz. Note length varied between 40.9 to 68.4 ms (averagely 0.553 ± 0.087 seconds), while the pulse rate ranged from 74.91 to 86.54 seconds (averagely 80.61 ± 4.16).

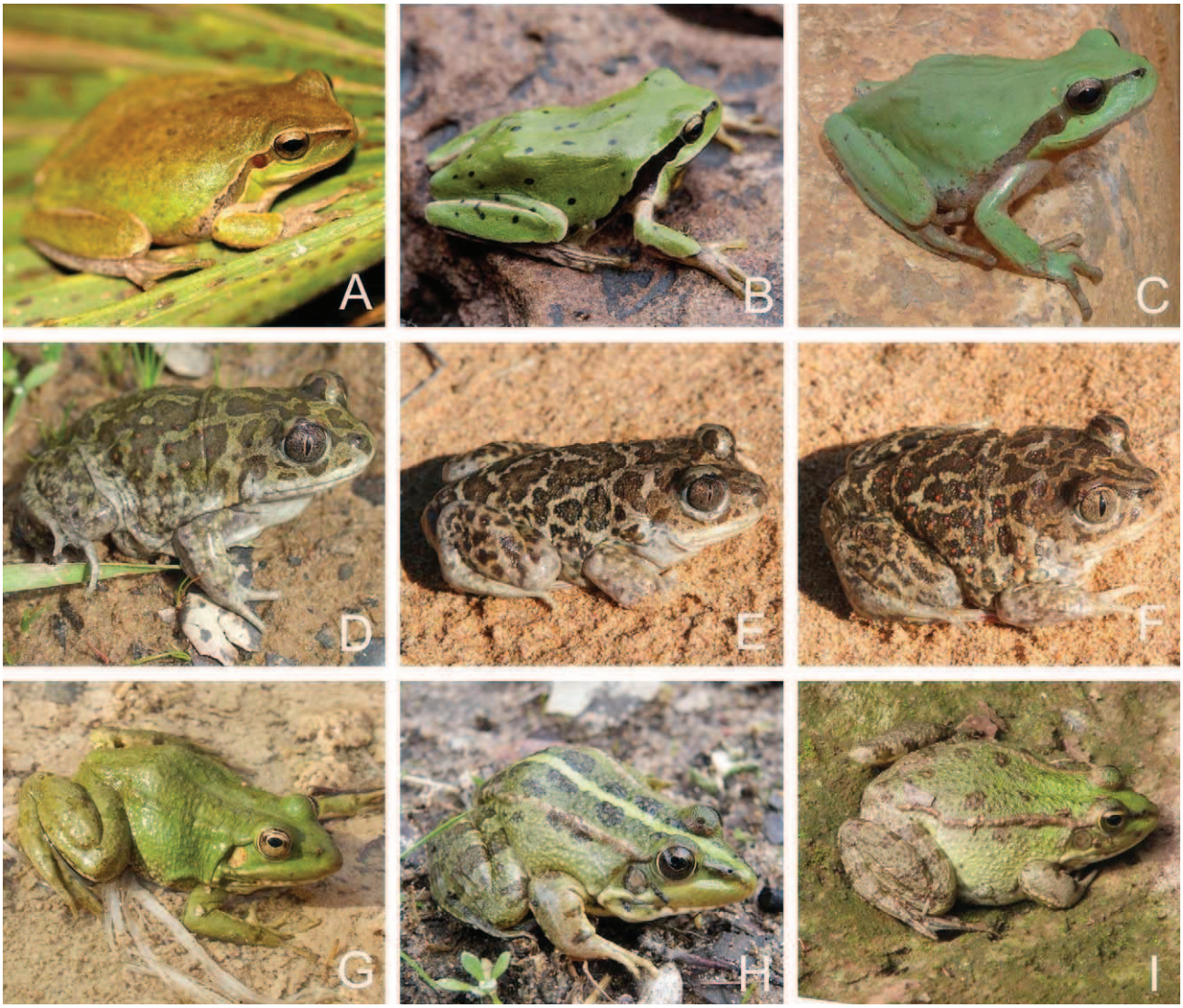


**FIGURE 15.** Members of the Hylidae, Pelobatidae and Ranidae families native to Morocco. A-C: *Hyla meridionalis*; A: Casablanca (Gabriel Martínez del Mármol); B: Oukaimeden (Frank Deschandol); C: Sidi Ifni (SB). D-F: *Pelobates varaldii*; D: Forêt de Mamora (WB); E-F: Forêt de Mamora (PdP). G-I: *Pelophylax saharicus*; G: High Atlas (WB); H: Forêt de Mamora (WB); I: Rabat (PdP).

**Natural history.** *Hyla meridionalis* can be found near permanent or temporary water bodies, often characterized by an abundance of aquatic vegetation (El Hamoumi *et al.* 2007). Throughout the north-western lowlands and the Rif- and Atlas Mountains, *H. meridionalis* is one of the most common species. In contrast to its common name, the Mediterranean Tree Frog can also be abundant in areas without significant tree cover, such as the stony Doukkala plain or high-altitude areas in the Middle- and High Atlas (Bons & Bons 1959). At the southern margins of the distribution such as the Anti-Atlas and the Ouarzazate depression, *H. meridionalis* occurs in arid terrain near temporary streams characterized by little vegetation. Doumergue (1901) describes reproduction to take place between the end of February and the end of April, while Bons (1970) observed reproduction in the Middle Atlas at the end of March. In the Mamora forest region near Rabat, reproduction starts in late January and continues until March (El Hamoumi 1988; pers. obs. P. de Pous). Individuals mainly consume insects (Doumergue 1901).

**Distribution.** The distribution map (Fig. 16A) is composed of records from Bons and Geniez (1996), Fahd *et al.* (2007), Harris *et al.* (2008) and Barnestein *et al.* (2010). New records reveal a continuous distribution from the north Atlantic Coast to the Rif Mountains, which is expanded along the Atlantic Coast and several areas of northern Morocco. The absence of this species in many parts of north-eastern Morocco and the Middle- and High Atlas is likely due to a lack of distributional research. Several new records have been found along the southern margin of the species distribution, where *H. meridionalis* occurs near (semi)permanent water bodies. The recently

discovered localities near Sidi Ifni (Donaire *et al.* 2004; Barnestein *et al.* 2010) represent the southernmost records of this species on the African continent.

**National Red List Status.** Least Concern.

**Taxonomic comment.** Following Recuero *et al.* (2007) and Stöck *et al.* (2008b) at least two *Hyla* taxa are present in the Maghreb. The boundary between these taxa has not yet been identified, but is presumed to be located in central Algeria.

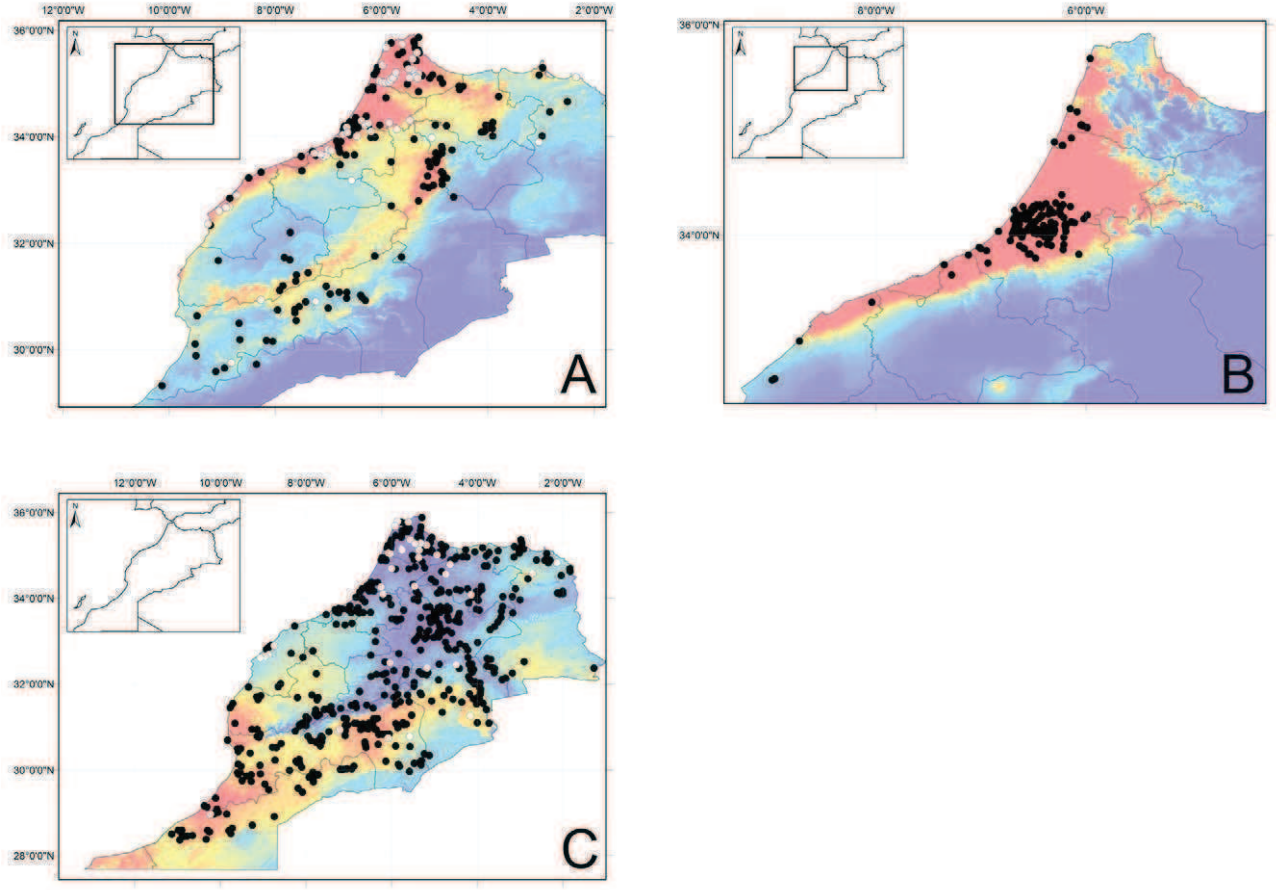


**FIGURE 16.** Niche models and distribution records of *Hyla meridionalis* (A), *Pelobates varaldii* (B) and *Pelophylax saharicus* (C) in Morocco. Warmer colours indicate higher climate suitability. Dark spots indicate records derived from literature, white spots show new records.

## Family Pelobatidae Bonaparte

### Moroccan Spadefoot Toad, *Pelobates varaldii* Pasteur and Bons 1959

Figs. 15D–F.

**Background information.** Information on *P. varaldii* remained scarce after the discovery of the species until Busack *et al.* (1985) provided data on the electrophoretic, immunological and morphological discrimination between *P. varaldii* and *P. cultripes,* which showed little morphological but considerable genetic differentiation. Recently, García-París *et al.* (2003) and Veith *et al.* (2006) found a well-supported sister taxa relationship between *P. varaldii* and *P. cultripes*, which dates back to the end of the Messinian Salinity Crisis, approximately 5.3 mya. Conclusively, both species likely diverged as a result of the collapse of the Gibraltar land bridge. Recent contributions by Crochet and Geniez (2003), Guzman *et al.* (2007), El Hamoumi *et al.* (2007), Guarino *et al.* (2011) and de Pous *et al.* (2012) have revealed details on the distribution, phenology and natural history of *P. varaldii*. Confusion regarding species-specific morphology however remained, as Pasteur and Bons (1959) described *P. varaldii* adults as well as larvae to be highly divergent from *P. cultripes* based on a large sample of

individuals, while Busack (1985) considered adults of both species to be highly similar in external morphology. However, the *P. varaldii* individuals analysed by Busack (1985) were partially substituted with small *P. cultripes* due to the low sample size of the former. Despite the fact that substrate is known to influence at least the size of *Pelobates* spp. (i.e. individuals located on sandy substrates tend to remain smaller; Marangoni & Tejedo 2008; Marangoni *et al*. 2008), descriptive statistics indicate that *P. varaldii* has a smaller mean and maximum SVL length compared to *P. cultripes* as reported in the available literature (Table 4). In addition to these results, we confirm the abundant presence of red dots (absent in *P. cultripes*), most pronounced on the upper eyelids but often also present on the forelimbs, hindlimbs, dorsum (sometimes including flanks) and head (Pasteur & Bons 1959, see Fig. 15D, F) in the majority of individuals (Table 5). Furthermore, larval morphology significantly differs between these two sister species as elaborately described by Pasteur and Bons (1959). Altogether, we regard the degree of morphological (larval and adult) and genetic divergence sufficient to recognize *P. varaldii* and *P. cultripes* as different species.

**TABLE 4.** Comparison of size differences (mm) in relation to substrate among *Pelobates cultripes* and *Pelobates varaldii*.

| | | | Mean SVL | | Max SVL | | |
|---|---|---|---|---|---|---|---|
| **Species** | **Locality** | **Substrate** | **Male (*N*)** | **Female (*N*)** | **Males** | **Females** | **Source** |
| *P. cultripes* | Arriça, P | Sandy | 52.8 (20) | 54.2 (40) | 62 | 72 | Leclair *et al*. 2005 |
| *P. cultripes* | Porto Covo, P | - | 71.5 (11) | 72.7 (4) | 80 | 84 | Talavera 1990 |
| *P. cultripes* | Montalvos, ES | - | 72.0 (76) | 74.6 (66) | 85 | 86 | Lizana *et al*. 1994 |
| *P. cultripes* | Madrid, ES | - | 79.8 (15) | 79.5 (14) | 87 | 101 | Talavera 1990 |
| *P. cultripes* | Cadiz, ES | - | 65.8 (30) | 72.1 (30) | 84 | 99 | S. Busack, unpublished data |
| *P. cultripes* | Pedroso, ES | Hercinic | 83.9 (56) | 90.4 (18) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Navas, ES | Hercinic | 83.8 (6) | 91.8 (5) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Gerena, ES | Hercinic | 93.3 (8) | - | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Aznalcóllar, ES | Hercinic | 88.1 (31) | 104.7 (14) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Lázaro, ES | Sandy | 58.4 (18) | 68.0 (1) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Bodogones, ES | Sandy | 71.6 (43) | 76.2 (40) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Abalario, ES | Sandy | 67.7 (83) | 71.8 (69) | - | - | Marangoni & Tejedo2008 |
| *P. cultripes* | Doñana, ES | Sandy | 66.1 (47) | 62.9 (24) | - | - | Marangoni & Tejedo2008 |
| *P. varaldii* | Foret Mamora, MAR | Sandy | 51.9 (98) | 53.6 (30) | 61 | 66 | Present study |

**Tadpole.** Pasteur and Bons (1959) gave an elaborate description of the tadpole of *P. varaldii* as support for specific status of this taxon. A summary of their results is presented here. A drawing of the tadpole is displayed in Fig. 12I. Fully developed tadpoles are characterized by their large size (up to 130 mm, occasionally larger), globular body and presence of black metatarsal tubercles. Eyes positioned laterally on head. Body length covers approximately 1.3 to 1.8 times body height and 1.6 times its width. Dorsum flat. Length of the tail ranges between 1.38 to 2.08 times body length. Tail length 1.8 to 2.4 times longer than deep. Dorsal and ventral tailfin highly extended, tapering to a point at the tip of the tail. Spiracle sinistral, pointing diagonally upwards. Oral disk oval shaped. Four or five tooth rows on the upper and five on the lower labium. Upper supralabial row highly reduced, large median gap in other supralabial rows, interspersed by mouth. Median gap in all infralabial rows except the lowest row. Labial rows with median gaps always asymmetrically divided. Colour uniform grey, the dorsum being somewhat darker in comparison with the rest of the body. Rarely limited pattern of pale brown spots on the tail. The black metatarsal tubercles already appear before full development of the posterior limbs.

**Bioacoustics.** The amplexus call of a single *P. varaldii* male (27 notes) and female (34 notes) originating from Forêt de Mamora were analysed and presented here for the first time (Fig. 7E&F). Calls were emitted at regular intervals in trains of up to seven notes. The notes were usually composed of three different pulses, of which the first two pulses are weak and brief whereas the third pulse is more powerful, longer and consists of a train of very short pulses. Occasionally, the first note is only composed of a single pulse (6.1% of analysed notes). Note length ranged

between 52 ms to 94 ms (averagely 85 ± 09 ms) in case of the male, and between 60 ms and 91 ms (averagely 83 ± 06 ms) for the female. The dominant frequency ranged from 589 to 1370 Hz for the male and from 675 to 981 Hz for the female. Compared to *P. cultripes*, the amplexus call of *P. varaldii* has a higher per-note pulse number and a higher frequency (Lizana *et al.* 1994).

**TABLE 5.** Morphological measurements (mm) of *Pelobates varaldii* from Fôret de Mamora, Rabat, Morocco. Numbers display average ± SD (range).

| Measurement | Female | Male |
|---|---|---|
| L | 5.36 ± 0.61 (4.5 – 6.6) | 5.17 ± 0.35 (4.39 – 6.06) |
| F | 2.52 ± 0.32 (1.8 – 3.05) | 2.44 ± 0.21 (1.95 – 2.8) |
| P | 3.44 ± 0.38 (2.79 – 4.12) | 3.39 ± 0.44 (0 – 3.98) |
| Lpa | 3.44 ± 0.43 (2.74 – 4.33) | 3.35 ± 0.29 (2.69 – 4.1) |
| Lpp | 7.99 ± 0.97 (6.3 – 9.57) | 7.87 ± 0.59 (6.67 – 9.03) |
| DpPp | 0.41 ± 0.06 (0.29 – 0.51) | 0.41 ± 0.05 (0.26 – 0.55) |
| Cint | 0.41 ± 0.05 (0.33 – 0.5) | 0.4 ± 0.04 (0.3 – 0.48) |
| Spi | 0.32 ± 0.05 (0.21 – 0.4) | 0.31 ± 0.04 (0.21 – 0.4) |
| LO | 0.67 ± 0.07 (0.52 – 0.81) | 0.67 ± 0.06 (0.56 – 0.81) |
| Dno | 0.52 ± 0.1 (0.41 – 0.94) | 0.47 ± 0.05 (0.35 – 0.6) |
| HW | 2.18 ± 0.24 (1.8 – 2.68) | 2.06 ± 0.26 (0 – 2.4) |
| Weight | 17.7 ± 6.3 (10 – 31) | 15.43 ± 3.15 (9 – 24) |
| Eye | 76.7 % | 81.8 % |
| FL | 43.3 % | 30.3 % |
| HL | 23.3 % | 16.2 % |
| D | 53.3 % | 51.5 % |
| H | 13.3 % | 10.1 % |

As reviewed by Lizana *et al.* (1994), *Pelobates* sp. are characterized by a rich vocal repertoire, whereas the advertisement call and release call of *P. varaldii* remain undescribed. Further study is therefore required in order to describe and assess the degree of differentiation between the calls of *P. varaldii* and other members of the genus.

**Natural history.** *Pelobates varaldii* is the most stenoecious Moroccan amphibian species, of which the occurrence is limited to the vicinity of Mediterranean temporary ponds located on loose, sandy soils at low altitudes. Additionally, the presence of significant tree cover (*Quercus suber* forests, although planted *Pinus*, *Acacia* and *Eucalyptes* forests are also occupied) seems to be of high importance (de Pous *et al.* 2012). *Pelobates varaldii* can be extremely common within suitable habitat; de Pous *et al.* (2012) encountered the species in 95% of more than 150 ponds located in and around Forêt de Mamora near Rabat. Natural history of the southernmost populations on the stony Doukkala plain (i.e. near Oualidia, Crochet & Geniez 2003) considerably differs, with individuals being highly localized, occupying fragmented, largely barren areas of loose sandy soil (de Pous *et al.* 2012).

Guarino *et al.* (2011) analysed age structure and growth of a population located in Forêt de Mamora. The age of the analysed individuals ranged between 2–7 years in males (mean 4.5±1.2, n = 66) and 2–10 years in females (4.7 ± 2.4, n = 20). *Pelobates varaldii* aestivates in the soil from late spring until the first winter rains. Activity is strictly nocturnal during the reproductive period, although aquatic individuals might remain day-active for several days during the activity peak (P. de Pous & W. Beukema pers. obs.). Reproduction takes place from the end of October to the end of January, with larvae being observed between November and April (El Hamoumi 1988; El Hamoumi & Himmi 2010; Lapeña *et al.* 2011; P. de Pous pers. obs.). Recently metamorphosed juveniles have been observed in May and June (Pasteur & Bons 1959). General activity continues up to May (Crochet & Geniez 2003; Guarino *et al.* 2011).

**Distribution.** Distribution data on this cryptic species were limited until recently, notwithstanding the contributions of Crochet and Geniez (2003), Guzman *et al.* (2007) and El Hamoumi and Himmi (2010). The

description of abundant calling *P. varaldii* in the High Atlas Mountains by Malkmus (1983) should be considered erroneous. Lapeña *et al.* (2011) provided a record from the northern Moroccan coast between Asilah and Tanger, thereby extending the distribution some 50 kilometres to the north, confirming predictions made by Pasteur and Bons (1959). de Pous *et al.* (2012) provided a detailed distributional review and threat assessment of the species, confirming the occurrence of *P. varaldii* in four isolated areas along the Atlantic coast, of which at least two are highly threatened. The distribution map (Fig. 16B) is composed of records from Bons and Geniez (1996), Crochet and Geniez (2003), Guzman *et al.* (2007), El Hamoumi and Himmi (2010), Lapeña *et al.* (2011) and de Pous *et al.* (2012).

**National Red List Status.** Endangered.

## Family: Ranidae Rafinesque

### North African Frog, *Pelophylax saharicus* (Boulenger 1913)

Figs. 15G–I.

**Background information.** The systematic history of *Pelophylax* in North Africa has been subject of considerable confusion. Benhachem and Benazzou (1992) summarized relevant information concerning this topic for the Moroccan populations. *Pelophylax saharicus* has been suggested to be the sister taxon of either *Pelophylax perezi* (López-Seoane 1885) (e.g. Uzzell 1982), or *Pelophylax ridibundus* (Pallas 1771) (e.g. Pasteur & Bons 1959). Recent phylogenetic analyses have confirmed the sister relation with *P. perezi* (e.g. Plötner 1998; Pyron & Wiens 2011), while considerable differentiation shown by means of mitochondrial and allozyme evidence suggest that these species diverged as a result of the Gibraltar Strait opening (Busack & Lawson 2008). The low degree of morphological differentiation between *P. perezi* and *P. saharicus* recovered by Busack and Lawson (2008) can be explained by sampling bias, with most analysed individuals originating from north-western Morocco. A similar pattern was found by Benhachem (1988), who found high similarity between *P. perezi* and northern *P. saharicus*, thus suggesting the presence of *P. perezi* in northern Morocco. In contrast, the occurrence of different phenotypes within Moroccan *P. saharicus* has been repeatedly described (e.g. Bons & Pasteur 1959; Bons & Geniez 1996), which led some authors to suggest the existence of multiple taxa. However, these phenotypes do not show any genetic differentiation (Buckley *et al.* 1996; Harris & Carretero 2003), and most likely represent ecotypes shaped by different environmental factors. A similar situation has been described in Tunisian populations of *P. saharicus* by Amor *et al.* (2009).

Recent phylogenetic analyses have shown that *P. saharicus* occurs in entire North Africa (e.g. Arano *et al.* 1998; Harris & Carretero 2003). Hemmer *et al.* (1980) showed a wide hybrid zone between two morphologically indistinguishable 'forms' to be present across the Maghreb, based on electrophoretic analyses. Buckley *et al.* (1994) recovered similar results as the former study. This led to the designation of the subspecies *P. s. riodeoroi* for the western form by Arano *et al.* (1998), which was subsequently shown to occur in entire Morocco by Harris and Carretero (2003).

**Tadpole.** The tadpole of *P. saharicus*, based on Moroccan material, was described by Llorente *et al.* (1996). A drawing of the tadpole is shown in Fig. 12J. Fully developed tadpoles are characterized by an elongated body shape and tail which is more than twice as long as the body. Eyes positioned dorsally on head. Spiracle sinistral. Oral disc ventral. Mouth almost completely surrounded by papillae, except for the upper part. One tooth row on the upper and three on the lower labium. Upper row on infralabial occasionally with narrow median gap. Colour ivory, brown or reddish with small, light brown patches on the dorsum and tail, which fade at the edges. Conspicuous single dark brown or black line runs from the anterior part of the body along the sides of the tail, to approximately two-thirds of its length.

**Bioacoustics.** Steinwarz and Schneider (1991) provided a comprehensive description of the advertisement call and various territorial calls of Tunisian *P. saharicus*. A summary of the advertisement call is presented here. Call characteristics are highly dependent of water temperature. At 19ºC, average call duration is 514.83 ms, while the call consists of 10.64 notes averaging 7.35 pulses per note. Note duration is 34.45 ms, note interval 16.62 ms. Calls can be emitted separately or in series. Although the advertisement call significantly differs from that of *P. perezi* in several characteristics, calls of both species are overall highly similar (Steinwarz & Schneider 1991).

**Natural history.** *Pelophylax saharicus* can be found near mostly permanent water bodies, ranging from ponds, rivers, (mountain)brooks, oases, sources or artificial basins (Le Berre 1989). Tolerance to human impact or alteration of habitats is particularly high. Individuals generally bask in the sun on the edge of water bodies during the day, while they venture into the surrounding terrain at night to forage (Doumergue 1901). Insects, other frogs (including *H. meridionalis*) and fishes comprise prey items (Doumergue 1901). Bons and Bons (1959) encountered large tadpoles during the first week of April on the Doukkala plain. P. de Pous and W. Beukema developed tadpoles in a large spring within the Beni Snassen Massif in early April 2009 as well as December 2012, and near Oualidia on the Doukkala Plain in mid February 2009. Donaire-Barroso and Bogaerts (2003b) encountered tadpoles during January in the western Rif Mountains, while tadpoles were observed in the same region during September (D. Donaire-Barroso pers. obs.). Schweiger (1992) observed recently metamorphosed juveniles near Ouarzazate during late summer and autumn. These limited data suggest that at least in the northern part of the country or within the main mountain ranges reproductive activity takes place in late spring and summer, while larvae generally hibernate. Populations in the arid southern part of Morocco remain active year-round (pers. obs.).

**Distribution.** *Pelophylax saharicus* is the most commonly recorded amphibian species in Morocco. The distribution map (Fig. 16C) is composed of records from Bons and Geniez (1996), Brito (2003), Fahd *et al.* (2007), Harris *et al.* (2008), Ramos and Díaz-Portero (2008), Harris *et al.* (2010) and Barata *et al.* (2011). New records fill in gaps within the already vast range of *P. saharicus* in Morocco.

**National Red List Status.** Least Concern.

## Discussion

### Distribution

While the current paper presents a significant number of new amphibian records from Morocco and for the first time summarizes all published distribution data for these species, we should emphasize that the data are biased to the northern and western part of the country. Additional new records can be especially expected in parts of the High- and Middle Atlas Mountains, and temporary streams in the southern and eastern arid part of the country (see e.g. Barata *et al.* 2011). The relative low amount of records from these latter areas might be explained by the restricted, seasonal dependent activity patterns of amphibians in these regions. Also, despite a lack of knowledge regarding phenological differences between Moroccan, let alone North African amphibian species (but see e.g. El Hamoumi 1988; Gallix 2002; El Hamoumi *et al.* 2007) it has become clear that significant differences exist that should be taken into account. For instance, research undertaken in Forêt de Mamora near Rabat by Hamoumi (1988) and Bons (1967) showed that *P. waltl* reproduced from October until April, *D. scovazzi* only in November (possibly in February), *A. mauritanicus* from March until the beginning of May, *B. boulengeri* only in April, *P. varaldii* from December until the beginning of March, *H. meridionalis* during February and March and finally *P. saharicus* from April until the beginning of July. The cryptic nature of several Moroccan amphibian species outside of their reproductive season makes these especially difficult to observe.

### Biogeographical overview

As noted before, Moroccan amphibians have long been considered as poor in diversity while populations were suggested to be closely related to their European counterparts. From a biogeographical viewpoint, the situation has proven to be much more complex. Although fossil records prior to the Pliocene are not particularly abundant, it is known that at least the families Bufonidae, Discoglossidae, Pipidae, Ranidae and the genus *Ptychadena* Boulenger 1917 occurred in central Morocco during the Miocene (Schleich *et al.* 1996). The westward migration of *B. boulengeri* from Eurasia into Africa (Stöck *et al.* 2006) and the range of *B. brongersmai* have likely been restricted by the Sahara desert during the Miocene (e.g. Douady *et al.* 2003), although the origin of the latter taxon has not been thoroughly clarified. Prior migration through, and subsequent collapse of the Gibraltar land bridge at the end of the Miocene (Krijgsman *et al.* 1999) was a major vicariant event which caused divergence among the genera *Alytes, Discoglossus, Pelobates, Pelophylax, Pleurodeles* and *Salamandra* (Carranza & Arnold 2004; Martínez-Solano *et al.* 2004; Veith *et al.* 2006; Zangari *et al.* 2006; Busack & Lawson 2008; Beukema *et al.* 2010) and potentially *Bufo* (Recuero *et al.* 2012).

During the succeeding Pliocene, transmarine migration from the Iberian Peninsula has additionally been suggested as cause for the African colonization by *B. bufo* at about 3 mya (Garcia-Porta *et al.* 2012). Additionally,

the presence of *Hyla* during this period has been suggested based on fossil remains (Bailon 2000). Marine transgressions and climate oscillations took place until the Lower Pleistocene at approximately 1.7 mya (Thompson & Fleming 1996; Rouchy *et al*. 2003), and have been the main cause for divergence among North African populations of at least *Discoglossus*, *Pleurodeles* and *Salamandra* (Carranza & Arnold 2004; Zangari *et al.* 2006; Beukema *et al.* 2010). Divergence within *Pelophylax* and *Hyla* could have occurred during the same period (Harris & Carretero 2003; Stöck *et al.* 2008b). The role of the Moulouya Basin, which has been identified as a major geographical barrier for Palearctic herpetofauna, seems to be of less importance in shaping amphibian divergence than initially thought. Of species which occur on both sides of the Moulouya Basin, at least *A. mauritanicus*, *B. boulengeri*, *H. meridionalis* and *P. saharicus* have migrated freely across (Harris & Carretero 2003; Batista *et al.* 2006; Harris & Perera 2009), while the situation for *B. bufo* and the genus *Discoglossus* is uncertain due to a relative low amount of samples (Zangari *et al.* 2006; Garcia-Porta *et al.* 2012; Recuero *et al.* 2012).

Divergence as a result of climate oscillations during the Upper Pleistocene has been shown for the genus *Salamandra* (Beukema *et al.* 2010), while at least *A. maurus* and *B. bufo* experienced range contractions as shown by fossil evidence (Ould Sabar & Michel 1996; Hossini 2001; Bailon & Aouraghe 2002; Stoetzel *et al.* 2010a). Humid periods up to the Holocene promoted dispersal within the northern Sahara desert for at least several bufonids (Batista *et al.* 2006; Harris & Perera 2009) by means of an interlinked waterway (Drake *et al.* 2011). A second, recent colonization of Morocco by the genus *Pleurodeles* took place during the Holocene (Batista *et al.* 2003; Carranza & Arnold 2004). The exact origins of both *H. meridionalis* and *B. brongersmai* remain thus far unresolved.

**Conservation remarks**

Close to a century ago, Werner (1931) noted that the herpetofaunal composition of several well-known collection sites near the Atlantic Coast mentioned by previous authors (e.g. Boulenger, Boettger) had considerably decreased as a result of urbanization. Throughout the decades, general land cover change and accompanying habitat destruction and pollution remained the most severe threats to Moroccan amphibians (Stuart *et al.* 2004). Habitat destruction is especially severe along the Atlantic Coast as a result of tourism development, which in some cases threatens entire metapopulations (de Pous *et al.* 2012). Several Moroccan amphibians have been categorized under both global and national IUCN regulations (Salvador *et al.* 2006a,b; Donaire-Barroso *et al.* 2008; Pleguezuelos *et al.* 2010). Unfortunately, the current system of national parks does not provide sufficient coverage to ensure future persistence of most–if not all–amphibian species (de Pous *et al.* 2011). Additionally, the amphibian pathogen *Batrachochytrium dendrobatidis* Longcore, Pessier and Nichols has recently been identified in northern Morocco (El Mouden *et al.* 2011) and might especially pose a threat to populations in the Rif- and Atlas Mountains where the climate is highly similar to that of mountain ranges in central Spain in which *B. dendrobatidis* has caused extensive mortality (e.g. Bosch & Martínez-Solano 2006).

The recent increase of national parks in Morocco (in comparison to those analysed by de Pous *et al.* 2011) is a solid step towards better amphibian conservation. However, we suggest that species-specific conservation effort is highly needed in several cases, such as coastal occurrence sites of *P. varaldii* (de Pous *et al.* 2012) and marginal populations of *S. algira* and *A. maurus*.

**Prospects**

Despite the recent increase and total accumulation of more than a century of studies directed at Moroccan amphibians, various knowledge gaps are readily identified. Concerning distribution data, especially eastern Morocco was already known to be poorly surveyed (Bons 1960), and half a century later still is (Barata *et al.* 2011). Large sections of the High Atlas Mountains, of which major parts are uninhabited and remote, have not been prospected. Finally, searches along the coastline ranging from Tanger in the north to Agadir in the south should focus on recording extant relict populations of lowland species such as *P. varaldii*.

From a systematic viewpoint, several missing tadpole descriptions and bioacoustic analyses represent major knowledge gaps. Tadpole descriptions of *D. scovazzi* and the North African *B. bufo* are lacking, which would be interesting comparative material in regard to other members of their respective genera. Intraspecific genetic variation of the fragmented distribution of *A. maurus* and *B. bufo* has not been assessed, while reanalyses and additional sampling of *H. meridionalis* and both *Discoglossus* spp. might reveal the evolutionary history of the former, while narrowing the potential contact zone of the latter two species.

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# Chapter 5

## Taxonomy and biogeography of *Bunopus spatalurus* (Reptilia; Gekkonidae) from the Arabian Peninsula

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In the last decade, taxonomic studies have drastically increased the number of species known to inhabit the Arabian deserts. While ongoing phylogenetic studies continue to identify new species and high levels of intraspecific genetic diversity, few studies have yet explored the biogeographic patterns in this arid region using an integrative approach. In the present work, we apply different phylogenetic methods in order to infer relationships within the Palearctic naked-toed geckos. We specifically address for the first time the taxonomy and biogeography of *Bunopus spatalurus* from Arabia using multilocus concatenated and species tree phylogenies, haplotype networks and morphology. We also use species distribution modelling and phylogeographic interpolation to explore the phylogeographic structure of *Bunopus spatalurus hajarensis* in the Hajar Mountains and the roles of climatic stability and possible biogeographic barriers on lineage occurrence and contact zones in this arid mountain endemism hotspot. According to the inferred topology recovered using concatenated and species tree methods, the genus "*Bunopus*" is polyphyletic. *Bunopus tuberculatus* and *B. blanfordii* form a highly supported clade closely related to *Crossobamon orientalis*, while the two subspecies of *"Bunopus" spatalurus* branch together as an independent highly supported clade that diverged during the Miocene. Within *B. s. hajarensis*, three geographically structured clades can be recognized that diverged during the late Miocene to Pliocene. The paleodistribution models indicate climatic stability during the Late Pleistocene and the lineage occurrence and predicted contact zones obtained from phylogeographic interpolation therefore probably result from the older splits of the groups when these lineages originated in allopatry. As demonstrated by the results of the multilocus molecular phylogenetic analyses

and the topological test carried out in the present study, the genus "*Bunopus*" is not monophyletic. To resolve this, we resurrect the genus *Trachydactylus* Haas and Battersby, 1959 fro the species formerly referred to as *Bunopus spatalurus*. Considering the morphological differences, the high level of genetic differentiation in the *12S* mitochondrial gene and the results of the phylogenetic and the *cmos* haplotype network analysis, we elevate *Trachydactylus spatalurus hajarensis* to the species level: *Trachydactylus hajarensis* (Arnold, 1980).

**Author contribution**: First authorship reflects that I was the main contributor to the paper. I have developed the concept together with Salvador Carranza, written the first draft, contributed to geographical sampling design and fieldwork. I also conducted the spatial modelling and phylogeographic interpolation.

# Taxonomy and biogeography of *Bunopus spatalurus* (Reptilia; Gekkonidae) from the Arabian Peninsula

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**Abstract**

In the last decade, taxonomic studies have drastically increased the number of species known to inhabit the Arabian deserts. While ongoing phylogenetic studies continue to identify new

species and high levels of intraspecific genetic diversity, few studies have yet explored the biogeographic patterns in this arid region using an integrative approach. In the present work, we apply different phylogenetic methods in order to infer relationships within the Palearctic naked-toed geckos. We specifically address for the first time the taxonomy and biogeography of *Bunopus spatalurus* from Arabia using multilocus concatenated and species tree phylogenies, haplotype networks and morphology. We also use species distribution modelling and phylogeographic interpolation to explore the phylogeographic structure of *Bunopus spatalurus hajarensis* in the Hajar Mountains and the roles of climatic stability and possible biogeographic barriers on lineage occurrence and contact zones in this arid mountain endemism hotspot. According to the inferred topology recovered using concatenated and species tree methods, the genus "*Bunopus"* is polyphyletic. *Bunopus tuberculatus* and *B. blanfordii* form a highly supported clade closely related to *Crossobamon orientalis*, while the two subspecies of *"Bunopus" spatalurus* branch together as an independent highly supported clade that diverged during the Miocene. Within *B. s. hajarensis*, three geographically structured clades can be recognized that diverged during the late Miocene to Pliocene. The paleodistribution models indicate climatic stability during the Late Pleistocene and the lineage occurrence and predicted contact zones obtained from phylogeographic interpolation therefore probably result from the older splits of the groups when these lineages originated in allopatry. As demonstrated by the results of the multilocus molecular phylogenetic analyses and the topological test carried out in the present study, the genus "*Bunopus"* is not monophyletic. To resolve this, we resurrect the genus *Trachydactylus* Haas and Battersby, 1959 fro the species formerly referred to as *Bunopus spatalurus*. Considering the morphological differences, the high level of genetic differentiation in the *12S* mitochondrial gene and the results of the phylogenetic and the *cmos* haplotype network analysis, we elevate *Trachydactylus spatalurus hajarensis* to the species level: *Trachydactylus hajarensis* (Arnold, 1980).

**Keywords:** paleodistribution modelling, phylogeography, multilocus phylogeny, spatial interpolation, contact zone, Palearctic naked-toed gecko

## Introduction

Deserts encompass a large portion (12%) of the Earth's land surface and are important for understanding global biodiversity patterns. While deserts are thought to have relative low species richness compared to other biomes, they are often inhabited by many specialized species and clades possessing a wide array of unique adaptations to arid conditions. For example, lizard communities in deserts might be richer compared to warm temperate and tropical regions and they can contain as many as 70 different species co-occurring at single localities (Pianka 1973; Rabosky et al. 2011). In the past, extensive work on desert reptiles has mainly focused on Australian, South African and North American deserts (e.g. Pianka 1986), while fewer studies exist for the Arabian deserts (e.g. Anderson 1896; Haas 1957; Arnold 1972, 1980, 1986; Gasperetti 1988; Schätti and Gasperetti 1994; Schätti and Desvoignes 1999; Carranza and Arnold 2012; Gardner 2013). Recently, however, an increasing number of taxonomic, biogeographic and phylogenetic studies have focused on lizards inhabiting these deserts (e.g. Carranza and Arnold 2012; Metallinou and Carranza, 2013; Metallinou et al. 2012, 2015; Šmíd et al. 2013a). In the last decade, taxonomic studies have drastically increased the number of species inhabiting the Arabian desert, while ongoing phylogenetic studies continue to identify new species, especially in the southern Arabian Peninsula (Babocsay 2004; Busais and Joger 2011; Carranza and Arnold 2012; Šmíd et al. 2013b, 2015; Metallinou and Carranza 2013; Vasconcelos and Carranza 2014) as predicted by Ficetola et al. (2013). Likewise, several studies have revealed high levels of intraspecific genetic structure of Arabian lizards, mostly in species inhabiting mountainous areas such as the Hajar Mountains in northern Oman and the UAE and the mountains in southern Oman and Yemen (e.g. Carranza and Arnold 2012; Šmíd et al. 2013a), but also in species occurring in lowlands (Metallinou et al. 2012; 2015). Two biodiversity rich areas with high levels of endemicity are recognized within Oman: the Hajar Mountains in North Oman and the Dhofar Mountains in South Oman and East Yemen, as reported previously (Carranza and Arnold 2012). These two mountainous regions have their own unique and complex geologic histories (reviewed in Carranza and Arnold 2012 and Gardner 2013; see also Arnold 1977, 1980 and other articles in the same volumes) but share distinct climatic conditions and vegetation that differentiate them from the much more arid surrounding lowland desert. Although the Hajar and the Dhofar Mountains are inhabited by partially distinct reptile communities with unique endemic species (e.g. *Hemidactylus*, *Asaccus*

geckos;) and even genera (*Omanosaura*, Lacertidae) (see Arnold 1986; Arnold and Gardner 1994; Carranza and Arnold 2012), several species occur in both regions while being absent from the lowland areas between them (*Bunopus spatalurus*, *Chalcides ocellatus*, *Trachylepis tessellata*, and *Platyceps rhodorachis*; see Gardner 2013).

Among the species exhibiting this notable North-South distribution pattern, *Bunopus spatalurus* is an interesting representative, as previously mentioned by Arnold (1980). This species is widely distributed in the southern Arabian Peninsula, ranging from western Yemen to the mountains of northern Oman and the UAE, but with some areas of absence (Sindaco and Jeremčenko 2008; Gardner 2013). Arnold (1980) described the subspecies *Bunopus spatalurus hajarensis* from the Hajar Mountain range and Masirah Island based on clear morphological differences. However, despite some recent phylogenetic studies on Palearctic naked-toed geckos (Červenka et al. 2008; Bauer et al. 2013), no molecular studies have yet addressed the status of *Bunopus spatalurus.* This is possibly the result of the difficulty in finding specimens of *B. s. spatalurus* compared with the relatively abundant *B. s. hajarensis* (Haas and Battersby 1959; Arnold 1980; Gardner 2013) as well as the current political instability in Yemen that obstructs fieldwork. Clarifying the phylogenetic position of *B. s. spatalurus* and *B. s. hajarensis* should provide important insights into Arabian reptile biogeography in general, while it would also contribute to clarifying the taxonomy of the non-monophyletic genus *Bunopus*.

The geologically complex Hajar Mountains in Northern Oman and the UAE are known for their numerous endemics and recent studies have identified high genetic variation of reptile species inhabiting this massif (Papenfuss et al. 2010; Carranza and Arnold 2012). However, no studies have explored the possible causes of this genetic structuring or investigated the historical biogeography of this arid region in detail using an integrative approach. Paleodistribution modelling using climatic datasets from the Last Glacial Maximum and the Mid-Holocene can help elucidate the roles of climate oscillations and stability, and has been used successfully in other interesting biodiversity hotspots (e.g. Waltari et al. 2007; Carnaval et al. 2010). Furthermore, promising methodological advances in spatial phylogeographic interpolation (e.g. Tarroso et al. 2014) further expand the possibilities to explore lineage occurrence and identify contact zones between lineages. Such spatial

modelling methods should provide interesting new insights into the roles of climatic fluctuations and refugia on phylogeographic structure in this high endemism area.

In the present work we revise the systematics of the genus *Bunopus* using multilocus concatenated and species tree phylogenies, haplotype networks and morphology. We also use species distribution modelling and phylogeographic interpolation to explore the phylogeographic structure of *Bunopus spatalurus* in the Hajar Mountains and the roles of climatic stability and possible biogeographic barriers on lineage occurrence and contact zones in this arid mountain endemism hotspot.

We considered the status of the OTUs in the present study within the framework of the General Lineage Species Concept (de Queiroz 1998, 2007), which uses the term 'species' for separately evolving metapopulation lineages. We apply two secondary recognition criteria to assess species limits within *Bunopus spatalurus* using the following approach: 1) identification of lineages based on the analyses of mitochondrial and nuclear data as indicators of distinct evolutionary trajectories; 2) presence of diagnostic morphological characters.

## Materials and Methods

### *Molecular samples, DNA extraction and amplification*

A total of 42 specimens were used in the phylogenetic analyses, including members of the Palearctic naked-toed geckos most closely related to the genus *Bunopus* (Bauer et al. 2013), as well as three out of the four species of this genus (*B. crassicauda* missing) and two outgroups (*Hemidactylus brasilianus* and *H. haitianus*). Given the focus of the study, sampling was especially intensive in *Bunopus spatalurus*. A list of all the specimens with their GenBank accession numbers, voucher codes and locality information is presented in Supplementary Table S1.

Genomic DNA was extracted from ethanol-preserved tissue samples using the standard high salt method (Sambrook et al. 1989). Up to six genetic markers were PCR-amplified and sequenced in both directions: one fragment of the mitochondrial gene encoding the ribosomal 12S rRNA (*12S*; primers 12SaGekko and 12SbGekko – Metallinou et al. 2015),

and five fragments of the nuclear genes encoding the oocyte maturation factor Mos (*cmos*; primers FUF and FUR – Gamble et al. 2008), the recombination-activating gene 1 (*rag1*; primers F700, R700 – Bauer et al. 2007, and R13 and R18 – Groth and Barrowclough 1999), the recombination-activating gene 2 (*rag2*; primers PyF1 and PyR – Gamble et al. 2008), the acetylcholinergic receptor M4 (*acm4*; primers int-F and int-R – Gamble et al. 2008), and a short fragment of phosphoducin (*pdc*; primers PHOF2 and PHOR1 – Bauer et al. 2007). PCR conditions used for the amplification of the *12S* mitochondrial fragment are as in Metallinou et al (2015) and for nuclear genes *cmos*, *rag1* and *rag2* as in Šmíd et al. (2013a), for the nuclear gene *acm4* in Barata et al. (2012) and for *pdc* in Bauer et al. (2007).

*Sequence analysis*

Geneious v. R6.1.6 (Biomatters Ltd.) was used for assembling and editing the chromatographs. Heterozygous positions for the nuclear coding gene fragments were identified based on the presence of two peaks of approximately equal height at a single nucleotide site in both strands and were coded using IUPAC ambiguity codes. The nuclear coding fragments were translated into amino acids and no stop codons were observed. DNA sequences were aligned for each gene independently using the online application of MAFFT v.7 (Katoh and Standley 2013) with default parameters (Auto strategy, Gap opening penalty: 1.53, Offset value: 0.0). For the *12S* ribosomal fragment the Q-INS-i strategy was applied, in which information on the secondary structure of the RNA is considered. Poorly aligned regions in the *12S* alignment were eliminated with Gblocks (Castresana 2000) under low stringency options (Talavera and Castresana 2007). In the case of the *cmos* gene used in the network analyses (see below), the software PHASE v. 2.1.1 was used to resolve heterozygous positions (Stephens et al. 2001) and SEQPHASE (Flot 2010) was used to convert the input and output files. Default settings of PHASE were used except for phase probabilities that were set as ≥ 0.7 (see Harrigan et al. 2008). Uncorrected mean genetic distances between and within groups for the mitochondrial gene fragment were calculated with MEGA 5 (Tamura et al. 2011), using the *p*-distance model.

*Phylogenetic and network analyses and estimation of divergence times*

Two datasets were used for the phylogenetic analyses. Dataset 1 was assembled with the aim of testing the monophyly of the genus *Bunopus* and its relationships with the most closely related genera of naked-toed geckos (Červenka et al. 2008; Bauer et al. 2013). This dataset consisted of 12 representatives of the Palearctic naked-toed geckos of the genera *Agamura*, *Crossobamon*, *Cyrtopodion*, *Tenuidactylus* and *Bunopus* and two representatives of the genus *Hemidactylus* selected as outgroups based on Gamble et al. (2012) and Bauer et al. (2013). We included members of all the species and subspecies of *Bunopus* with the only exception of *B. crassicauda*, which is closely related to *Bunopus tuberculatus* sensu lato (Červenka et al. 2008). Dataset 1 consisted of an alignment of 3020 base pairs (bp) of concatenated mitochondrial (379 bp of *12S*) and nuclear (742 bp of *cmos*; 1038 of *rag1*; 363 of *rag2*; 444 bp of *acm4*; 394 of *pdc*) DNA sequences. Dataset 2 was assembled with the aim of studying in detail the phylogeographic relationships of the two endemic Arabian subspecies of the genus *Bunopus* (*Bunopus s. spatalurus* and *Bunopus s. hajarensis*) and to assess their subspecific status. This dataset consisted of 32 samples, four *Bunopus s. spatalurus* and 28 *Bunopus s. hajarensis*, from 30 localities across the entire distribution range of the species in Arabia (Fig. 1). In the face of uncertainty over the sister group to *Bunopus spatalurus* (see results below) and in order to optimize the alignment of the *12S* gene, we did not include any outgroups in dataset 2 and use Bayesian methods for inferring the root of the phylogenetic tree (Huelsenbeck et al. 2002). Dataset 2 consisted of an alignment of 396 bp of the *12S* gene.

Best-fitting models of nucleotide evolution for dataset 1 were inferred using PartitionFinder v.1.1.1 (Lanfear et al. 2012) with the following settings: branch lengths linked, only models available in BEAST evaluated, AIC model selection criterion applied, all partition schemes analyzed. Each gene was set as an independent partition. A two-partition scheme was selected: p1, *12S* gene and the GTR+G model of sequence evolution; p2, all five nuclear genes and the HKY+G model of sequence evolution. For dataset 2 we used jModeltest v.0.1.1 (Guindon and Gascuel 2003; Darriba et al. 2012) under the Akaike information criterion (AIC) (Akaike 1973) and the HKY+I+G model of sequence evolution was selected. For both datasets we performed for each partition a Likelihood-ratio test implemented in MEGA v.5 (Tamura et al. 2011) to test whether a strict or a relaxed molecular clock fit our data best. The hypothesis

that the sequences evolve in a clock-like manner could not be rejected at a 5% significance level for all nuclear genes of dataset 1, while it was rejected for the *12S* gene in both datasets.

Phylogenetic analyses for dataset 1 were performed with maximum likelihood (ML) and Bayesian inference (BI) methods. ML analyses were performed in RAxML v.7.4.2 (Stamatakis 2006) as implemented in raxmlGUI (Silvestro and Michalak 2012) with 100 random addition replicates, using the GTR+G model of sequence evolution and independent model parameters for the two partitions (see above). Reliability of the ML tree was assessed by bootstrap analysis (Felsenstein 1985) including 1000 replications. The software BEAST v.1.7.5 (Drummond and Rambaut 2007) was used for BI analysis of the concatenated dataset as well as species tree analysis employing *BEAST (Heled and Drummond, 2010). Three individual runs of $5x10^7$ generations were carried out, sampling at intervals of 10000 generations. Models and prior specifications applied were as follows (otherwise by default): model of sequence evolution for each of the two partitions of dataset 1, as indicated above; Yule process tree prior; random starting tree; base substitution prior Uniform (0,100); alpha prior Uniform (0,10). Partitions and clock models were unlinked and the xml file was manually modified to set Ambiguities="true" for the nuclear genes in order to account for variability in the heterozygous positions, instead of treating them as missing data. Posterior trace plots and effective sample sizes (ESS) of the runs were monitored in Tracer v1.5 (Rambaut and Drummond 2007) to ensure convergence. The results of the individual runs were combined in LogCombiner discarding 10% of the samples and the maximum clade credibility (MCC) ultrametric tree was produced with TreeAnnotator (both provided with the BEAST package). Phylogenetic analyses for dataset 2 were performed with BI only, with the same parameters as above but using a Coalescent Constant Size process tree prior. Nodes were considered strongly supported if they received posterior probability (pp) support values ≥ 0.95 (Wilcox et al. 2002; Huelsenbeck and Rannala 2004)

Absolute divergence times were estimated concurrently in the Bayesian analyses of both datasets, applying a previously calculated mean rate of molecular evolution for the same *12S* gene fragment (mean: 0.00755, stdev: 0.00247) (Carranza and Arnold 2012). Despite the problems associated with using evolutionary rates from other organisms for time tree calibration, the rate of the *12S* rRNA gene applied here has been corroborated by independent studies that used different calibration points and different taxa to the ones employed by

Carranza and Arnold (2012) (Sindaco et al. 2012; Metallinou et al. 2012). Moreover, the rates by Carranza and Arnold (2012) have already been applied to several different studies of lizards for which reliable internal calibration points based on geographic or fossils evidence do not exist (Hawlitschek and Glaw 2012; Milá et al. 2013; Vasconcelos and Carranza 2014; Šmíd et al. 2013a; Metallinou et al. 2015).

A dataset including sequences of the nuclear gene *cmos* for the same 32 specimens included in dataset 2 (see above and Table 1) were used to infer the genealogical relationships within *Bunopus spatalurus* using a haplotype network inferred with statistical parsimony as implemented in the program TCS v.1.21 (Clement et al. 2000). Phased sequences were used (see above) and a connection limit of 95% was applied.

*Topology test*

In order to assess the polyphyletic status of the genus *Bunopus* (see below) a tree with the alternative topology (topological constraint) in which the members of the genus *Bunopus* were forced to form a monophyletic group was reconstructed using dataset 1. The constrained topology was compared to the unconstrained, best ML, tree using the Approximately-Unbiased (AU; Shimodaira 2002) and Shimodaira-Hasegawa (SH; Shimodaira and Hasegawa 1999) tests. Per-site log likelihoods were estimated using raxmlGUI and *p*-values were calculated using CONSEL (Shimodaira and Hasegawa 2001).

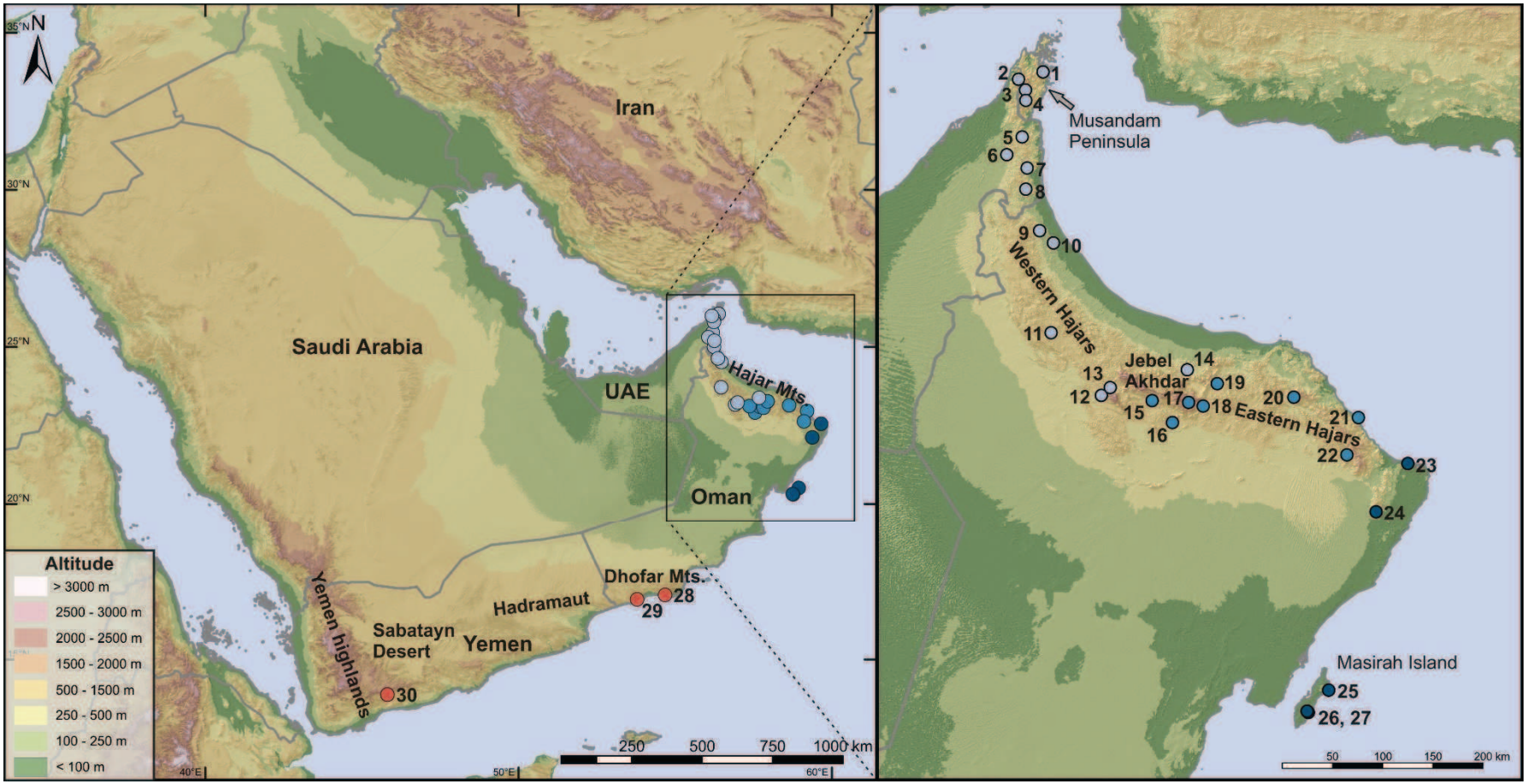


**Figure 1.** Map of localities of material examined in this study. Circles indicate samples used in the genetic analyses (red = *Trachydactylus spatalurus*, blue = *T. hajarensis*), with different shades of blue representing the distinct clades of *T. hajarensis* as shown in Fig. 2. A list of all the specimens with their taxonomic identification, sample code, voucher code, country, and corresponding geographical distribution data and GenBank accession numbers for the sequenced genes is presented in Supplementary Table S1. Taxon names correspond to changes proposed in this paper.

*Species distribution modelling*

A total of 113 distribution records of *Bunopus spatalurus hajarensis* were assembled from literature and fieldwork. Spatially autocorrelated distribution records were removed using a spatial rarefying protocol as implemented in SDMtoolbox (Brown 2014). After spatial rarefying, 65 distribution records were retained for species distribution modelling (SDM) (Supplementary Fig. S1A and Table S2). Bioclimatic variables were downloaded from the WorldClim database version 1.4 (Hijmans et al. 2005) at a resolution of 2.5 arc minutes (nearly 5x5 km). Past climate data for the Last Glacial Maximum (LGM) and Mid-Holocene (MH) were obtained from the WorldClim database as well at a similar resolution. The average of three (LGM: CCSM4, MIROC-ESM and MPI-ESM) and nine (MH: BCC-CSM1-1, CCSM4, CNRM-CM5, HadGEM2-CC, HadGEM2-ES, IPSL-CM5A-LR, MIROC-ESM, MPI-ESM-P, MRI-CGCM3) Global Climate Models was used for each time period respectively. The data was projected to the Asia South Albers Equal Area Conic projection and resampled to a resolution of 5 km. Collinearity of the initial variables was measured by Pearson's correlation coefficient in ENMtools 1.3 (Warren et al. 2010). A total of five bioclimatic variables, all of which had a correlation degree lower than 0.75 (Pearson´s coefficient), were retained. The final set of variables used for the *Bunopus s. hajarensis* SDMs consisted of: Temperature Seasonality (BIO4), Max Temperature of Warmest Month (BIO5), Annual Precipitation (BIO12), Precipitation of Driest Month (BIO14) and Precipitation of Warmest Quarter (BIO18).

Species distribution models were generated using the presence/background algorithm Maxent, version 3.3.3k (Phillips et al. 2006). The ENMeval R package (Muscarella et al. 2015) was used to tune and evaluate the SDMs for *Bunopus s. hajarensis* based on the "checkerboard2" method under a wide variety of settings and regularization parameters in a criterion-based model selection framework. Competing models were compared using the AICc as suggested by Warren and Seifert (2011). Maxent was subsequently used with the following settings: convergence threshold = 0.00001, maximum number of iterations = 500 and $\beta_j = 3.0$) while using Linear, Quadratic and Hinge features. We followed the suggestion of VanDerWal et al. (2009) and used an exploratory analysis to define the most appropriate calibration region. Final models were calibrated in a background region that encompassed all known localities and included areas that have been accessible to the species via dispersal over relevant time periods (Merow et al. 2013). The average of ten pseudo-replicated models with

randomly selected test samples was used to produce SDMs, which were plotted in logistic format. The final models were reclassified in ArcGIS 10 (ESRI) into binary presence-absence maps using the maximum training sensitivity plus specificity threshold (MTSPS), which maximizes the sum of sensitivity (proportion of actual positives that are correctly identified) and specificity (proportion of negatives that are correctly identified) and has been shown to produce highly accurate predictions (Liu et al. 2005; Jiménez-Valverde and Lobo 2007). All models were tested with receiver operating characteristics (ROC) curve plots, which plot the true-positive rate against the false-positive rate. The average area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model. Comparisons of the environmental variables used for projection to those used for training the model were made using visual interpretation of multivariate similarity surface (MESS) pictures and the most dissimilar variable (MoD) (Elith et al. 2010).

*Phylogeographic interpolation*

The recently developed PHYLIN R package (Tarroso et al. 2014) was used to spatially interpolate the phylogeographic data of *Bunopus s. hajarensis*. These interpolations can be used to predict the spatial occurrence of different lineages within a phylogeny using a modified method of kriging. We used only *Bunopus s. hajarensis* haplotypes from dataset 2 to create an ultrametric BI tree in BEAST, as above. The same extent and resolution as used for the SDM was used as study region for interpolation. A total variogram was produced with the default values (lag = 0.6°, $lag_{tol}$ = 0.3°), and a model was fitted with sill and range estimated by nonlinear least squares with nugget forced to 0. Additionally, we investigated the spatial dependence of the three main clades (1–3) using cluster variograms with fitted model by nonlinear least squares or forced range size. All variograms were created and fitted with a spherical model. PHYLIN was used to interpolate clade occurrence following the 0.95 probability and to identify potential contact zones between the three main clades recognized in the phylogenetic analyses (see results) using a single threshold (hs = 6).

## Results

*Molecular analyses*

The results of the concatenated ML analysis of the selected Palearctic naked-toed geckos using dataset 1 are presented in Figure 2. The BI concatenated (BEAST) and species tree (*BEAST) analyses supported the same phylogenetic relationships except for the position of *Agamura persica*, which is inferred as sister taxon to *Bunopus spatalurus* in the ML analysis and as sister taxon to the other members of the naked-toed geckos in the Bayesian analyses, although this relationship is supported with a high pp value only in the BEAST analysis (Fig. 2 and Supplementary Figure S2). Diversification in this clade of geckos is estimated to have initiated approximately 22.7 Mya (13.9–32.2, 95% HPD) or 25.3 (15.5–35.6, 95% HPD) based on *BEAST analyses. According to the topology inferred using concatenated (ML and BEAST) and species tree (*BEAST) methods, the genus "*Bunopus*" is polyphyletic. *Bunopus tuberculatus* and *B. blanfordii* form a highly supported clade closely related to *Crossobamon orientalis*, while the two subspecies of *"Bunopus" spatalurus* cluster together in an independent highly supported clade that originated in the late Miocene, approximately 7.6 Mya (4.4–11.1, 95% HPD) or 7.9 Mya (3.9–12.4, 95% HPD) based on *BEAST analyses. There is also limited support for the monophyly of the group comprising *Cyrtopodion scabrum*, the two species of *Tenuidactylus* and the clade formed by *Crossobamon orientalis*, *B. tuberculatus* and *B. blanfordii* (Fig. 2 and Supplementary Figure S2). In all the analyses, the specimen of *B. tuberculatus* from UAE is more closely related to *B. blanfordii* than to the other specimen of *B. tuberculatus* from Iran included in the phylogenetic analyses (Fig. 2 and Table S1).

The results of the topology test carried out with dataset 1 in which all seven specimens of the genus "*Bunopus*" included in the analyses were forced to form a monophyletic group, indicated that the constrained topology was statistically significantly different from the best ML topology presented in Fig. 2, rejecting the monophyly of the genus "*Bunopus*" (AU $p < 0.00001$; SH $p < 0.00001$).

The BI tree inferred using dataset 2 is presented in Fig. 3 and, like in Fig. 2, it shows that the two taxa form two well-supported reciprocally monophyletic groups. Within *"Bunopus" s. spatalurus*, there are two well-supported clades, one with representatives from Yemen (loc. 30 in Fig.1) and the other from Dhofar, Oman (locs. 28–29), with a relatively recent

divergence, approximately 2.7 Mya (0.8–8.0, 95% HPD). Within *"Bunopus" s. hajarensis*, three geographically structured clades can be recognized: clade 1, sister clade to the other two clades and restricted to the northeastern tip of Oman, in the isolated massifs of the Jebel Khamis, Jebel Qahwan and surrounding areas and in Masirah Island (locs. 23–27 in Fig. 1); clade 2, distributed across the Eastern Hajars and the Jebel Akhdar (locs. 15–22 in Fig. 1); and clade 3, distributed from the western foothills of the Jebel Akhdar, across the Western Hajars and up to the northernmost tip of the Hajar mountain range (Musandam Peninsula, Oman) (locs. 1–14 in Fig. 1). Although monophyly of each of these three clades is well supported, their inter-relationships are not totally resolved (Fig. 3). Diversification in this taxon initiated approximately 5.7 Mya (2.4–13.2, 95% HPD) and in each of the three clades it took place during the Pleistocene. The uncorrected genetic distances (*p*-distance) for the *12S* mitochondrial gene among the 32 samples included in dataset 2 are presented in Supplementary Table S3. The level of genetic divergence in the *12S* between the two subspecies of *"Bunopus" spatalurus* is 13.0± 2%. The genetic divergence within *"Bunopus" s. spatalurus* is only 2.0± 0.6%, while it is 4.5± 0.7% for *"Bunopus" s. hajarensis*. The genetic divergence between the three clades of *"Bunopus" s. hajarensis* is 6.9± 1.2% between clades 1 and 2; 6.6± 1.2% between clades 1 and 3; and 6.2± 1.1% between clades 2 and 3. The level of genetic variability within each one of the clades is 0.5± 0.3%, 1.7± 0.4%, and 1.1± 0.3% for clades 1, 2 and 3, respectively.

The nuclear *cmos* haplotype network analysis shows that the two subspecies of *"Bunopus" spatalurus* do not share haplotypes (Fig. 4). The three haplotypes revealed in *"Bunopus" s. spatalurus* are separated by a maximum of two mutations. The high level of genetic variability within *"Bunopus" s. hajarensis*, as observed in the *12S* mitochondrial gene, with clades 1–3 diverging by a *p*-distance above 6% in all three comparisons (see above), is also apparent in the *cmos* nuclear gene, for which 12 haplotypes separated by 1–4 mutational steps were recovered (Fig. 4). Despite the high level of genetic variability at both mitochondrial and nuclear levels and the geographic coherence of the three mitochondrial clades of *"Bunopus" s. hajarensis* across its distribution range, all three clades share *cmos* nuclear haplotypes. Clades 1 and 2 present two private haplotypes each and clade 3, which is represented in total by six haplotypes, has five private haplotypes.

*Species distribution modelling*

Maxent produced SDMs of good predictive accuracy according to the average test AUC (0.796±0.063). The present and past SDMs for *"Bunopus" spatalurus hajarensis* reveal large suitable areas in the Hajar Mountains and surrounding areas that have remained stable and extend well beyond the current distribution range (Supplementary Figure S1). Although the SDMs predict stability in all areas in the Hajar Mountains, there is a large area of unsuitable climate between the Hajars and Masirah Island in the Sharqiyah Sands region. This gap exists throughout all time periods and indicates that these populations have been isolated. The LGM model, however, predicts unsuitable climatic conditions at Masirah Island. Both MESS and MoD pictures depict several areas outside the training range. For example, most areas in the Musandam Peninsula and the UAE as well as parts of the Jebel Akhdar have areas with non-analog climatic conditions, mainly for BIO4 but also BIO5, BIO12 and BIO18 (see Supplementary Figure S3).

*Phylogeographic interpolation*

The spherical model fitted to the total variogram obtained from PHYLIN had a good fit as suggested by nonlinear least squares ($R^2$=0.957) (Supplementary Figure S4). The model indicates a clear isolation-by-distance pattern, with small genetic differences at short distances and stabilization of the semi-variance at larger distances The cluster variograms for clades 1–3, however, indicate only a good fit for clade 3 ($R^2$ = 0.8540) and a very poor fit of clades 1 and 2 ($R^2$ = NA and $R^2$ = 0.096). The maps of predicted occurrence of each lineage predicted well the spatial pattern of the three clades in North Oman, despite the low fit models of clades 1 and 2 (Supplementary Figure S4 and S5). Clade 1 occurs only in the extreme south-eastern tip of the Hajar Mountains and on Masirah Island and is predicted to occur only in suitable lowland areas in this region. Clade 2 is predicted to occur from the south and eastern Jebel Akhdar to the western Hajars, including the gap between these two mountain ranges. Clade 3 has the largest range and is predicted to occur widespread in the north-western Hajar Mountains in Oman and the UAE. The eastern border of this clade is predicted to occur along the western flanks and north of the Jebel Akhdar (Supplementary Figure S5). The potential contact zones as represented by the average probability of the presence of multiple clades were identified at the margins of the spatial occurrence of the clades as given

by the phylogenetic tree using a single threshold (Fig. 5). The potential contact zones with the highest probabilities are located in the centre of the Jebel Akhdar (clade 2 and 3) as well as in the Eastern Hajars (clade 1 and 2).

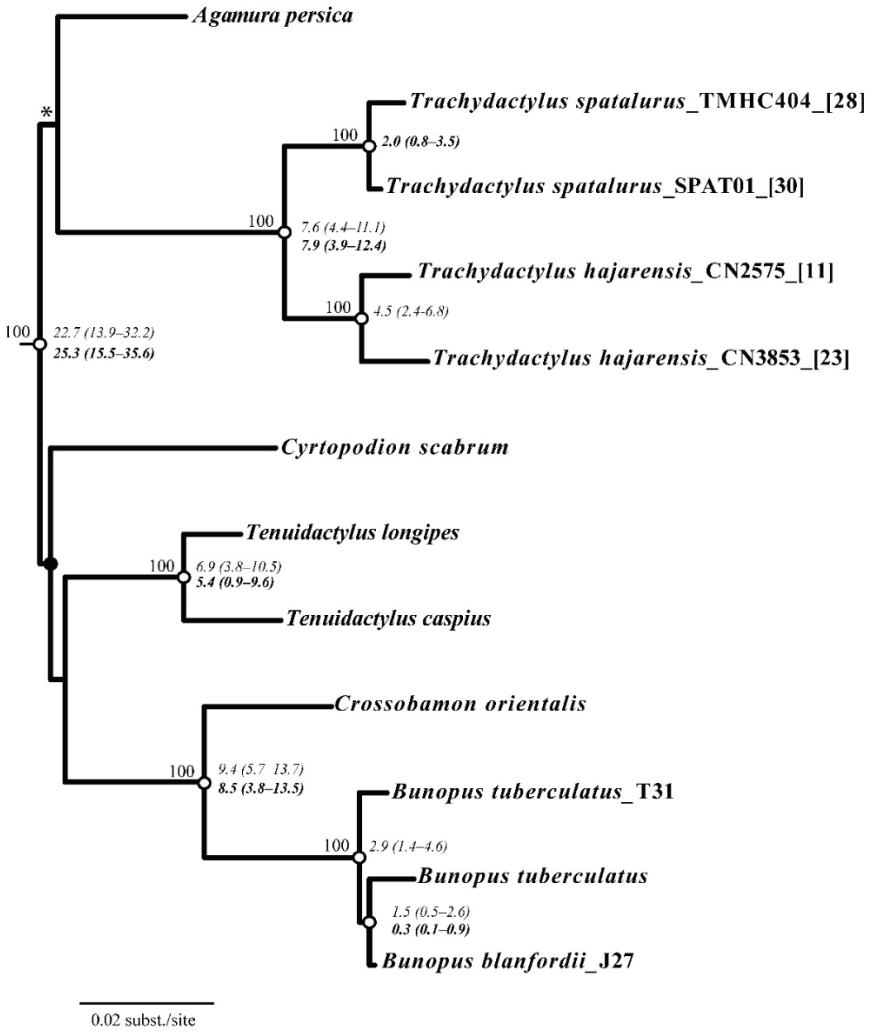


**Figure 2.** ML tree of the Palearctic naked-toed geckos related to *Bunopus* and *Trachydactylus*, inferred using the concatenated dataset (*12S*, *cmos*, *rag1*, *rag2*, *acm4*, *pdc*). The two *Hemidactylus* used to root the tree are not shown. Bootstrap values > 70% in the ML analysis are shown next to the corresponding nodes. Empty circles indicate pp > 0.95 in the concatenated (BEAST) and species tree (*BEAST) analyses; black-filled circle indicates pp > 0.95 in the BEAST analysis only. An asterisk by the node (*) of the ML tree indicates that this topological relationship was not supported by the BEAST or *BEAST analyses (see Supplementary Fig. 2). Ages of all well-supported relationships inferred using concatenated BI analysis (BEAST) or species tree analysis (*BEAST, in bold letters) are shown by the nodes in italics with the 95% HPD between brackets. Taxon names correspond to changes proposed in this paper.

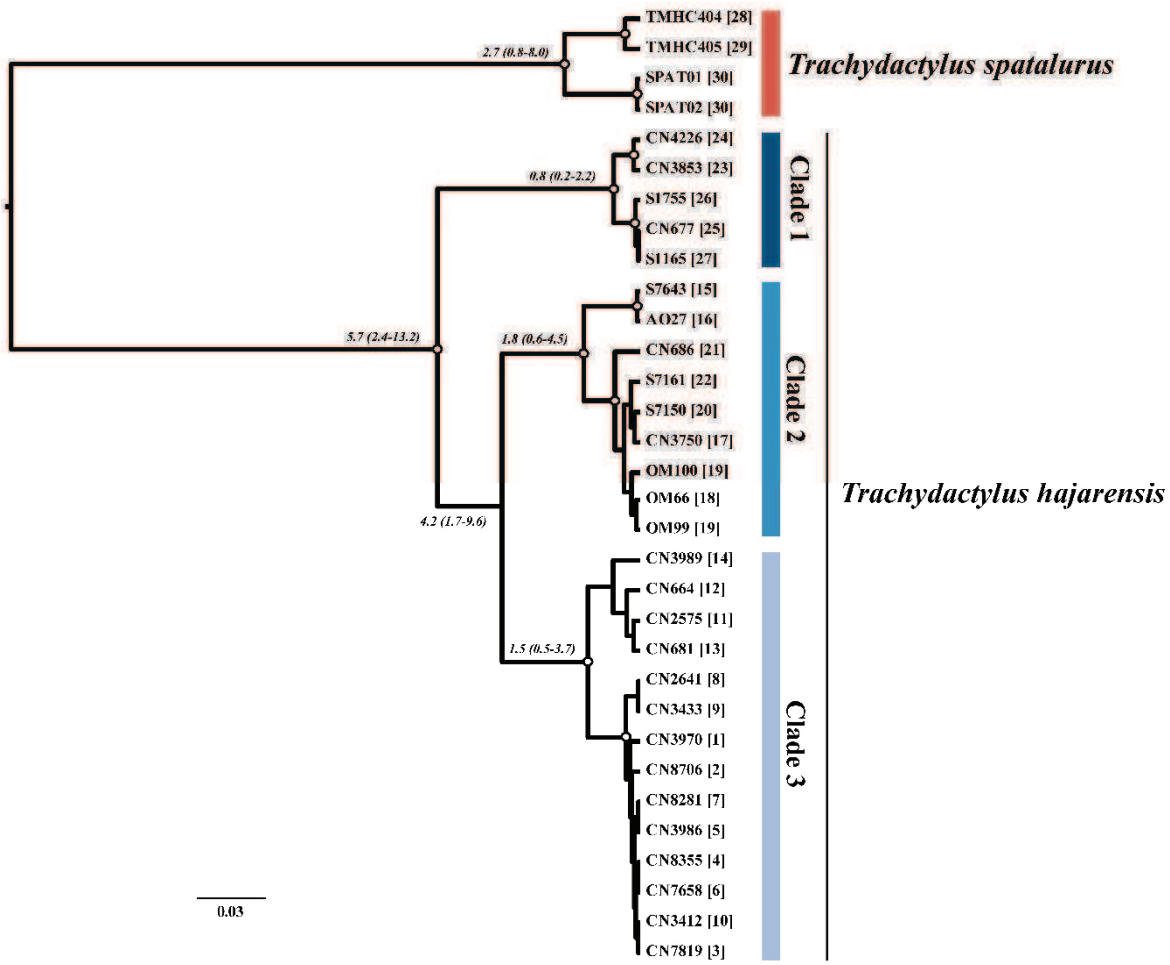


**Figure 3.** BI MCC tree of *Trachydactylus spatalurus* and *T. hajarensis* inferred using BEAST on dataset 2 (*12S* mtDNA gene). A list of all the specimens with corresponding sample code, voucher code, country, locality code and GenBank accession numbers is presented in Supplementary Table S1. Ages of some relevant nodes are shown by the nodes in italics with the 95% HPD between brackets. Taxon names correspond to changes proposed in this paper.

*Taxonomic account*

As demonstrated by the results of the multilocus molecular phylogenetic analyses and the topological test carried out in the present study, the genus "*Bunopus*" is not monophyletic. *Bunopus tuberculatus* Blanford, 1874 is the type species of the genus *Bunopus* Blanford, 1874 and therefore, the clade including *B. tuberculatus* and *B. blanfordii* should retain the generic name (and would also most probably include *Bunopus crassicauda* Nikolsky, 1907; Červenka et al. 2008) and the clade formed by the two members of the species "*Bunopus*" *spatalurus* should be assigned a different generic name. Haas and Battersby (1959), using distinct

morphological characters described a new genus and species *Trachydactylus jolensis* Haas and Battersby, 1959. *Trachydactylus jolensis* was found to be morphologically similar to *Bunopus spatalurus* Anderson, 1901 by Leviton and Anderson (1967), but because the authors were not convinced of the generic allocation of the species they suggested the combination *Trachydactylus spatalurus*. Later, Arnold (1977) argued that the generic characters among the Arabian naked-toed geckos were rather arbitrary and not good indicators of relationships and returned the species tentatively to the genus *Bunopus*, a taxonomic arrangement followed by all subsequent authors (Uetz 2014). Based on our molecular data, in order to resolve the polyphly of "*Bunopus"*, we resurrect the genus *Trachydactylus* Haas and Battersby, 1959 (type species *Trachydactylus jolensis* Haas and Battersby, 1959 by original designation; considered a junior subjective synonym of *Trachydactylus spatalurus* (Anderson, 1901) by Arnold 1977) for the clade formed by the two subspecies of *Trachydactylus spatalurus*: *Trachydactylus spatalurus spatalurus* (Anderson, 1901) and *Trachydactylus spatalurus hajarensis* (Arnold, 1980).

As already stated by Arnold (1980) in the original description of *Trachydactylus spatalurus hajarensis*, the two subspecies of *Trachydactylus spatalurus* present some differences at the morphological level. *Trachydactylus s. hajarensis* is differentiated from *Trachydactylus s. spatalurus* by its smaller size (up to 50 mm of SVL compared with up to 67 mm in *T. s. spatalurus*); the presence in the dorsum of neck and body of about eight (at mid-body) and six (between the hind legs) longitudinal rows of irregular, enlarged scales with a strong medial keel that increases in height to the posterior border, compared with unkeeled or feebly keeled dorsal scales in *T. s. spatalurus* (Fig. 6A,B,C,D); strongly keeled scales also on the dorsal part of the hind limbs, compared with unkeeled or feebly keeled in *T. s. spatalurus* (Fig. 6G,J); presence of protruding (convex) scales on top of the head, compared with flattened scales in *T. s. spatalurus* (Fig. 6E,H); presence of a pair of enlarged postmental scales, each situated laterally to the posterior section of the mental, compared with no clearly differentiated chin shields in *T. s. spatalurus* (Fig. 6K–N). As a result of the morphological differences, the high level of genetic differentiation in the *12S* mitochondrial gene (13.0±2%), and the results of the phylogenetic and the *cmos* haplotype network analysis (Figs. 2–4,

Supplementary Fig. S2; Supplementary Table S3), we elevate *Trachydactylus spatalurus hajarensis* to the species level: *Trachydactylus hajarensis* (Arnold, 1980).

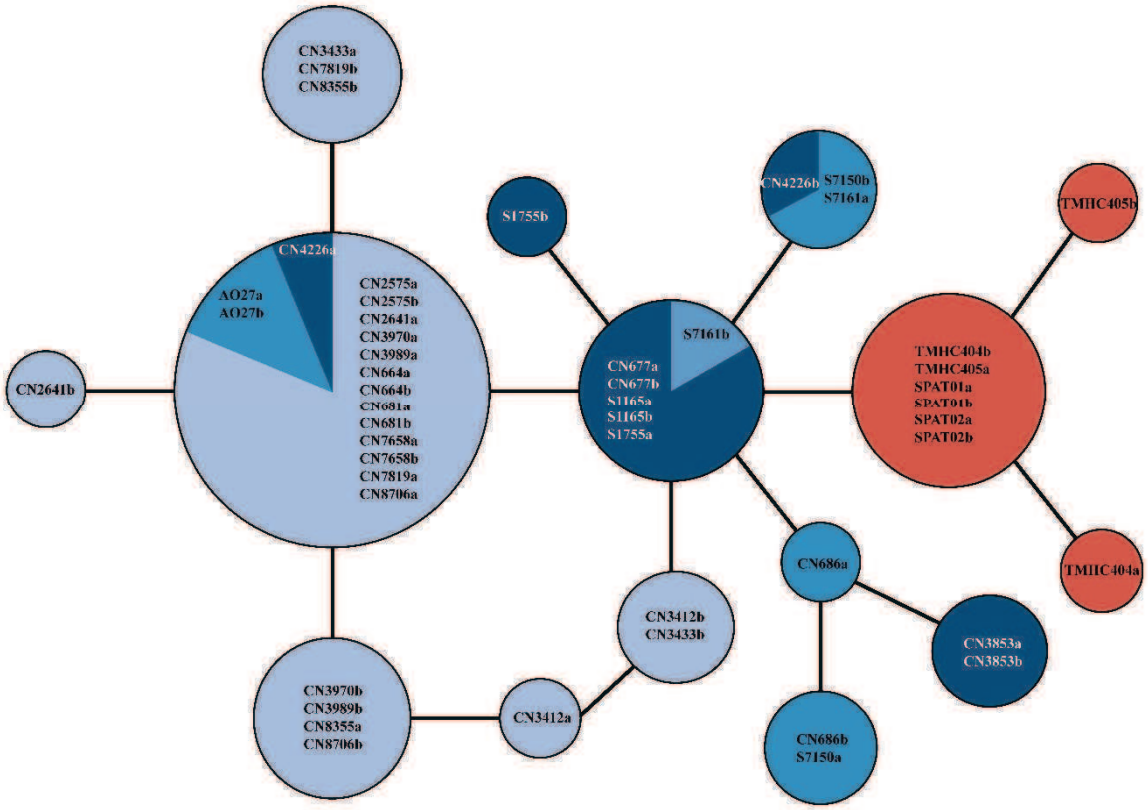


**Figure 4.** Haplotype network of the phased sequences of the nuclear marker *cmos*. Colors correspond to Fig. 3. Phase probabilities were set as ≥0.7. A list of all the specimens with corresponding sample code, voucher code, country, locality code and GenBank accession numbers is presented in Supplementary Table S1.

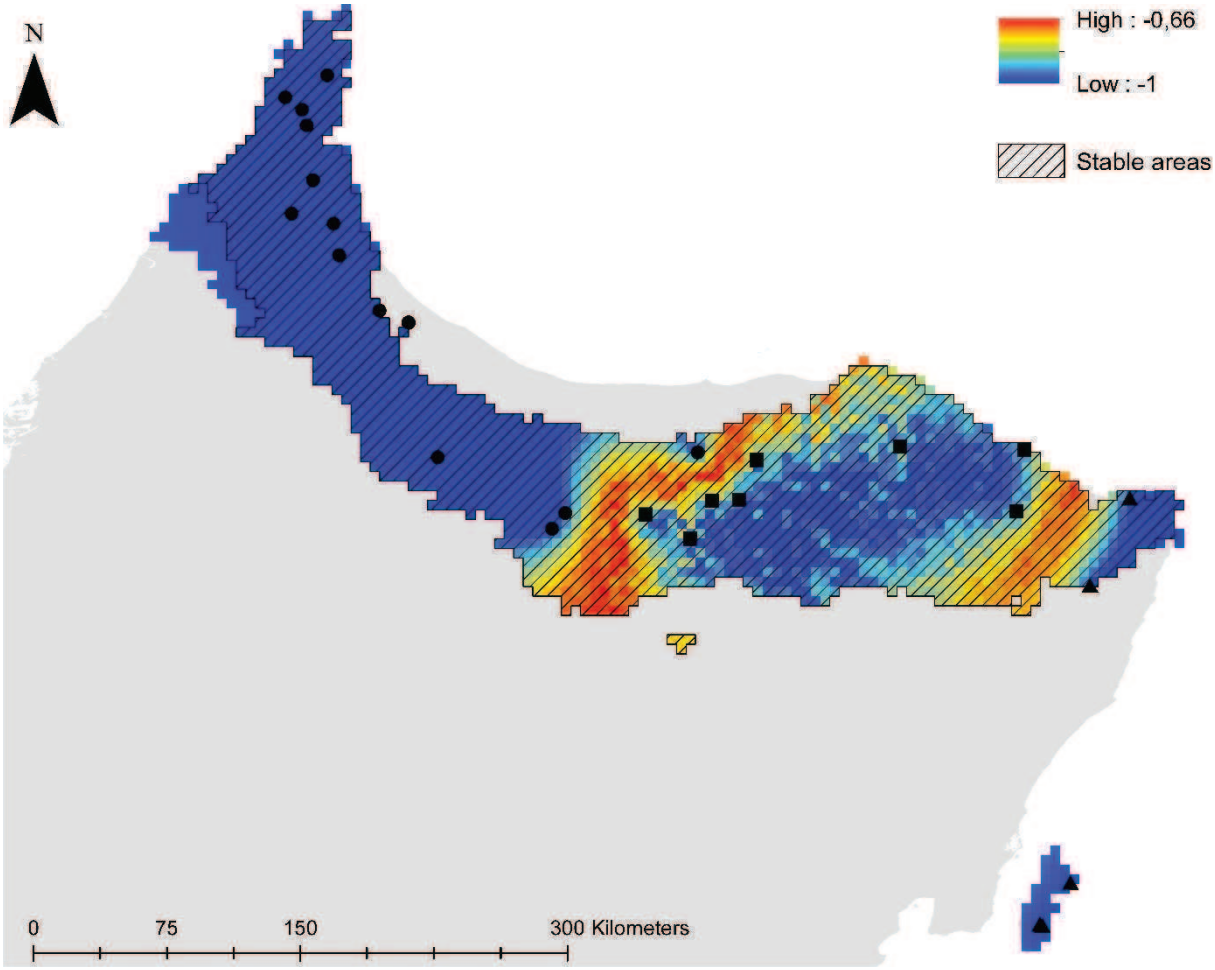


**Figure 5.** Potential contact zones of three main clades of *Trachydactylus hajarensis* from the Hajar Mountains in Oman and the UAE. Contact zones are represented by the average probability of presence of multiple clades. The dashed areas show climatic stability as inferred using paleodistribution modelling (Last Glacial Maximum, Mid-Holocene) using the software Maxent.

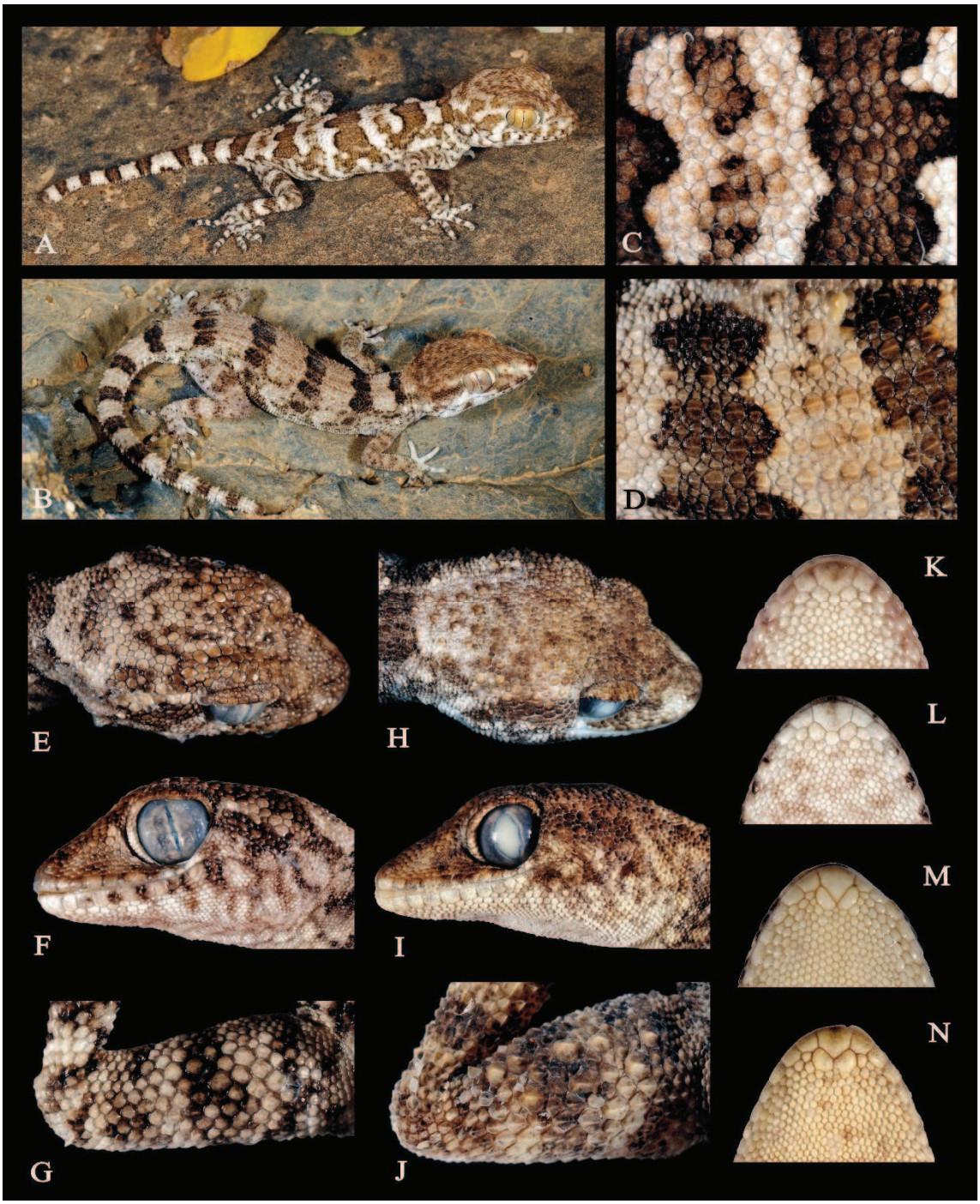


**Figure 6.** Pictures of (A) *Trachydactylus spatalurus* (TMHC 2013.10.404**,** male) and (B) *Trachydactylus hajarensis* (TMHC 2013.10.407). Close up detail of the dorsal scales of (C) *Trachydactylus spatalurus* (TMHC 2013.10.404) and (D) *Trachydactylus hajarensis* (TMHC 2013.10.408). Details of the dorsal side of head (E), lateral side of head (F), and dorsal side of the right hind limb (G) of *Trachydactylus spatalurus* (TMHC 2013.10.404); of the dorsal side of head (H), lateral side of head (I), and of the dorsal side of the right hind limb (J) of *Trachydactylus hajarensis* (TMHC 2013.10.407). Underside of head (gular region showing the arrangement of mental and postmental scales and chin shields) of (K) *Trachydactylus spatalurus* (TMHC 2013.10.405**,** male), (L) *Trachydactylus spatalurus* (TMHC 2013.10.404), (M) *Trachydactylus hajarensis* (TMHC 2013.10.407), and (N) *Trachydactylus hajarensis* (TMHC 2013.10.409). Data for *Trachydactylus spatalurus* specimens TMHC 2013.10.404 and TMHC 2013.10.405 are presented in Supplementary Table 1. *Trachydactylus hajarensis* TMHC 2013.10.407, male, 3 km S. of Al-Hamra, Nizwa, Oman (23.057 57.288, 671 m a.s.l.); TMHC 2013.10.408, male, same data as TMHC 2013.10.407; TMHC 2013.10.409, male, Surrounding of Jebel Shams Resort, Oman (23.208 57.200, 1983 m a.s.l.).

**Discussion**

The knowledge on the systematics and phylogenetic relationships of the Palearctic naked-toed geckos has increased in recent years thanks to the use of molecular data (Červenka et al. 2008, 2010; Gamble et al. 2012; Bauer et al. 2013). However, despite the effort in including the maximum number of taxa, neither *Trachydactylus spatalurus* nor *Trachydactylus hajarensis* have been included in any molecular phylogenetic analysis before. As a result of that, the taxonomic status of "*Bunopus"* had not been evaluated. The phylogenetic relationships recovered in our analyses of dataset 1 (Fig. 2) are very similar to those presented by Gamble et al. (2012), with which we share all five nuclear genes of dataset 1. The sister taxa relationship between *Bunopus* and *Crossobamon* received high support in all the analyses. However, we observe conflicting levels of support between different analysis for the phylogenetic position of the other naked-toed gecko genera (i.e. *Agamura*, *Cyrtopodion*, *Tenuidactylus*) and we consider that it remains unresolved (Gamble et al. 2012; Bauer et al. 2013). As already suggested by Červenka et al. (2008, 2010) and highlighted by Bauer et al. (2013), it seems that there are several cryptic taxa masquerading under *B. tuberculatus*. According to our analyses, *B. tuberculatus* is paraphyletic with respect to *B. blanfordii*, further supporting this hypothesis. However, until a full assessment of the systematics of *B. tuberculatus* and related species (*B. blanfordii* and *B. crassicauda*) is carried out, including an extensive sampling across its distribution range and multilocus phylogenies and morphological analyses, it will not be possible to reach taxonomic conclusions.

The present study shows that *Trachydactylus spatalurus* and *T. hajarensis* form two well-supported reciprocally monophyletic groups that diverged during the Miocene. Regarding *T. spatalurus*, the scarcity of samples makes this still a very preliminary study. However, with the three samples available two well supported groups can be identified, 1 – locality [30], Yemen; and 2 – localities [28,29], Oman (Fig. 1) separated by an uncorrected genetic distance in the *12S* of 2.3–3.2% (see Supplementary Table S3). According to our calibrations, these two lineages split approximately 2.0 Ma according to dataset 1 and 2.7 Ma ago according to dataset 2 (Figs. 2–3). In the south of the Arabian Peninsula there are two main mountain ranges, one in western Yemen, in the area called Western Highlands/Yemen Highlands, characterized by being geologically closely related to the African side of the Red Sea, and another mountain range that extends from central Yemen, in the Central

Highlands/Hadramout uplands, to south Oman (Dhofar Mountains), with geological affinities to the majority of the Arabian peninsula (Sahil and Alsina 1999; Bosworth et al. 2005; As-Saruri et al. 2010). Between these two mountain ranges there is the Sabatayn Basin and more to the North the Sabatayn desert, and within each mountain range a complex mosaic of valleys. These two areas are important reservoirs of endemism and harbor significant levels of intraspecific genetic variability in geckos (Carranza and Arnold 2012; Šmíd et al. 2013a; Metallinou et al. 2012, 2015). The phylogeographic pattern within *T. spatalurus* is similar to that reported between the species *Uromastyx yemenensis* and *U. benti*, and with similar genetic distances, 2.3–3.3% in the ribosomal mitochondrial gene *16S* (Wilms and Schmitz 2007). With an increased sampling effort in *T. spatalurus* it would be interesting to see if this phylogeographic pattern persists and use this and other relevant groups to investigate whether those two mountain ranges acted as major independent refugia during climate shifts, potentially with the Sabatayn Basin as a hydrological (advancing of sea water through the basin) or climatic barrier.

By elevating *Trachydactylus hajarensis* to the specific status, we add a new species to the already long and varied list of endemic reptiles of the Hajar Mountains, which include one snake (*Echis omanensis*), one endemic genus of lacertid lizards with two species (*Omanosaura jayakari* and *O. cyanura*), four species of the genus *Asaccus* (*A. montanus*, *A. platyrhynchus*, *A. gallagheri* and *A. caudivolvulus*), two species of *Pristurus* (*P. gallagheri* and *P. celerrimus*) and three *Hemidactylus* (*H. luqueorum, H. hajarensis,* and *H. endophis*) (Gardner 2013). Moreover, ongoing phylogeographic research in this mountain range suggests that the levels of genetic variability in some other taxa, including the diurnal gecko *Pristurus rupestris* and the nocturnal geckos of the genera *Ptyodactylus* and *Asaccus* are much higher, with several undescribed species (work in progress). The old age and specific situation of the Hajar Mountains, surrounded by the sea in the northwest, north and east and by a very large arid desert in the south and west (Edgell 2006), have probably played a crucial role in the origin and maintenance of its unique reptile fauna (Arnold and Gallagher 1977). The Hajar Mountains have relatively lower vegetation diversity in terms of number of endemic species compared to the Dhofar Mountains (Ghazanfar 1998), but contain some rather isolated highland areas like the Jebel Akhdar and much lower areas like the Western Hajars with different climatic conditions, which probably played and still play a very important role in the speciation

dynamics of most of the lineages. One of the best examples are the geckos of the genus *Asaccus*, with *A. platyrhynchus* restricted to the Jebel Akhdar massif, *A. caudivolvulus* distributed across the Western Hajars, and the much smaller-sized *A. gallagheri* distributed across the whole mountain range and found in sympatry with the other two species (Arnold and Gardner 1994; Papenfuss et al. 2010). Another endemic *Asaccus* species, *A. montanus*, is restricted to the highlands of the Jebel Akhdar, but, as suggested by phylogenetic analyses using morphological and molecular data, it is most probably the result of an independent colonization from Iran (Arnold and Gardner 1994; Papenfuss et al. 2010). Similar patterns are found in the genus *Pristurus*, with *P. gallagheri* being restricted to the Jebel Akhdar and *P. celerrimus* found in the Jebel Akhdar but also across the Western Hajars and up to the Musandam Peninsula (Arnold 2009), although these two species are not sister taxa (Papenfuss et al. 2009; Badiane et al. 2014). The two *Hemidactylus* endemic to the Hajar Mountains and its foothills (*H. hajarensis* and *H. luqueorum*) present almost non-overlapping distribution ranges within the massif. While *H. luqueorum* is restricted to the highlands of the Jebel Akhdar, its sister species, *H. hajarensis*, is only found in the lowland areas of the Jebel Akhdar and across the Eastern Hajars (Carranza and Arnold 2012).

The present work on *Trachydactylus hajarensis* reveals a high level of genetic diversity in both mtDNA (*12S*) and nDNA (*cmos*) genes (Figs. 3 and 4) and places its intraspecific diversification during the Late Miocene to Pleistocene. The three highly divergent clades reported in the present study are geographically structured along the Hajar Mountains and the total variogram obtained from PHYLIN indicates a pattern of isolation-by-distance (Supplementary Figure S4). The cluster variograms suggested by nonlinear least squares show a low fit for clades 1 and 2 which is likely the result of reduced sampling for these clades that hampers the variogram to depict the spatial dependence for these lineages. An increased future sampling effort would possibly decrease the width of the contact zones and will also clarify the predicted occurrence of each of the three clades with more certainty. The maps of the predicted occurrence (Supplementary Figure S5) depict the spatial pattern of the three clades quite well except in the under sampled eastern border areas of clade 2 near the contact zone with clade three. Clade 3 occupies the largest area of the distribution, whereas clade 1 is restricted to the easternmost part of the Hajar Mountains, as well as Masirah Island. We have only used three main clades in the present PHYLIN sampling scheme while there is

substantial genetic variation within these clades that is not depicted in both the lineage occurrence and contact zone maps. Future work using a broader sampling and a species delimitation framework (e.g. GMYC; Pons et al. 2006) should provide further insights into lineage occupancy while simultaneously narrowing the contact zones between clades. In comparison to many other regions globally, the Hajar Mountains have remained stable throughout the late Quaternary (Supplementary Figure S1) and paleodistribution modelling indicates continued suitability throughout the distribution range of *T. hajarensis*. The continued connection between the ranges of the three clades may have enhanced dispersal (gene flow) throughout the late Quaternary. The predicted contact zones probably result from the older splits of the groups when these lineages originated in allopatry while the maintenance of these contact zones could be the result of reproductive isolation between the clades. Given the distribution of the different clades of *T. hajarensis*, it can be assumed that they originated through allopatric isolation caused by a combination of past geographic and climatic events during the Miocene and Pliocene epoch. A similar pattern of distinctly divergent clades across the Hajar Mountains was already found in *Hemidactylus hajarensis* (Carranza and Arnold 2012), where two clades occur in allopatry in the eastern and central Hajar Mountain ranges. Nevertheless, a case similar to *T. hajarensis* with the presence of three highly divergent clades across the Hajar Mountain range has not been reported yet, but preliminary data on some other groups (Papenfuss et al. 2010), and especially ongoing studies using molecular phylogenies for all the reptile endemics of this massif indicate that this could be a common pattern. Additional studies using other taxa will help to understand the processes that have shaped the distribution patterns of the taxa of the Hajar Mountains.

Although the mitochondrial and nuclear results presented in Figs. 3 and 4 and in Supplementary Table 3 seem to suggest that *T. hajarensis* in the Hajar Mountains can represent a species complex, more detailed phylogenetic and morphological analyses will be necessary to investigate this issue. The most divergent clade (clade 1) includes specimens from the easternmost part of Oman and a population on Masirah Island. These populations seem to be isolated by the Sharqiyah Sands, a sand dune desert with no records of *T. hajarensis* (Arnold 1980; Gallagher and Arnold 1988; Gardner 2013; pers. observ.) According to our dating analyses (Fig. 3) and *p*-distance values (Supplementary Table 3), the three specimens of clade 1 from Masirah Island (locs. 25–27 in Fig. 1) are differentiated from the rest of

specimens from clade 1 (locs. 23–24 in Fig. 1) by a *p*-distance in the *12S* of 0.9%, having diverged around 0.8 Mya (0.2–2.2, 95% HPD), during the Pleistocene. These results, together with the widespread distribution range of *T. hajarensis* within Masirah Island (pers. observ.), suggest that the population from Masirah Island may have been established by natural colonization instead of being the result of a human-mediated introduction (*cf*. to lizards of the genus *Chamaeleo*; Gardner 2013). The LGM model shows unsuitable conditions on Masirah Islands and it is therefore hypothesized that this population persisted in suitable microrefugia and subsequently recolonized most parts of the island. The relative coarse resolution used for SDM has probably resulted in the unsuitability of the island but using finer grained GCM would probably show that heterogeneity in the mountainous areas on Masirah Islands provided temporary microrefugia for *T. hajarensis* and other species (see Hannah et al. 2014). The predictive power of paleodistribution models has been recently criticized when applying such models to species that violate the basic assumption of the environment as main driver of their distribution patterns (Tonini et al. 2013). *Trachydactylus hajarensis* is a widespread generalist species that is largely dependent on the presence of rocky substrates in areas with sufficient landscape heterogeneity and precipitation. The climate in most deserts is relatively homogeneous compared to tropic or temperate regions and most reptile species in Oman have mainly specialized in microhabitat use such as sandy vs. rocky plains (Metallinou et al. 2012, 2015). In the lowland regions of Oman, *T. hajarensis* is directly replaced by the competing *B. tuberculatus* and the absence of the latter species on Masirah Island could well explain the continued persistence of populations of the former. Nevertheless, more sampling in northeast Oman will be necessary in order to assess the actual level of genetic differentiation between *T. hajarensis* from this area and from Masirah Island. Other taxa, like for instance the amphibian *Duttaphrynus dhufarensis*, and the reptiles *Telescopus dhara dhara*, *Spalerosophis diadema cliffordii*, *Platyceps rhodorachis*, seem to present a similar disjunct distribution to *T. hajarensis* with a hiatus between Masirah Island and the easternmost Hajar Mountains (Gardner 2013). Geomorphological studies and dating estimates of the presence of endemic sand dune specialist taxa like *Stenodactylus sharqiyahensis* suggest that the Sharqiyah Sands have been in place for a relatively long period, which could date back to the Middle Miocene to Plio-Pleistocene (Radies et al. 2004; Metallinou and Carranza 2013). If the connection between the populations of *T. hajarensis* from the Hajar Mountains and Masirah Island had been cut off at that time, we would expect

that the level of genetic variability would be maintained despite increased sampling in extreme northeastern Oman.

The present study indicates that the combination of morphology, molecular phylogenetics, paleodistribution modelling and phylogeographic interpolation are very powerful tools for use in taxonomy, biogeography and phylogeography. Future studies comparing the patterns of the several endemic reptiles of the geologically complex Hajar Mountains using an integrative approach that combines spatial modelling and genetics will be very valuable in order to obtain a better understanding of the patterns and processes that have shaped their unique diversity and distribution.

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**Table S1.** Specimens included in the molecular analyses. For all 34 specimens newly sequenced in the present study we provide their taxonomic identification, sample code, voucher reference, corresponding geographical distribution data (latitude, longitude and country), locality code as shown in the map from Fig. 1 and GenBank accession numbers. For the specimens not sequenced in the present study we only provide the GenBank accession numbers for the genes used and in one case that all the genes correspond to the same specimen we also provide the country. IBE: Institute of Evolutionary Biology, Barcelona, Spain; JEM: Department of Ecology, Charles University in Prague, Czech Republic; NMP: National Museum, Prague, Czech Republic; TMHC: Tomas Mazuch Herpetological Collection, Czech Republic.

| Species | Sample code | Voucher | Lat. | Long. | Country | Locality code | *12S* | *cmos* | *rag1* | *rag2* | *acm4* | *pdc* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Agamura persica* | | | | | | | DQ852726 | JQ945528 | JQ945281 | JQ945420 | JQ945634 | JQ945349 |
| *Bunopos blanfordii* | J27 | | 31.583 | 37.25 | Jordan | | To be added | To be added | To be added | To be added | To be added | To be added |
| *Bunopus tuberculatus* | T31 | | 29.633 | 50.433 | Iran | | To be added | To be added | To be added | To be added | To be added | To be added |
| *Bunopus tuberculatus* | | | | | UAE | | - | JQ945535 | JQ945287 | JQ945427 | JQ945641 | JQ945355 |
| *Crossobamon orientalis* | | | | | | | DQ852715 | JQ945547 | JQ945299 | JQ945440 | JQ945653 | JQ945368 |
| *Cyrtopodion scabrum* | | | | | | | EU589172 | HQ426532 | HQ426275 | HQ426448 | HQ426355 | HQ426186 |
| *Hemidactylus brasilianus* | | | | | | | DQ120428 | HQ426523 | EU268290 | HQ426439 | HQ426346 | EU268320 |
| *Hemidactylus haitianus* | | | | | | | DQ120388 | HQ426543 | HM559700 | - | HQ426368 | HM559667 |
| *Tenuidactylus caspius* | | | | | | | EU589164 | JQ945620 | JQ945340 | JQ945514 | JQ945727 | JQ945409 |
| *Tenuidactylus longipes* | | | | | | | EU589170 | JQ945621 | JQ945341 | JQ945515 | JQ945728 | JQ945410 |
| *Trachydactylus spatalurus* | SPAT01 | JEM SPAT01 | 13.877 | 45.800 | Yemen | 30 | To be added | To be added | To be added | - | To be added | To be added |
| *Trachydactylus spatalurus* | SPAT02 | JEM SPAT02 | 13.877 | 45.800 | Yemen | 30 | To be added | To be added | To be added | To be added | To be added | To be added |
| *Trachydactylus spatalurus* | TMHC404 | TMHC 2013.10.404 | 17.0289 | 54.6665 | Oman | 28 | To be added | To be added | To be added | To be added | To be added | To be added |
| *Trachydactylus spatalurus* | TMHC405 | TMHC 2013.10.405 | 16.8844 | 53.7731 | Oman | 29 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN4226 | | 22.107 | 59.357 | Oman | 24 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3853 | | 22.5401 | 59.6408 | Oman | 23 | To be added | To be added | To be added | To be added | To be added | To be added |
| *Trachydactylus hajarensis* | S1755 | | 20.3118 | 58.7366 | Oman | 26 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN677 | | 20.4981 | 58.9306 | Oman | 25 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | S1165 | | 20.2995 | 58.7497 | Oman | 27 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | S7643 | | 23.101 | 57.3496 | Oman | 15 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | AO27 | | 22.905 | 57.53 | Oman | 16 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN686 | IBE CN686 | 22.9492 | 59.1983 | Oman | 17 | To be added | To be added | - | - | - | - |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Trachydactylus hajarensis* | S7161 | | 22.6161 | 59.0937 | Oman | 22 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | S7150 | | 23.1317 | 58.6189 | Oman | 20 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3750 | IBE CN3750 | 23.0865 | 57.6762 | Oman | 17 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | OM100 | NMP 74270/2 | 23.2543 | 57.9315 | Oman | 19 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | OM66 | NMP 74268 | 23.0535 | 57.805 | Oman | 18 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | OM99 | NMP 74270/1 | 23.2543 | 57.9315 | Oman | 19 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3989 | IBE CN3989 | 23.377 | 57.6644 | Oman | 14 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN664 | | 23.1498 | 56.8942 | Oman | 12 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN2575 | | 23.7102 | 56.4431 | Oman | 11 | To be added | To be added | To be added | To be added | To be added | To be added |
| *Trachydactylus hajarensis* | CN681 | | 23.22 | 56.9736 | Oman | 13 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN2641 | | 24.9936 | 56.2167 | UAE | 8 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3433 | | 24.6208 | 56.3398 | Oman | 9 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3970 | IBE CN3970 | 26.0421 | 56.3697 | Oman | 1 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN8706 | | 25.9758 | 56.1504 | Oman | 2 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN8281 | | 25.1823 | 56.229 | UAE | 7 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3986 | IBE CN3986 | 25.459 | 56.1834 | UAE | 5 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN8355 | | 25.7863 | 56.2169 | Oman | 4 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN7658 | | 25.3001 | 56.0453 | UAE | 6 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN3412 | IBE CN3412 | 24.5131 | 56.4634 | Oman | 10 | To be added | To be added | - | - | - | - |
| *Trachydactylus hajarensis* | CN7819 | | 25.8798 | 56.2144 | Oman | 3 | To be added | To be added | - | - | - | - |

**Table S2.** Distribution database of *Trachydactylus hajarensis* with latitude, longitude, as used for species distribution modelling in the present study.

Species,Lat,Long
Trachydactylus_hajarensis,3280202.7761,-7532487.3804
Trachydactylus_hajarensis,3231209.5397,-7554738.8005
Trachydactylus_hajarensis,3064566.1726,-7565693.468
Trachydactylus_hajarensis,3040367.0931,-7581372.7552
Trachydactylus_hajarensis,3257284.7792,-7779619.587
Trachydactylus_hajarensis,3278523.733,-7767290.5958
Trachydactylus_hajarensis,3307176.1835,-7591839.6166
Trachydactylus_hajarensis,3301442.7013,-7742270.7396
Trachydactylus_hajarensis,3308718.4751,-7661615.6955
Trachydactylus_hajarensis,3272691.5366,-7596113.5738
Trachydactylus_hajarensis,3270829.0658,-7804580.0293
Trachydactylus_hajarensis,3302839.2581,-7921388.1982
Trachydactylus_hajarensis,3416116.4791,-7976965.6299
Trachydactylus_hajarensis,3378491.7183,-7937809.956
Trachydactylus_hajarensis,3517020.7973,-7983689.999
Trachydactylus_hajarensis,3458153.1504,-7991511.9652
Trachydactylus_hajarensis,3305635.8531,-7775345.3866
Trachydactylus_hajarensis,3262733.3723,-7857223.229
Trachydactylus_hajarensis,3439570.3321,-8003551.6101
Trachydactylus_hajarensis,3497725.5047,-7997667.1796
Trachydactylus_hajarensis,3433922.4495,-7979941.786
Trachydactylus_hajarensis,3275426.4664,-7823853.9284
Trachydactylus_hajarensis,3338514.0841,-7682654.7623
Trachydactylus_hajarensis,3477600.3992,-7990827.8911
Trachydactylus_hajarensis,3291267.2681,-7609297.6795
Trachydactylus_hajarensis,3320295.9883,-8007284.3403
Trachydactylus_hajarensis,3411615.7907,-8024236.6987
Trachydactylus_hajarensis,3452097.2425,-8012174.5411
Trachydactylus_hajarensis,3323157.9055,-7978542.6578
Trachydactylus_hajarensis,3346418.2113,-7981669.7205
Trachydactylus_hajarensis,3364237.4847,-7985973.6116
Trachydactylus_hajarensis,3493809.9959,-8014532.322
Trachydactylus_hajarensis,3436018.0471,-7998106.4727
Trachydactylus_hajarensis,3489218.9796,-7995232.5976
Trachydactylus_hajarensis,3439245.2708,-7974816.003
Trachydactylus_hajarensis,3385014.7954,-7954337.927
Trachydactylus_hajarensis,3466674.377,-7973499.8413
Trachydactylus_hajarensis,3517020.7973,-7983689.999
Trachydactylus_hajarensis,3302839.2581,-7921388.1982
Trachydactylus_hajarensis,3289098.8539,-7899106.519
Trachydactylus_hajarensis,3262733.3723,-7857223.229
Trachydactylus_hajarensis,3275225.205,-7824044.3434
Trachydactylus_hajarensis,3296554.885,-7814819.119
Trachydactylus_hajarensis,3270829.0658,-7804580.0293
Trachydactylus_hajarensis,3196399.1115,-7786227.8985
Trachydactylus_hajarensis,3257284.7792,-7779619.587
Trachydactylus_hajarensis,3295202.291,-7786517.7345
Trachydactylus_hajarensis,3275620.9311,-7774498.5987
Trachydactylus_hajarensis,3278961.3423,-7751956.8365
Trachydactylus_hajarensis,3320673.8755,-7757579.3045
Trachydactylus_hajarensis,3298636.0963,-7744953.2282
Trachydactylus_hajarensis,3338471.1687,-7725057.3169
Trachydactylus_hajarensis,3344974.3221,-7684623.2602
Trachydactylus_hajarensis,3315131.9735,-7663388.6481
Trachydactylus_hajarensis,3031202.6721,-7591006.1892
Trachydactylus_hajarensis,3056671.0326,-7585256.6537
Trachydactylus_hajarensis,3081693.6093,-7573436.2121
Trachydactylus_hajarensis,3064566.1726,-7565693.468
Trachydactylus_hajarensis,3257476.7444,-7603676.258
Trachydactylus_hajarensis,3291267.2681,-7609297.6795
Trachydactylus_hajarensis,3316993.3582,-7613988.135
Trachydactylus_hajarensis,3308929.8422,-7596501.7468
Trachydactylus_hajarensis,3231209.5397,-7554738.8005
Trachydactylus_hajarensis,3280202.7761,-7532487.3804
Trachydactylus_hajarensis,3256781.5692,-7507920.8667

**Table S3**. *12S* uncorrected genetic distances (*p*-distances; complete deletion) between all the specimens of *Trachydactylus spatalurus* and *Trachydactylus hajarensis* included in the present study. Colors are according to the different clades highlighted in Figure 3. In red: *Trachydactylus spatalurus*; dark blue, clade 1 of *Trachydactylus hajarensis*; blue, clade 2 of *Trachydactylus hajarensis*; light blue, clade 3 of *Trachydactylus hajarensis*. Genetic distances are given in % and are shown below the diagonal. The Standard Errors are shown above the diagonal.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TMHC404 | | 0.5 | 0.8 | 0.8 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| 2 | TMHC405 | 0.9 | | 0.9 | 0.9 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| 3 | SPAT01 | 2.3 | 3.2 | | 0.0 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.7 | 1.7 | 1.6 | 1.7 | 1.7 | 1.7 | 1.6 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| 4 | SPAT02 | 2.3 | 3.2 | 0.0 | | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.8 | 1.8 | 1.8 | 1.8 | 1.7 | 1.7 | 1.7 | 1.6 | 1.7 | 1.7 | 1.7 | 1.6 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| 5 | CN4226 | 12.6 | 12.9 | 13.5 | 13.5 | | 0.0 | 0.5 | 0.5 | 0.5 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.2 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 6 | CN3853 | 12.6 | 12.9 | 13.5 | 13.5 | 0.0 | | 0.5 | 0.5 | 0.5 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.2 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 7 | S1755 | 12.3 | 12.6 | 13.5 | 13.5 | 0.9 | 0.9 | | 0.0 | 0.0 | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 8 | CN677 | 12.3 | 12.6 | 13.5 | 13.5 | 0.9 | 0.9 | 0.0 | | 0.0 | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 9 | S1165 | 12.3 | 12.6 | 13.5 | 13.5 | 0.9 | 0.9 | 0.0 | 0.0 | | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.2 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 10 | S7643 | 14.0 | 14.0 | 14.3 | 14.3 | 7.0 | 7.0 | 7.3 | 7.3 | 7.3 | | 0.0 | 0.9 | 0.8 | 0.8 | 0.9 | 0.9 | 0.9 | 0.9 | 1.1 | 1.1 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 11 | AO27 | 14.0 | 14.0 | 14.3 | 14.3 | 7.0 | 7.0 | 7.3 | 7.3 | 7.3 | 0.0 | | 0.9 | 0.8 | 0.8 | 0.9 | 0.9 | 0.9 | 0.9 | 1.1 | 1.1 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 12 | CN686 | 12.6 | 12.6 | 13.5 | 13.5 | 6.4 | 6.4 | 6.7 | 6.7 | 6.7 | 3.2 | 3.2 | | 0.6 | 0.5 | 0.6 | 0.6 | 0.7 | 0.7 | 1.2 | 1.2 | 1.2 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 13 | S7161 | 13.2 | 13.2 | 13.5 | 13.5 | 6.4 | 6.4 | 6.7 | 6.7 | 6.7 | 2.9 | 2.9 | 1.2 | | 0.3 | 0.4 | 0.4 | 0.5 | 0.4 | 1.2 | 1.2 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 14 | S7150 | 12.9 | 12.9 | 13.2 | 13.2 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 2.9 | 2.9 | 0.9 | 0.3 | | 0.3 | 0.4 | 0.5 | 0.4 | 1.2 | 1.2 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 15 | CN3750 | 12.6 | 12.6 | 13.5 | 13.5 | 5.8 | 5.8 | 6.1 | 6.1 | 6.1 | 3.2 | 3.2 | 1.2 | 0.6 | 0.3 | | 0.5 | 0.5 | 0.5 | 1.2 | 1.2 | 1.2 | 1.1 | 1.4 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 16 | OM100 | 13.5 | 13.5 | 13.7 | 13.7 | 6.7 | 6.7 | 7.0 | 7.0 | 7.0 | 3.2 | 3.2 | 1.5 | 0.9 | 0.6 | 0.9 | | 0.5 | 0.4 | 1.2 | 1.2 | 1.2 | 1.2 | 1.4 | 1.4 | 1.3 | 1.4 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 17 | OM66 | 13.7 | 13.7 | 14.0 | 14.0 | 6.7 | 6.7 | 7.0 | 7.0 | 7.0 | 3.2 | 3.2 | 1.8 | 1.2 | 0.9 | 1.2 | 0.9 | | 0.3 | 1.2 | 1.1 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 18 | OM99 | 13.5 | 13.5 | 13.7 | 13.7 | 6.7 | 6.7 | 7.0 | 7.0 | 7.0 | 2.9 | 2.9 | 1.5 | 0.9 | 0.6 | 0.9 | 0.6 | 0.3 | | 1.2 | 1.1 | 1.1 | 1.1 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| 19 | CN3989 | 12.6 | 12.6 | 12.6 | 12.6 | 6.7 | 6.7 | 7.0 | 7.0 | 7.0 | 5.3 | 5.3 | 5.8 | 5.6 | 5.6 | 5.8 | 6.1 | 5.6 | 5.6 | | 0.6 | 0.6 | 0.6 | 0.8 | 0.8 | 0.7 | 0.8 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| 20 | CN664 | 13.2 | 13.2 | 12.6 | 12.6 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 5.3 | 5.3 | 5.8 | 5.6 | 5.6 | 5.8 | 6.1 | 5.6 | 5.6 | 1.5 | | 0.3 | 0.4 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| 21 | CN2575 | 12.9 | 12.9 | 12.3 | 12.3 | 5.8 | 5.8 | 6.1 | 6.1 | 6.1 | 5.0 | 5.0 | 5.6 | 5.3 | 5.3 | 5.6 | 5.8 | 5.3 | 5.3 | 1.2 | 0.3 | | 0.3 | 0.8 | 0.8 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| 22 | CN681 | 13.2 | 13.2 | 12.6 | 12.6 | 5.6 | 5.6 | 5.8 | 5.8 | 5.8 | 4.7 | 4.7 | 5.3 | 5.0 | 5.0 | 5.3 | 5.6 | 5.0 | 5.0 | 1.5 | 0.6 | 0.3 | | 0.8 | 0.8 | 0.7 | 0.8 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| 23 | CN2641 | 12.6 | 12.6 | 12.6 | 12.6 | 6.4 | 6.4 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 7.0 | 6.7 | 6.7 | 7.0 | 7.3 | 6.7 | 6.7 | 2.3 | 2.3 | 2.0 | 2.3 | | 0.0 | 0.4 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 24 | CN3433 | 12.6 | 12.6 | 12.6 | 12.6 | 6.4 | 6.4 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 7.0 | 6.7 | 6.7 | 7.0 | 7.3 | 6.7 | 6.7 | 2.3 | 2.3 | 2.0 | 2.3 | 0.0 | | 0.4 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 25 | CN3970 | 12.6 | 12.6 | 12.6 | 12.6 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.6 | 0.6 | | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 26 | CN8706 | 12.0 | 12.0 | 12.0 | 12.0 | 6.4 | 6.4 | 6.7 | 6.7 | 6.7 | 6.7 | 6.7 | 7.0 | 6.7 | 6.7 | 7.0 | 7.3 | 6.7 | 6.7 | 2.3 | 2.3 | 2.0 | 2.3 | 0.6 | 0.6 | 0.6 | | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| 27 | CN8281 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 28 | CN3986 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | | 0.0 | 0.0 | 0.0 | 0.0 |
| 29 | CN8355 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | | 0.0 | 0.0 | 0.0 |
| 30 | CN7658 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | | 0.0 | 0.0 |
| 31 | CN3412 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | | 0.0 |
| 32 | CN7819 | 12.3 | 12.3 | 12.3 | 12.3 | 6.1 | 6.1 | 6.4 | 6.4 | 6.4 | 6.4 | 6.4 | 6.7 | 6.4 | 6.4 | 6.7 | 7.0 | 6.4 | 6.4 | 2.0 | 2.0 | 1.8 | 2.0 | 0.3 | 0.3 | 0.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | |

## Supporting information

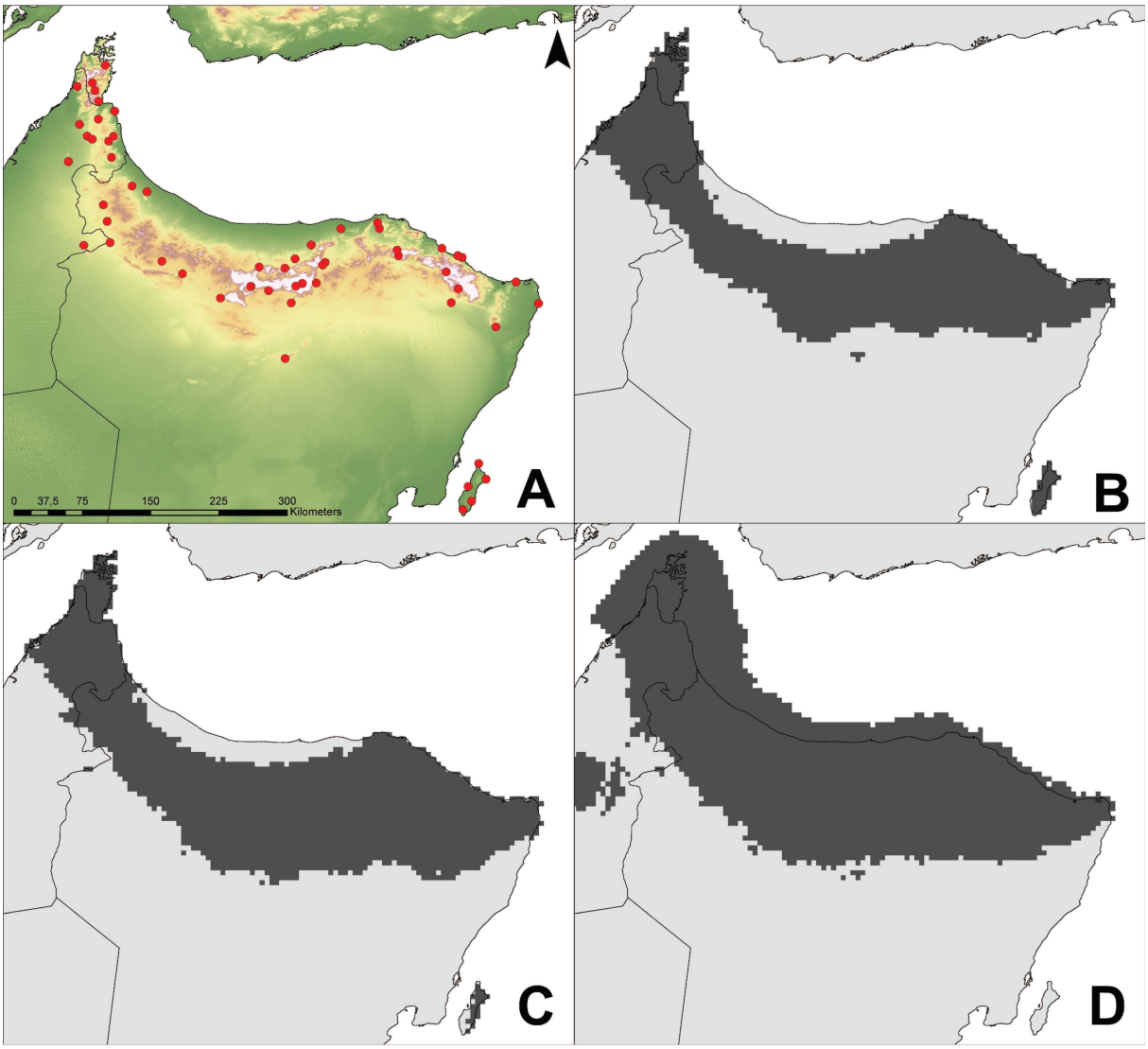


**Figure S1.** Potential species distribution models of *Trachydactylus hajarensis*. A) The available distribution records used for SDM, B) present, C), Mid-Holocene and D) Last Glacial Maximum. All models are above the MTSPS threshold.

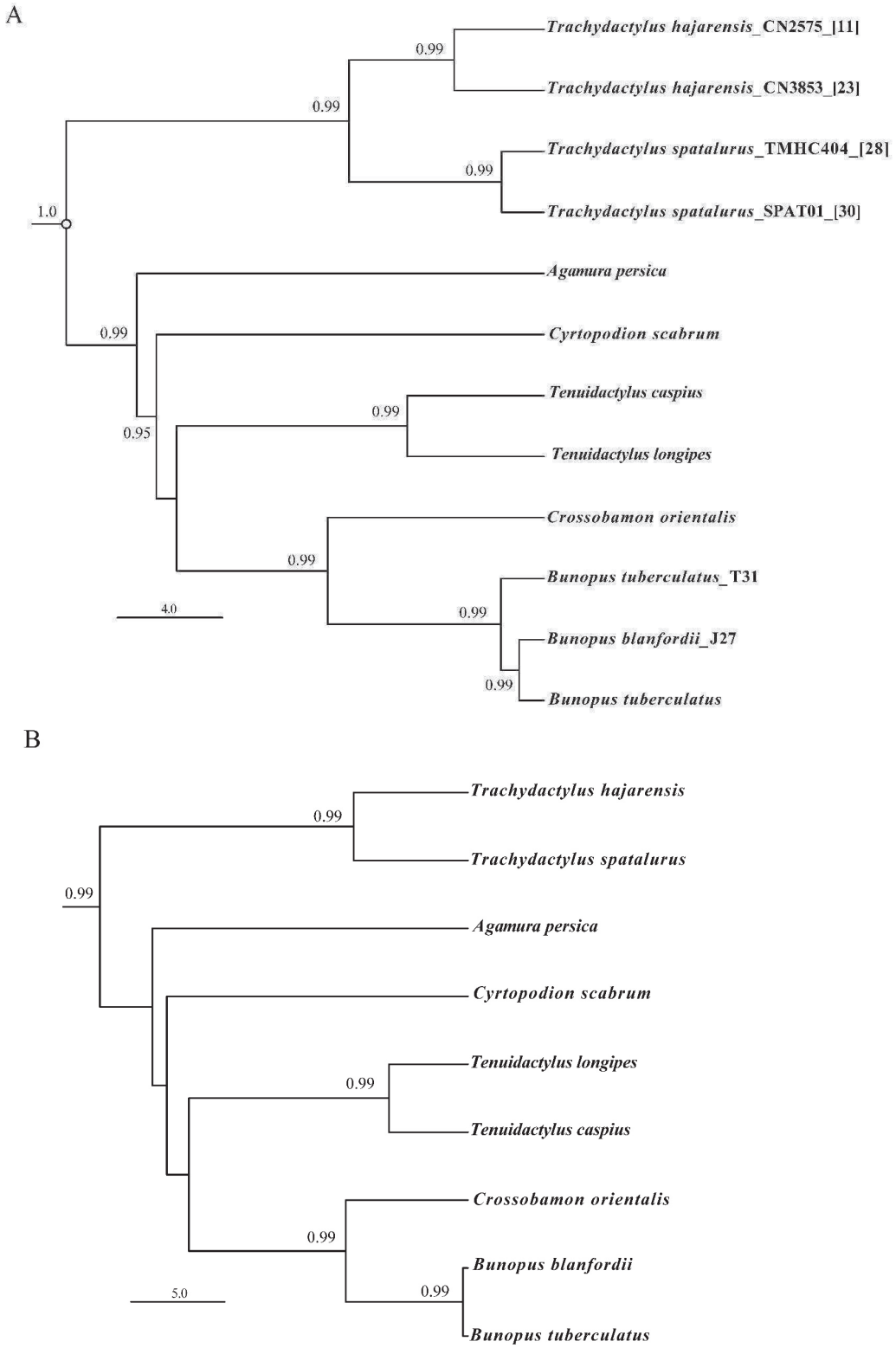


**Figure S2**. A) Bayesian tree inferred using BEAST on dataset 1 (*12S, cmos, rag1, rag2, acm4*, and *pdc* genes); B) species tree inferred with *BEAST using dataset 1. The two *Hemidactylus* used to root the tree are not shown. Empty circles indicate pp > 0.95. Taxon names correspond to changes proposed in this paper.

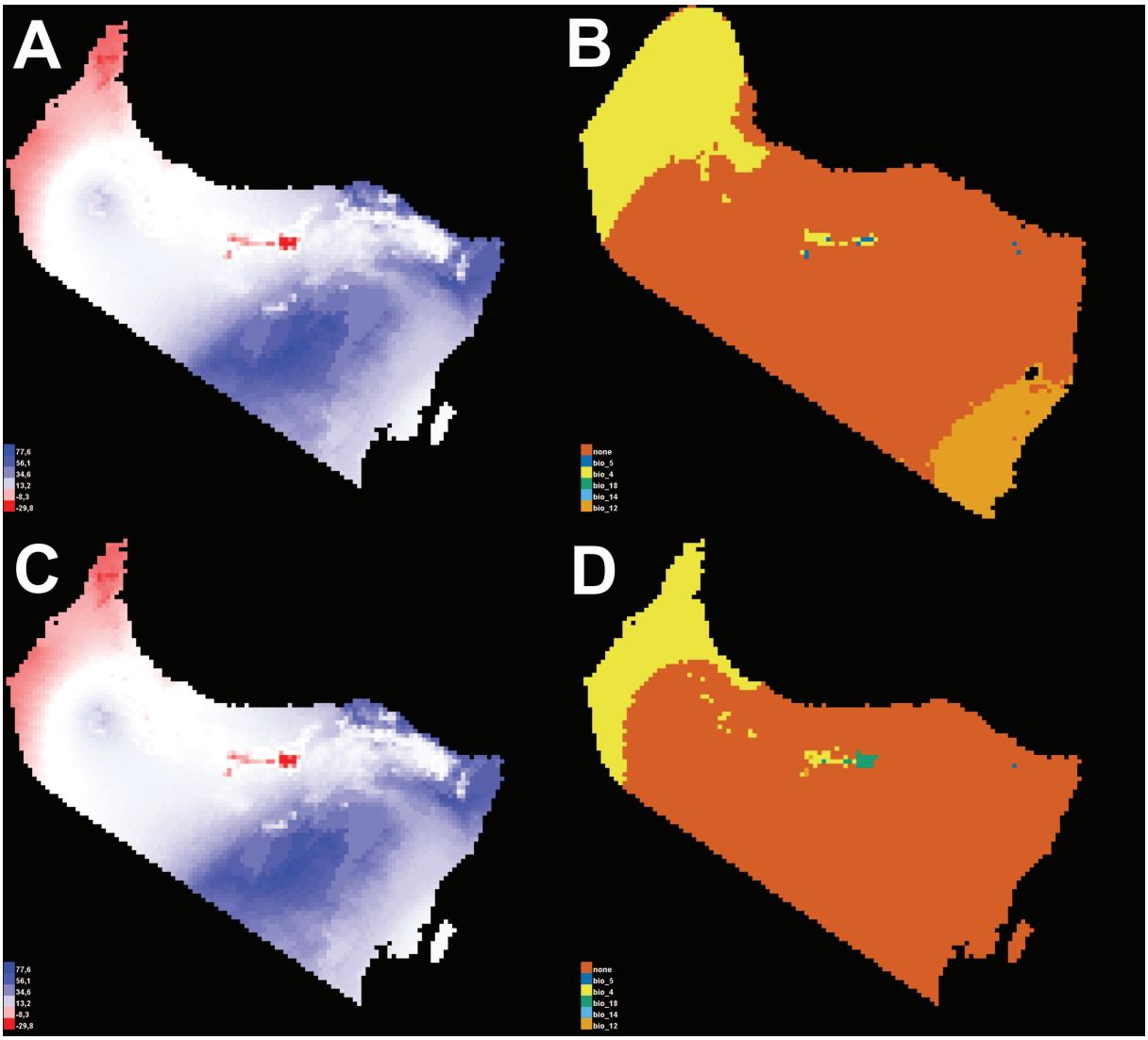


**Figure S3**. MESS (A and C) and MoD (B and D) pictures of the projected SDMs for the (A-B) Mid-Holocene and (C-D) Last Glacial Maximum.

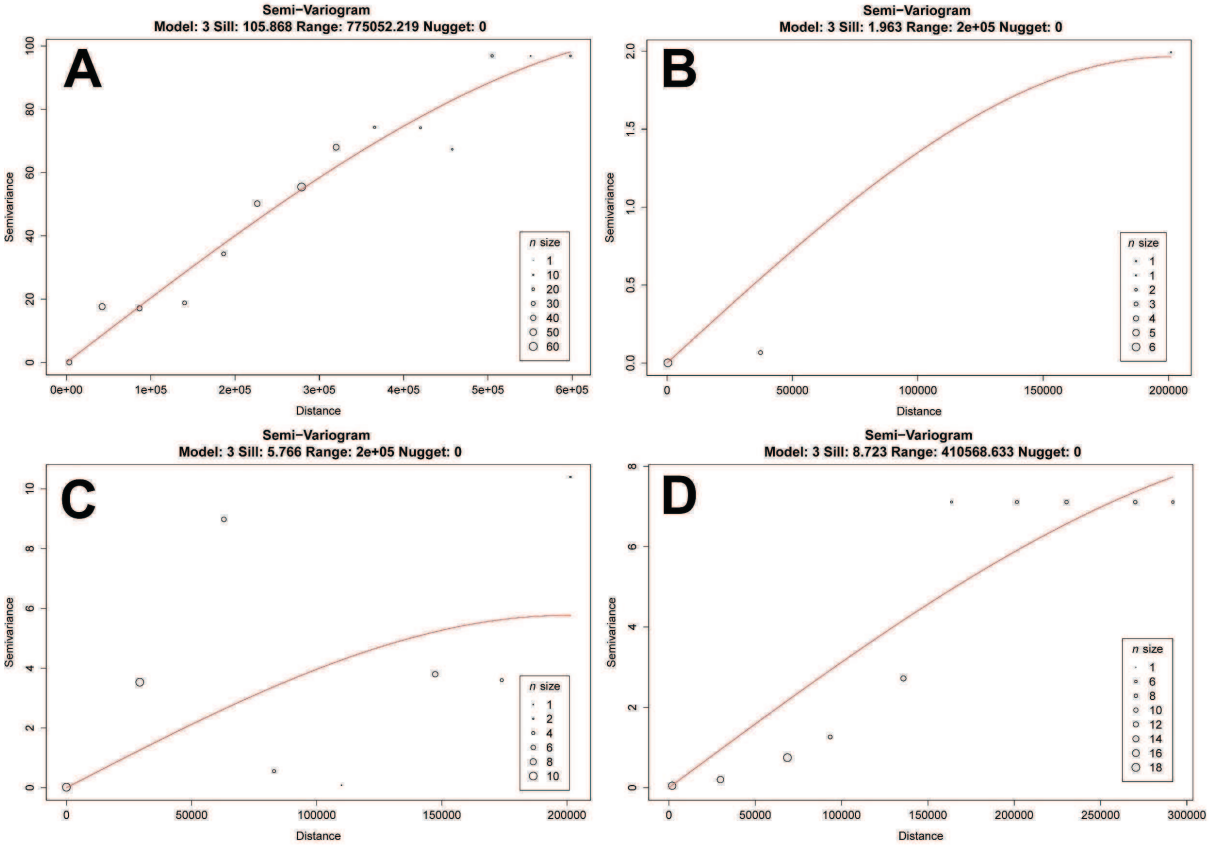


**Figure S4**. Total (A) and cluster variograms (B clade 1, C clade 2 and D clade 3) with fitted models. The number of pairwise samples within each distance class is represented with different circle size.

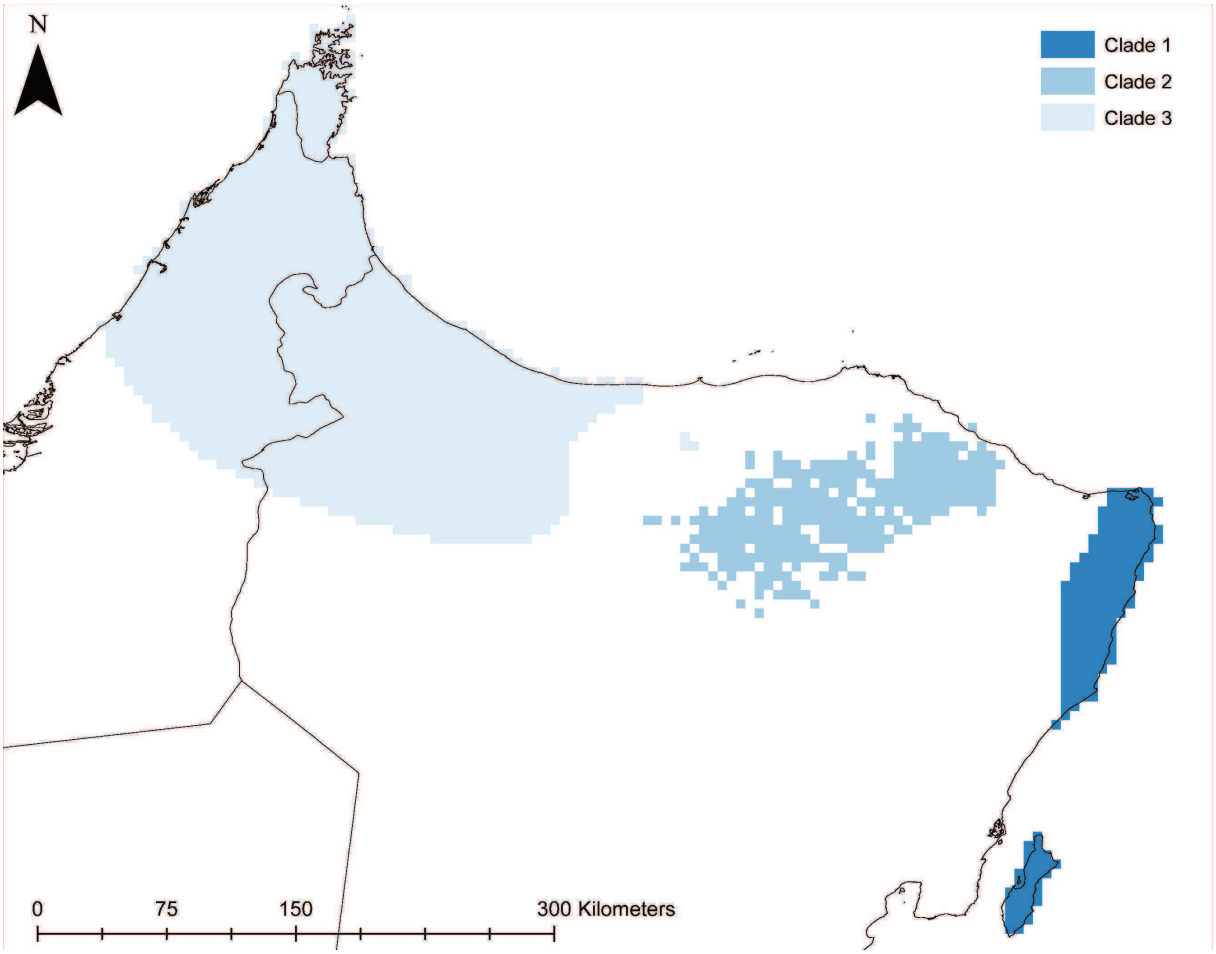


**Figure S5**. Predicted occurrence of the three main clades of *Trachydactylus hajarensis* using phylogeographic interpolation using the PHYLIN R package

# Chapter 6

## Range contraction and loss of genetic variation of the Pyrenean endemic newt *Calotriton asper* due to climate change

Journal: Published (early view) in Regional Environmental Change

Authors: Philip de Pous, Albert Montori, Fèlix Amat and Delfi Sanuy

Many studies have identified climate warming to be among the most important threats to biodiversity. Climate change is expected to have stronger effects on species with low genetic diversity, ectothermic physiology, small-ranges, low effective populations sizes, specific habitat requirements and limited dispersal capabilities. Despite an ever increasing number of studies reporting climate change induced range shifts, few of these have incorporated species' specific dispersal constraints into their models. Moreover, the impacts of climate change on genetic variation within populations and species have rarely been assessed while this is a promising direction for future research.

Here we explore the effects of climate change on the potential distribution and genetic variation of the endemic Pyrenean newt *Calotriton asper* over the period 2020-2080. We use species distribution modelling in combination with high-resolution gridded climate data while subsequently applying four different dispersal scenarios. We furthermore use published data on genetic variation of both mtDNA and AFLP loci to test if populations with high genetic diversity (nucleotide diversity and expected heterozygosity) or evolutionary history (unique haplotypes and *K* clusters have an increased extinction risk from climate change.

The present study indicates that climate change drastically reduces the potential distribution range of *C. asper*, and reveals dispersal possibilities to be minimal under the most realistic dispersal scenarios. Despite the major loss in suitable climate, the models highlight relative large stable areas throughout the species core distribution area indicating persistence of populations over time. The results, however, show a major loss of genetic diversity and evolutionary history. This highlights the importance of accounting for intraspecific genetic variation in climate change impact studies. Likewise, the integration of species' specific

dispersal constraints into projections of species distribution models is an important step to fully explore the effects of climate change on species potential distributions.

**Author contribution**: First authorship reflects that I was the main contributor to the paper. I have developed the concept, written the first draft and I conducted all analyses.

Reg Environ Change
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ORIGINAL ARTICLE

# Range contraction and loss of genetic variation of the Pyrenean endemic newt *Calotriton asper* due to climate change

**Philip de Pous[1,2] · Albert Montori[3] · Fèlix Amat[4] · Delfí Sanuy[1]**

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**Abstract** Many studies have identified climate warming to be among the most important threats to biodiversity. Climate change is expected to have stronger effects on species with low genetic diversity, ectothermic physiology, small ranges, low effective populations sizes, specific habitat requirements and limited dispersal capabilities. Despite an ever-increasing number of studies reporting climate change-induced range shifts, few of these have incorporated species' specific dispersal constraints into their models. Moreover, the impacts of climate change on genetic variation within populations and species have rarely been assessed, while this is a promising direction for future research. Here we explore the effects of climate change on the potential distribution and genetic variation of the endemic Pyrenean newt *Calotriton asper* over the period 2020–2080. We use species distribution modelling in combination with high-resolution gridded climate data while subsequently applying four different dispersal scenarios. We furthermore use published data on genetic variation of both mtDNA and AFLP loci to test whether populations with high genetic diversity (nucleotide diversity and expected heterozygosity) or evolutionary history (unique haplotypes and *K* clusters) have an increased extinction risk from climate change. The present study indicates that climate change drastically reduces the potential distribution range of *C. asper* and reveals dispersal possibilities to be minimal under the most realistic dispersal scenarios. Despite the major loss in suitable climate, the models highlight relatively large stable areas throughout the species core distribution area indicating persistence of populations over time. The results, however, show a major loss of genetic diversity and evolutionary history. This highlights the importance of accounting for intraspecific genetic variation in climate change impact studies. Likewise, the integration of species' specific dispersal constraints into projections of species distribution models is an important step to fully explore the effects of climate change on species potential distributions.

**Keywords** Amphibian · Maxent · Fragmentation · Conservation · Species distribution modelling · Dispersal

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## Introduction

Climate change has affected and changed global biodiversity throughout Earth's history. Although rapid climatic changes have occurred in the past, current climate warming is occurring very rapidly by comparison, and recent studies have identified climate warming to be among the most

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important threats to biodiversity (e.g. Parmesan and Yohe 2003; Root et al. 2003; Thomas et al. 2004). Climate change is predicted to have effects on different levels and components of biodiversity. At the species level, climate change has been shown to alter physiology, distribution, phenology, behaviour and ecological interactions (Hughes 2000; McCarty 2001; Walther et al. 2002; Root et al. 2003; Parmesan 2006; Bellard et al. 2012). In fact, climate change is causing many species to shift their geographical ranges and phenologies at faster rates than previously expected (Staudinger et al. 2012). However, these rates are not uniform across species as climate change is expected to have stronger effects on species with low genetic diversity, ectothermic physiology, small ranges, low effective populations sizes, specific habitat requirements and limited dispersal capabilities (e.g. Thomas et al. 2004). Isolated populations are frequently at higher risk of extinction and have reduced genetic diversity that may reduce their adaptive potential (Leimu et al. 2010). Species that are able to disperse across fragmented landscapes may be stressed by translocation, reestablishment of territory and increased competition from other species (Vögeli et al. 2011).

The importance of genetic variation in determining species' ecological and evolutionary responses to climate change is just beginning to be revealed, but genetics and genomics are likely to play a large role in climate change biology in the coming years (Staudinger et al. 2012).

Amphibians are an important component of biodiversity. These species are, however, often underrepresented in conservation planning despite having the highest threat status of all terrestrial vertebrates, with significantly more species at risk than either birds or mammals (Temple and Cox 2009; Foden et al. 2013). Ectotherms, such as amphibians, are particularly suitable model organisms in terms of climate change sensitivity because they largely comply with their surrounding thermal environment (Angilletta 2009). As a result, amphibians are considered to be highly vulnerable to climate change. The effect of climate change on the distribution of European amphibians has been relatively well studied using correlative species distribution models (e.g. Araújo et al. 2006; Carvalho et al. 2010). Notwithstanding the different methodologies and resolutions of these studies, the results show significantly reduced potential distributions for many species, including completely lost ranges already by the year 2050 (see Carvalho et al. 2010). None of these studies, however, explored the consequences of including dispersal constraint in species distribution modelling to assess species' climate change vulnerability, and this remains an important topic for further research.

Genetic variation at the intraspecific level provides the basis for any evolutionary change and is the most fundamental level of biodiversity. In order to fully understand the evolutionary consequences of climate change and its long-term effects on biodiversity, it is necessary to assess the effects of climate change on intraspecific genetic diversity and evolutionary history (Crandall et al. 2000; Fraser and Bernatchez 2001; Moritz 2002; Pauls et al. 2013). The conservation of evolutionary history has received considerable attention, while fewer studies have focused on patterns of intraspecific genetic variation at the population level. For example, Bálint et al. (2011) showed that climate change impacts are drastically underestimated without accounting for intraspecific genetic variation and cryptic diversity. It is therefore not surprising that the incorporation of genetic diversity into climate change impact studies is considered an important and promising research topic that requires further development (Pauls et al. 2013; Anderson 2013; Franklin 2013).

Climate-induced range shifts and population declines are expected to increase the prevalence of population bottlenecks and reduce genetic diversity within and among species. Long-lived species are particularly vulnerable to climate changes because they experience longer generation times, lower population turnover rates and slower rates of evolution (Staudinger et al. 2012).

The Pyrenean brook newt *Calotriton asper* is a well-known example of a long-lived species (see Montori 1990; Miaud and Guillaume 2005). It is distributed along the Pyrenean mountain range (Sillero et al. 2014; France, Spain and Andorra) where it inhabits torrents, streams and mountain lakes, while occasionally occurring in caves systems. Over the years, *C. asper* has received considerable attention in the scientific literature and there is an increasing understanding of the species' natural history (e.g. Böhme et al. 1999; Miaud and Guillaume 2005; Montori et al. 2008a, 2012; Colomer et al. 2014; Oromi et al. 2014). There is still a limited understanding of the genetic structure of *C. asper*, and the available studies used a variety of genetic markers that showed contrasting results of genetic structuring (Carranza and Amat 2005; Montori et al. 2008b; Milá et al. 2010; Valbuena-Ureña et al. 2012). As an example, Milá et al. (2010) reported high genetic structuring using genome-wide AFLP but low genetic diversity for the mtDNA sequences. The latter pattern was also found by Valbuena-Ureña et al. (2012) who sequenced a large number of samples for mtDNA from the entire distribution range, as well as a smaller number of nuclear DNA samples. The results of the genome-wide AFLP analysis indicate extreme dispersal limitation in *C. asper*, which is corroborated by capture–recapture data that indicate a sedentary lifestyle (Montori et al. 2008a).

Araújo et al. (2006, 2011) and Carvalho et al. (2010) studied the effects of climate change on the distribution of *C. asper* and reported contrasting results ranging from an expanded distribution under unlimited dispersal to a complete loss of the distribution range by 2050. The reported

differences between these studies likely result from the use of different time periods, global circulation models (GCM), emission scenarios, climate resolutions, distribution sources and modelling techniques, as these are known to influence the results of correlative models (e.g. Heikkinen et al. 2006; Beaumont et al. 2008). Another important aspect in climate change studies relies on the dispersal ability of the modelled organism. All currently available studies that included *C. asper* applied the unrealistic unlimited and no dispersal scenarios, while it is known that especially partial dispersal models improve projections of altered distributions under climate change (Bateman et al. 2013). Assessing species abilities to disperse has been identified as a major decisive parameter in determining species resilience to climate change, especially in organisms with low vagility such as amphibians (Wells 2007). Despite the increasing number of available tools, few studies have integrated dispersal constraints into projections of species distribution models.

The Pyrenees are an interesting region to study the effects of climate change on biodiversity for several reasons. First of all, various studies have already revealed current climate and environmental changes throughout the region. As an example, López-Moreno et al. (2008a) showed that changes in precipitation, temperature and snow accumulation, together with an increase in vegetation density in headwater regions due to abandonment of traditional land uses such as grazing, have led to a marked reduction in water availability in the region. Second, future climate change predictions indicate major changes in precipitation, temperature and thickness and duration of snowpack in the next century (López-Moreno et al. 2008b, 2009). Third, research indicates that several endemic species with restricted ranges, which are primary targets for conservation efforts, will completely lose their distribution ranges already in 2050 (Carvalho et al. 2010). Altogether, these changes may increase the hydric stress on aquatic ecosystems. Our study species, *C. asper*, depends on streams and lakes for survival, and climate-induced changes in hydrology are potentially one of the greatest threats to most aquatic-breeding amphibians. For example, Montori et al. (2012) and Colomer et al. (2014) showed major population decreases following flooding due to extreme rainfall events.

The present study extends on the previous works by Araujo et al. (2006) and Carvalho et al. (2010) and aims to explore for the first time the effects of climate change on the potential distribution, genetic diversity and evolutionary history of *C. asper* over the period 2020–2080. To this aim, we use an integrative approach that combines species distribution modelling with high-resolution gridded climate data while subsequently applying four different dispersal scenarios. We furthermore use published data to test whether populations with high genetic diversity or evolutionary history have an increased extinction risk from climate change.

## Materials and methods

### Distribution data

A total of 1682 distribution records of *C. asper* were assembled from databases and the literature (supplementary information Table S1 in File S1). The distribution records went through a process of filtering to remove duplicate records within unique grid cells in ENMtools 1.3 (Warren et al. 2010) and to reduce sampling bias by using a kernel density grid as implemented in the Java program OccurrenceThinner version 1.0.4 (Verbruggen et al. 2013). After filtering, the final dataset consisted of 514 distribution records (Fig. 1).

### Climate data and variable selection

All present bioclimatic variables were downloaded from the WorldClim database version 1.4 (Hijmans et al. 2005). Three global circulation models (GCM: CCCMA, HADCM3 and CSIRO) and a single IPCC emission scenario (SRES A2; "business-as-usual scenario") were downloaded from http://www.ccafs-climate.org and used to generate scenarios for 2020–2080 with 10-year intervals. All bioclimatic variables were downloaded at a resolution of 30 arc seconds (nearly $1 \times 1$ km). We calculated the average of the three GCM using ArcGIS 10 (ESRI) as this filters out biases of individual models, retaining only those errors that are generally pervasive (Randall et al. 2007), and may compare better with the observed climatology than individual models. Collinearity of the initial variables was measured by Pearson's correlation coefficient in ENMtools 1.3 (Warren et al. 2010). A total of eight variables, all of which had a correlation degree lower than 0.75 (Pearson coefficient), were retained. Selection of predictor variables was based on ecological understanding of the species and represented both temperature variation and precipitation regimes such as precipitation of the wettest month, as suggested to be important for *C. asper* (e.g. Montori et al. 2012). The final set of environmental predictor variables used for SDM consisted of: mean diurnal range (BIO2), isothermality (BIO3), maximum temperature of warmest month (BIO5), minimum temperature of coldest month (BIO6), mean temperature of wettest quarter (BIO8), mean temperature of driest quarter (BIO9), precipitation of wettest month (BIO13) and precipitation seasonality (BIO15).

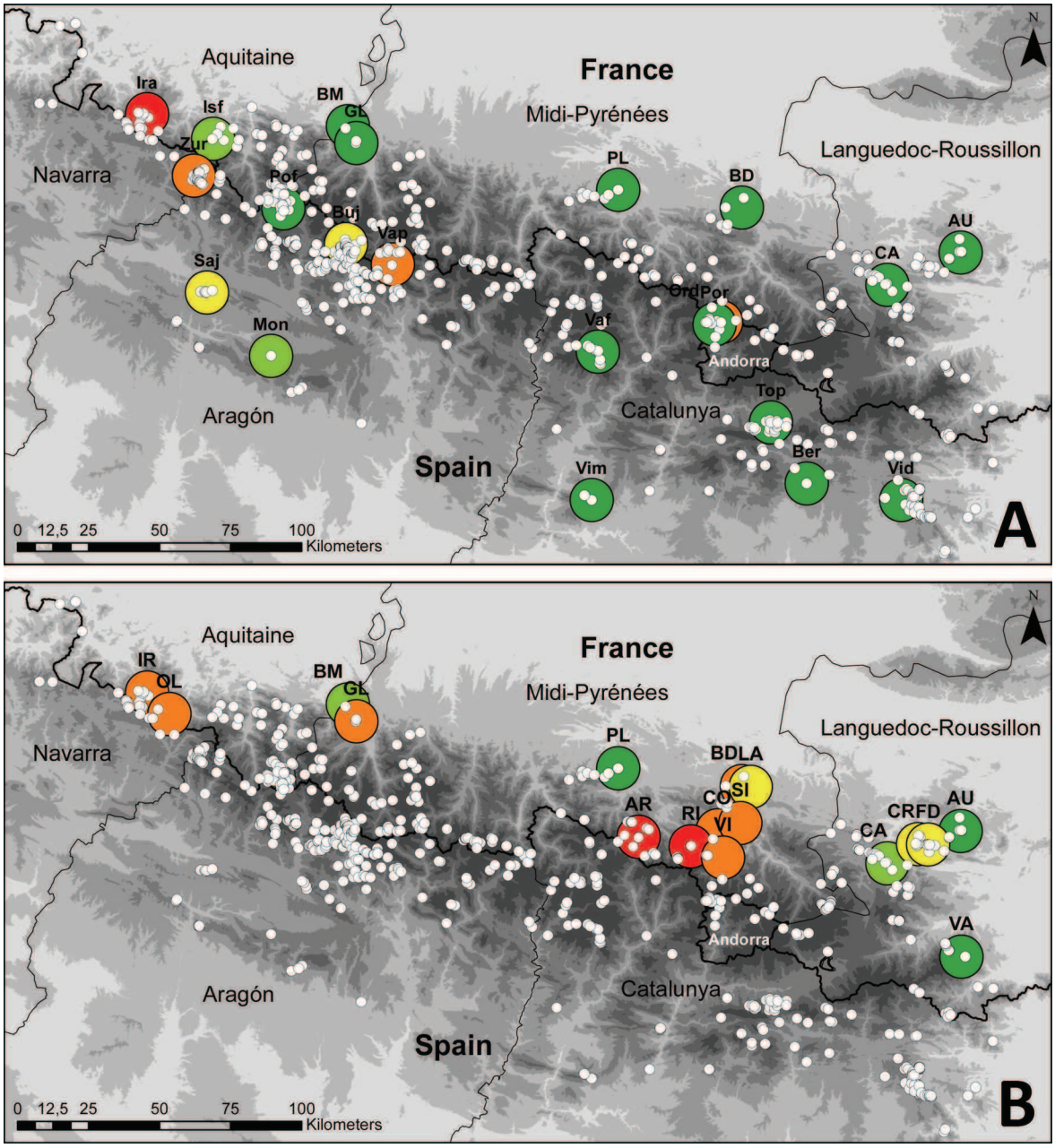


**Fig. 1** The Pyrenees with all distribution records of *Calotriton asper* (*white dots*) used for species distribution modelling. Coloured circles indicate **a** cytochrome *b* nucleotide diversity and **b** mean expected heterozygosity (AFLP) of each population (gradient where green is low and red is high diversity). *Darker grey* colours indicate higher altitude

## Species distribution modelling

The SDMs were generated by the presence/background algorithm Maxent, version 3.3.3 k (Phillips et al. 2006). It has been shown that Maxent produces high quality predictions that are often more successful when evaluated and compared with other predictive models (e.g. Hernandez et al. 2006; Giovanelli et al. 2010). Maxent was used with default settings (convergence threshold = 0.00001, maximum number of iterations = 500 and $\beta_j = 1$) while partitioning the geographical records between training and test samples. We followed the suggestion of VanDerWal et al. (2009) and used an exploratory analysis to define the most appropriate calibration region. Final models were calibrated in a background region defined by a buffer of 200 km that encompassed all known localities and included areas that have been accessible to the species via dispersal over relevant time periods (Merow et al. 2013). This approach has been applied by various authors working with aquatic salamanders in Europe (e.g. Wielstra et al.

2012). Subsequently, models were projected onto a larger area. The average of ten pseudo-replicated models with randomly selected test samples was used to produce SDMs, which were plotted in logistic format. The final models were reclassified in ArcGIS 10 (ESRI) into binary presence–absence maps using the maximum training sensitivity plus specificity threshold (MTSPS), which maximizes the sum of sensitivity (proportion of actual positives that are correctly identified) and specificity (proportion of negatives that are correctly identified) and has been shown to produce highly accurate predictions (Liu et al. 2005; Jiménez-Valverde and Lobo 2007). All models were tested with receiver operating characteristics (ROC) curve plots, which plot the true-positive rate against the false-positive rate. The average area under the curve (AUC) of the ROC plot of ten models was taken as a measure of the overall fit of each model. The threshold-dependent true skill statistic (TSS) was also used for model validation as this method is not influenced by prevalence (Allouche et al. 2006). Although the TSS values were lower compared with the AUC values (see results), they also indicated good to excellent model performance (Coetzee et al. 2009). Additionally, we used null models to test for significance of the SDM (Raes and ter Steege 2007). We generated 100 null distributions of random points in the study area using ENMtools (Warren et al. 2010) with the number of random points equal to the actual number of distribution records used for SDM. The null models were created and assessed following Raes and ter Steege (2007). Comparisons of the environmental variables used for projection to those used for training the model were made using visual interpretation of multivariate similarity surface (MESS) pictures and the most dissimilar variable (MoD) (Elith et al. 2010).

### Incorporating dispersal constraints

We used a cellular automaton model as implemented in the MIGCLIM R package (Engler and Guisan 2009; Engler et al. 2012), in order to incorporate dispersal potential for the period 2020–2080 at the most realistic scale for *C. asper*. MIGCLIM was initialized to model the dispersal over a period of 70 years (present-2080) under four different dispersal scenarios: (1) "unlimited dispersal" (species can disperse to any suitable cell); (2) "no dispersal"; (3) "no dispersal barrier" (species can disperse following the MIGCLIM simulation but are not affected by barriers); and (4) "strong dispersal barrier" (species can disperse following the MIGCLIM simulation but are affected by dispersal barriers such as certain land cover classes). The MIGLIM models were parameterized using the following settings: rcThreshold = 242, envChgSteps = 7, dispSteps = 7, dispKernel = 1, iniMatAge = 2, propaguleProd = 0.01, 0.08, 0.5, 0.92, lddFreq = 0, lddMinDist = 0, lddMaxDist = 0, replicates = 10 (see Engler et al. 2012 and supplementary information Table S2 in File S1). In order to model dispersal at a realistic scale (maximum 100 m per year based on capture–recapture data provided by Montori et al. 2008a), we assumed presence in the entire 1-km cell with predicted presence. All SDMs were therefore downscaled to 100-m resolutions using ArcGIS 10. Barrier cells were constructed using land cover and slope data. European Environment Agency (2013) was obtained at a resolution of 100 m (European Environment Agency) and reclassified in ArcGIS 10 by excluding artificial surfaces and most agricultural areas, while including forest and semi-natural areas and water bodies, based on expert knowledge (see supplementary information Table S3 in File S1). Elevation data were obtained from the ASTER Global Digital Elevation Model (at a resolution of 30 m) and interpolated to 100 m using a bilinear interpolation. Slope was created using the Spatial Analyst toolbox in ArcGIS 10 and reclassified to include cells >40° as barrier cells, based on an exploration of several settings. Subsequently, land cover and slope were merged into a single layer and used as dispersal barrier for the "strong barrier" scenario in MIGCLIM (see scheme in Fig. 2).

### Predicting the impacts of climate change on genetic diversity

A total of 384 DNA sequences of a single mitochondrial gene (cytochrome *b*) were obtained from GenBank (Carranza and Amat 2005; Milá et al. 2010; Valbuena-Ureña et al. 2012) and assembled and edited in Geneious 6.0.6 (Biomatters Ltd.). As a second dataset, we obtained published data on variation in 382 amplified fragment length polymorphism (AFLP) loci (Milá et al. 2010). We used two approaches to assess the impact of climate change on genetic variation. The first method (method A) used cytochrome *b* nucleotide diversity ($\pi$) and mean expected heterozygosity ($H_e$) based on AFLP loci as a measure of population genetic diversity. The second method (method B) used the distribution of unique haplotypes (cytochrome *b*) and optimal *K* of 5 obtained from Milá et al. (2010) as a measure of evolutionary history (Crandall et al. 2000; Frazer and Bernatchez 2001; Moritz 2002). In order to find the optimal *K*, Milá et al. (2010) used the Bayesian assignment probability test implemented in the program STRUCTURE 2.2 (Pritchard et al. 2000). This software uses a Bayesian approach to generate posterior probabilities of assignment of individuals to each of a given number of groups (*K*) independently of the sampling site of origin. Milá et al. (2010) applied a model of no admixture with correlated allele frequencies and calculated the optimal value of *K* following Evanno et al. (2005).

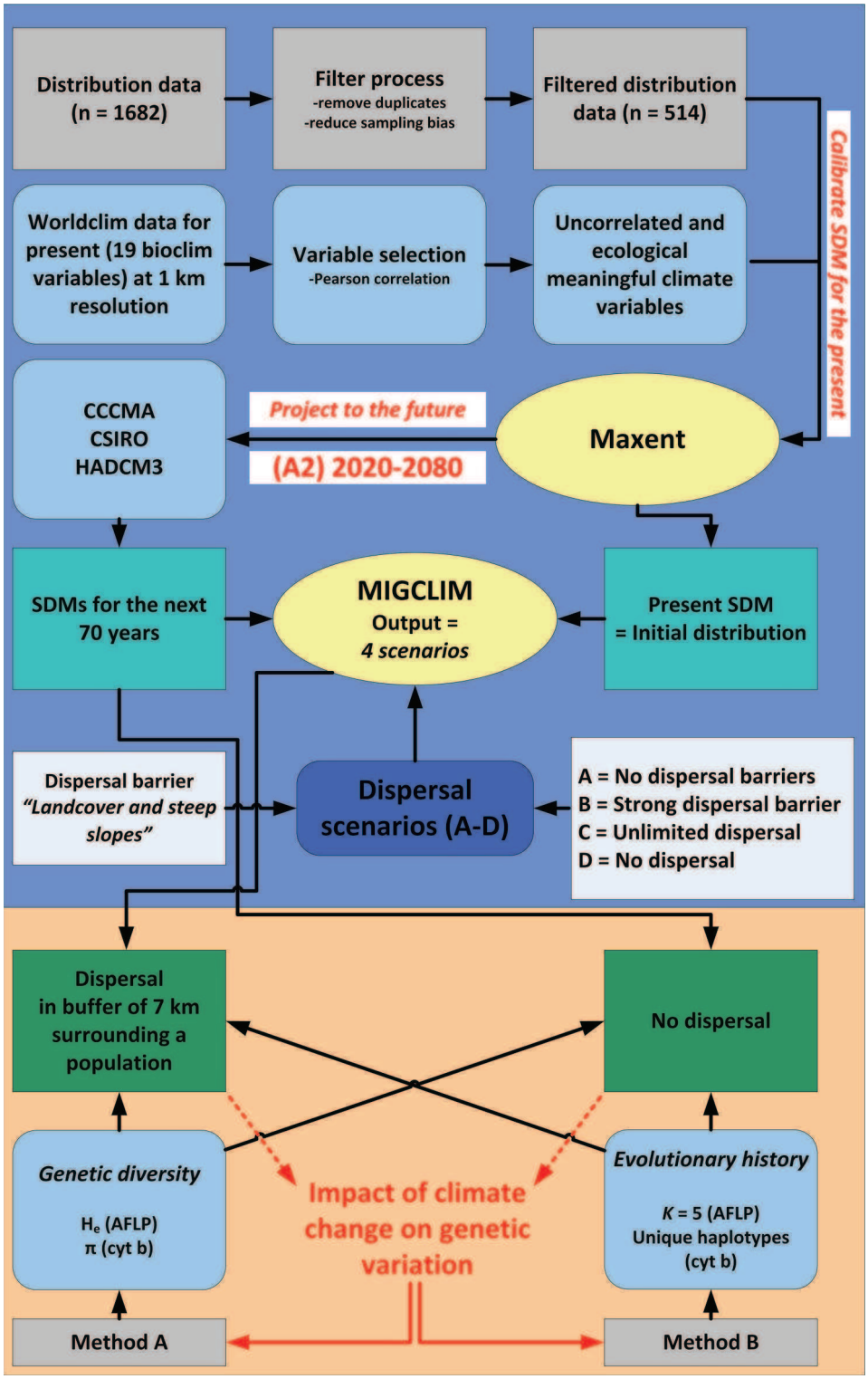


**Fig. 2** A schematic overview of the methodological framework used to study the effects of climate change on genetic variation of the Pyrenean endemic newt *Calotriton asper*

The effect of climate change on genetic variation was assessed using two methods. First we used the coordinates of each population to extract the logistic output values of Maxent for each future SDM at the initial 1-km resolution and assessed whether plotted trend lines drop below the MTSPS threshold obtained from the Maxent output. This method assesses whether genetic diversity decreases simply because suitable areas are lost without dispersal. For the second method, we used a buffer surrounding the populations with genetic diversity (or unique haplotypes)

to account for possible dispersal while assessing the loss of genetic diversity. We assumed a maximum annual dispersal of 100 m per year and extracted the MIGCLIM model output of each dispersal scenario using a buffer of 7 km (70 years times 100 m). Using this buffer, we analysed stability, dispersal potential and the loss of suitable cells to predict the effects of climate change in the 7-km area surrounding populations with genetic variability and/or unique haplotypes. In other words, this method assesses whether genetic diversity decreases because suitable areas are lost but allows for annual dispersal using the four MICCLIM scenarios (see scheme in Fig. 2).

## Results

Maxent produced SDMs of high predictive accuracy (Supplementary information Table S4 in File S1), according to the average test AUC (average AUC = 0.934 ± 0.011, range 0.931–0.938) and TSS values (average TSS = 0.824 ± 0.037, range 0.782–0.863). The SDMs performed statistically significantly better than random (Supplementary information Figure S1). The MESS pictures for the future SDMs revealed non-analogue climatic conditions in large parts of the distribution range (Supplementary information Figure S2). The pictures of the most dissimilar variable (MoD) showed the very low contributing predictor variable isothermality (BIO3) to be furthest outside its training range (Supplementary information Figure S3).

The SDMs reveal continuous decrease in suitable climate over time but show persistence of *C. asper* in large parts of the initial distribution (Figs. 3, 4). The decrease in the number of suitable cells is most prominent under the "no dispersal" scenario (75 %), while the "unlimited dispersal", "strong dispersal barrier" and "no dispersal barrier" scenarios all indicate a similar loss (70 %). There is a significant relationship between the number of suitable cells and the future time periods (2020–2080) under all four dispersal scenarios: "unlimited dispersal": $r = -.91$, $p$ (one tailed) <.001; "no dispersal": $r = -.98$, $p$ (one tailed) <.001; "no dispersal barrier": $r = -.93$, $p$ (one tailed) <.001; and "strong dispersal barrier": $r = -.93$, $p$ (one tailed) <.001. Barrier effects were overall minimal, and the "strong dispersal barrier" and "no dispersal barrier" scenarios had very similar model outcomes (Table 1; Fig. 3).

Method A: the populations with the highest nucleotide diversity are mainly located in the western and central part of the distribution, with the highest nucleotide diversity found in Irati (Fig. 1 and Supplementary information Table S6 in File S1). The expected heterozygosity was overall low with the highest values found in Arcouzan (AR) and Ribaui (RI) and the lowest in the Auriac population (AU). Method B: four haplotypes are restricted to single populations (Ira [h5], Isf [h4], Saj [h6] and Vaf [h7]), while two other haplotypes are only found in two populations (Buj-Vap [h3] and Vap-Zur [h8]). The structure analysis of all individuals resulted in the assignment to an optimal $K$ of 5 (results obtained from Mila et al. 2010); IR and OL $K = 1$, BM and GL $K = 2$, PL K = 3, BD-LA-AR-RI-CO-SI-VI-AU-CA-CR-FD $K = 4$ and VA $K = 5$. These clusters show geographical structure from the west to the east of the Pyrenees (Supplementary information Figure S4).

The SDMs and MIGCLIM models indicate a severe loss of genetic variation as a result of climate change. Using the method assuming no dispersal, 77.8 % of the populations with cytochrome *b* nucleotide diversity ($\pi > 0$) and 88.2 % of all the populations sampled using AFLP loci drop below the MTSPS threshold in at least one of the future models (Fig. 5a, b). The second method, accounting for dispersal in a 7-km buffer surrounding populations, revealed a likely extinction of six (cytochrome *b*; 67 %) and 15 populations (AFLP; 88.2 %), respectively (Fig. 6a, b and Supplementary information Table S7 and S8 in File S1). Importantly, the population with the highest expected heterozygosity (AR) is predicted to persist following climate change. Regarding the evolutionary history, three of the four haplotypes that are unique to a single population (Ira, Isf and Saj) and four ($K = 1$–3 and $K = 5$) of the five $K$ clusters will be lost under scenarios with no or limited dispersal. Only population AR and CO of the relative widespread $K = 4$ will persist. Additionally, there is a major loss of suitable cells with very limited dispersal opportunities in the buffer regions surrounding all populations with genetic data (Supplementary information Table S7 and S8 in File S1), indicating a decreased suitability of the 7-km-wide areas surrounding populations with genetic variation. The minimum dispersal distance for each population to reach a cell with continued suitability is high for both the cytochrome *b* (no dispersal barrier: average dispersal distance 6639 m, range 791–14,573 m; strong dispersal barrier 6769 m, range 1484–14,573 m) and AFLP datasets (no dispersal barrier: average dispersal distance 6567 m, range 573–24,634 m; strong dispersal barrier 6749 m, range 573–27,648 m).

These results indicate a major loss of genetic diversity and loss of evolutionary history in *C. asper* as a result of climate change under multiple dispersal scenarios.

## Discussion

Our study shows that future climate change will decrease the potential distribution range under four different dispersal scenarios. Despite these major losses, the models reveal several areas that remain stable throughout the

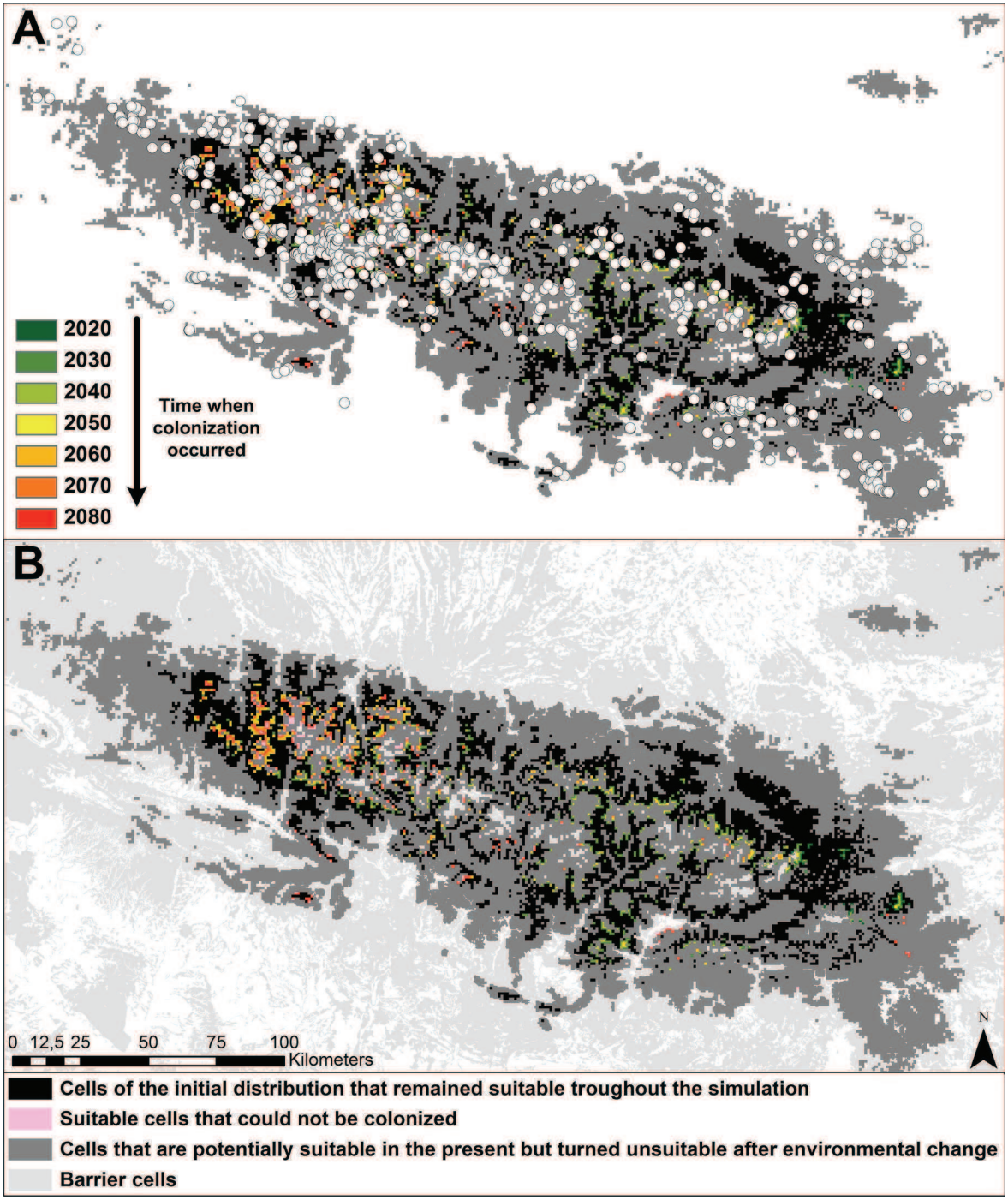


**Fig. 3** Future distribution of *Calotriton asper* under different dispersal scenarios. **a** "no dispersal barrier" and **b** "strong dispersal barrier" scenarios

simulation indicating persistence of *C. asper* over time. Despite the predicted persistence, our results indicate a major loss of genetic diversity and evolutionary history as a result of climate change.

Our study uses a novel integrative approach that has potential applications for amphibian conservation assessments and possibly other organisms. Below we discuss the importance and limitations of our method and provide recommendations for future research.

## Limitations of used methods

The results of this study should be interpreted with caution given the uncertainties in the modelling process: the

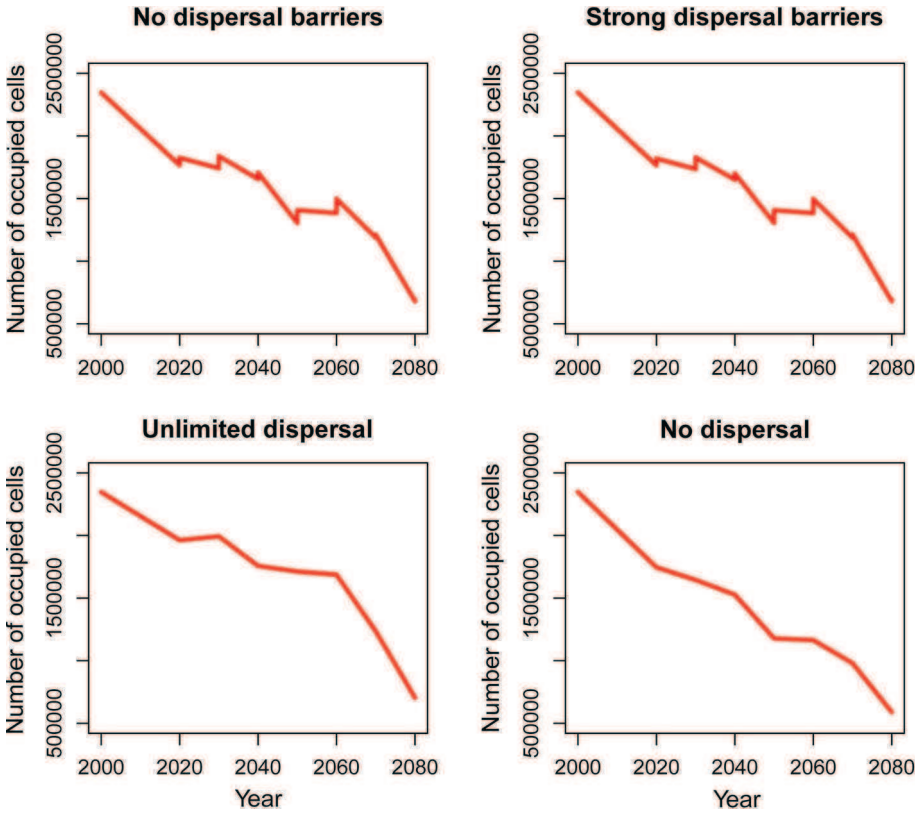


**Fig. 4** Number of occupied cells under present and future climate conditions for *Calotriton asper*

**Table 1** The results of the dispersal simulations using MIGCLIM with a cell resolution of 100 m

| Dispersal scenario | iniCount[a] | noDispCount[b] | univDispCount[c] | occupiedCount[d] | absentCount[e] | totColonized[f] | totDecolonized[g] |
|---|---|---|---|---|---|---|---|
| No barrier | 2346780 | 589900 | 704400 | 693142 | 6569993 | 596722 | 2250360 |
| Strong barrier | 2346780 | 589900 | 704294 | 692975 | 6570160 | 582745 | 2236550 |

[a] Number of cells of the species' initial distribution

[b] Number of cells colonized at the end of the simulation under the "no dispersal" scenario

[c] Number of cells colonized at the end of the simulation under the "unlimited dispersal" scenario

[d] Number of cells in an occupied state at the end of the simulation

[e] Number of cells in unoccupied state at the end of the simulation

[f] Total number of cells colonized during the entire simulation

[g] Total number of cells lost due to climate turning unfavourable during the entire simulation

statistical methods used for modelling species distributions, the input data, the GCM and emission scenarios (SRES) used to predict future ranges, the scale of the analysis and the specific dispersal abilities (Heikkinen et al. 2006; Araújo and New 2007; Beaumont et al. 2008; Seo et al. 2009; Buisson et al. 2010).

Range-shifting species create two additional problems when using correlative SDMs: (1) species records no longer reflect stable relationships with environment and (2) environmental combinations in future scenarios will not have been adequately sampled (Elith et al. 2010). Consequently, range-shifting species violate the equilibrium assumption and often require a certain degree of model extrapolation, making forecasting of species distributions extremely challenging (Elith et al. 2010). The MESS and MoD pictures indicate areas with non-analogue climate conditions within the distribution range (Supplementary Figures S2 and S3), and predictions in these areas should be treated with caution (Elith et al. 2010). The most dissimilar variable in the study region is isothermality (BIO3). As the Maxent analysis of variable contributions indicates this variable to have a minimal contribution to the models, we believe that non-analogue conditions have played a minor role in the present study (see Supplementary table S5 in File S1).

In addition to the uncertainties mentioned above, correlative SDM approaches are subject to a wide range of

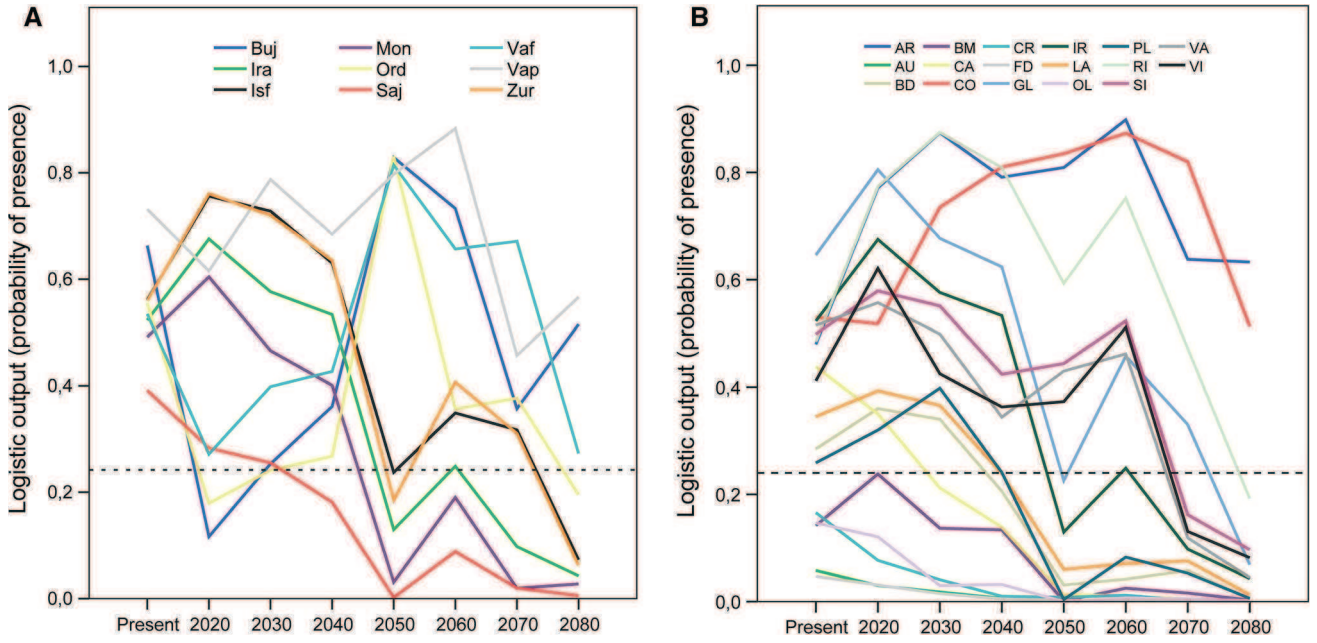


**Fig. 5** Trends in future suitability of populations under a scenario of no dispersal. The *dashed horizontal line* indicates the maximum test sensitivity plus specificity threshold. **a** cytochrome *b* and **b** populations sampled using AFLP loci

problems arising from sampling bias, the choice of predictor variables, the quality of species distribution data, spatial autocorrelation, thresholds and the choice of the extent of the study region (see e.g. Franklin 2010; Peterson et al. 2011). We have aimed to minimize the effect of these uncertainties by a careful examination of the data. For example, we have selected only records with high precision and also applied several methods to reduce sampling bias (see "Materials and methods" section). Furthermore, we have applied a threshold that has been shown to produce highly accurate predictions (Liu et al. 2005; Jiménez-Valverde and Lobo 2007) in comparison with other thresholds. The present study includes several parameters that are somewhat arbitrary due to a lack of data. For example, the barriers such a slope have been selected based on expert knowledge, while optimally these should have been based on proper field studies. As we report minimal differences between the dispersal scenarios, however, it is unlikely that these settings have not affected the present study.

### Incorporating dispersal and genetic variation

While many studies have raised the importance of incorporating dispersal in projecting changes in species' distributions (Davis et al. 1998; Guisan and Thuiller 2005; Araújo and Guisan 2006; Thuiller et al. 2008; Carvalho et al. 2010), the majority have only used the "unlimited dispersal" and/or "no dispersal" scenarios (e.g. Thomas et al. 2004; Araújo et al. 2006; Carvalho et al. 2010; Thuiller et al. 2011; see Bateman et al. 2013). Although there have been several methodological advances towards incorporating dispersal constraints (e.g. Lischke et al. 2006; Engler and Guisan 2009; Midgley et al. 2010; Engler et al. 2012), their application remains extremely limited (Engler et al. 2012). The present study represents one of the first applied case studies using a species-specific integration of SDM and dispersal modelling using four different dispersal scenarios. Surprisingly, the dispersal scenarios showed little differences in the number of cells occupied at the end of the simulation. As expected, the "no dispersal" scenario resulted in the highest loss (75 %) of suitable cells, whereas the remaining scenarios showed only minor differences (70 % decrease). This similarity is likely the result of the small number of remaining suitable cells and indicates that *C. asper* can potentially colonize most of the remaining suitable areas. The results of both genome-wide AFLP analysis (Milá et al. 2010) and capture–recapture (Montori et al. 2008a), however, indicate extremely low dispersal in *C. asper*, making the "no dispersal" or both the partial dispersal scenarios most realistic. This is in concordance with the literature on dispersal of other salamander species that mostly show limited dispersal capacity and home range sizes (Wells 2007). Although MIGCLIM was initially developed to study plant dispersal, the present study shows great promise for application on animal species that have been previously assessed using unrealistic dispersal scenarios. The broad literature on dispersal through telemetry data and mark recapture studies should help in the creation of realistic dispersal models for a wide variety of organism.

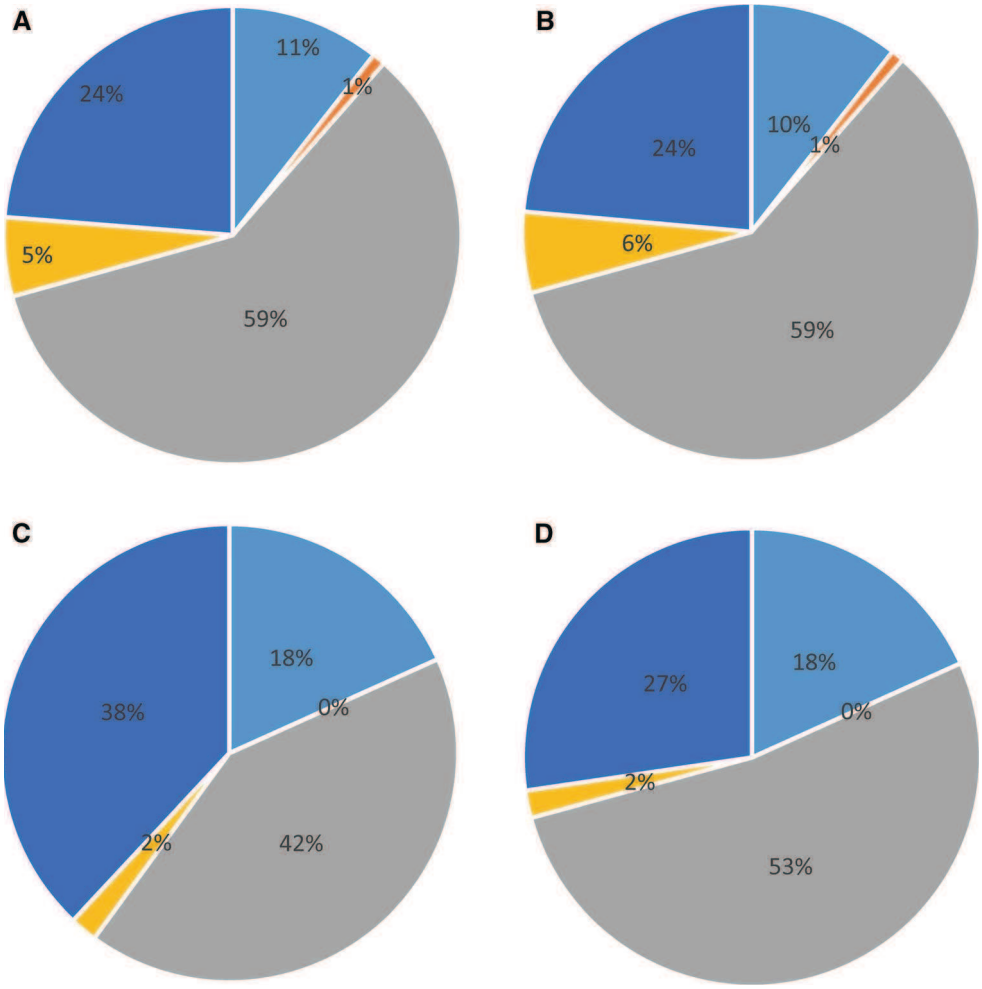


**Fig. 6** Combined results of loss of genetic diversity for populations of *Calotriton asper* from the Pyrenees when accounting for potential dispersal in a 7-km buffer surrounding populations using no dispersal (**a**–**c**) and strong dispersal barriers (**b**–**d**). **a**–**b** cytochrome *b* nucleotide diversity (>0) or unique haplotypes and **c**–**d** mean expected heterozygosity of populations sampled for AFLP loci. The colours indicate: *grey* = lost, *dark orange* = potential suitable, *light blue* = stable, *light orange* = dispersal and *dark blue* = unsuitable (colour figure online)

The incorporation of genetic variation into climate change impact studies is an important research topic (Anderson 2013; Franklin 2013), and the recent development of new methods allowing the integration of intraspecific genetic diversity appears to be promising (Espíndola et al. 2012; Pfenninger et al. 2012). Several of these methods, however, require extensive genetic datasets with a thorough range-wide sampling that is often beyond the possibilities for non-model organisms (Pfenninger et al. 2012) such as *C. asper*. In the present study, we have used both cytochrome *b* and an AFLP dataset (Milá et al. 2010).While the cytochrome *b* data are thoroughly sampled, the AFLP dataset is unevenly biased towards French side of the distribution range and lacks samples from Spain and Andorra. The contrasting variation in genetic structure detected using both AFLP (Milá et al. 2010) and cytochrome *b* (Valbuena-Ureña et al. 2012) markers indicates the importance of using a multilocus approach in detecting the true genetic structure in *C. asper*. While the cytochrome *b* provides a range-wide picture of the shallow genetic structure at the mtDNA level, the limited sampling of the available AFLP data indicates a vastly underestimated level of cryptic intraspecific genetic diversity in many parts of the distribution range that is missed within the present study.

While it is clear that the measured impacts of climate change of genetic diversity are largely influenced by the methodological framework (e.g. the choice of marker and the completeness and adequacy of spatial sampling; Pfenninger et al. 2012), the available studies have applied and explored several different techniques (e.g. Habel et al. 2011; Taubmann et al. 2011; Alsos et al. 2012; Velo-Antón et al. 2013; D'Amen et al. 2013). In general, however, climate change is expected to impact intraspecific diversity in many ways (reviewed in Pauls et al. 2013) including (1) changes in the distribution of genetic variants in space and time as the ranges of populations and species change, (2) changes in levels of phenotypic plasticity of individuals and populations as they respond to new environmental conditions and (3) evolutionary adaptation to changing environmental conditions (Hoffmann and Sgrò 2011). These changes will, in many cases, reduce genetic diversity

in populations and species, ultimately resulting in reduced population viability and possibly extinction due to genetic impoverishment (Pauls et al. 2013). Reductions in genetic diversity or changes in haplotypic diversity due to genetic drift can occur rapidly if population sizes are small and migration is curtailed (Lacy 1987; Peakall and Lindenmayer 2006), especially in species with low habitat availability and low dispersal capacity (Louy et al. 2007) such as *C. asper*.

Several studies have previously assessed the effects of climate change on *C. asper*, but all used coarse-resolution climate and distribution data. For example, Carvalho et al. (2010) assessed the impacts of climate change on the Iberian herpetofauna for three time periods using an ensemble of bioclimatic models and a combination of three GCM and two storylines at a resolution of ~10 km. The choice of the spatial resolution is an important factor in SDM and can have important effects on future suitability predictions (Randin et al. 2009; Bellard et al. 2012). Carvalho et al. (2010) predicted a complete loss of suitable climate for *C. asper* already in 2050, and these results are in sharp contrast with the present study despite using the same GCM and storyline (A2). These differences are likely the result of the different resolutions, as coarser resolutions often predict a complete loss of suitability in comparison with finer resolutions (Randin et al. 2009). Furthermore, Carvalho et al. (2010) used distribution data from a limited part of the distribution only which could result in an underestimation of suitable climate space (Barbet-Massin et al. 2010).

Our study extends on the previous works (Araújo and Guisan 2006; Carvalho et al. 2010) using a novel integrative approach that combines SDM and dispersal modelling while assessing the impacts on genetic diversity and evolutionary history. The conservation of populations and species is the main aim of conservation biology, and the present study underscores the importance of using different dispersal scenarios in climate change impact assessments. These methods should be widely applicable in conservation research on other salamander species, especially when detailed ecological data such as dispersal distances are available.

## Conclusions and recommendations

The present study indicates that climate change drastically reduces the potential distribution range of *C. asper* and reveals dispersal possibilities to be minimal under realistic dispersal scenarios. Despite the major loss in suitable climate, the models highlight relatively large stable areas throughout the species core distribution area indicating persistence of populations over time in comparison with previous studies. The results of the effects of climate change on genetic variation analyses, however, show a major loss of genetic diversity ($\pi$ and $H_e$) and evolutionary history. These results highlight the importance of accounting for intraspecific genetic variation and cryptic diversity in climate change impact studies. The use of higher resolution markers such as microsatellites (e.g. Drechsler et al. 2014; Valbuena-Ureña et al. 2014) and a range-wide sampling of all populations combined with promising new methods (e.g. Dubey et al. 2013; Pfenninger et al. 2012) should provide improved insights into the effects of climate change on the loss of genetic diversity and evolutionary history in *C. asper* from the Pyrenees. Future research should explore the increasing availability of genomic resources for non-model organisms (e.g. Ekblom and Galindo 2011; Ellegren 2014) as insights into the genetic response mechanisms of *C. asper* to climate change can only be understood using non-neutral markers (Pauls et al. 2013). Likewise, the integration of species' specific dispersal constraints into projections of species distribution models is an important step to fully explore the effects of climate change on species potential distributions. The exploration of new tools and frameworks (e.g. Anderson 2013; Fordham et al. 2013) should be conducted in order to clarify the contrasting results of the available studies (Araújo et al. 2006; Carvalho et al. 2010; present study). Finally, an increasing amount of studies have successfully used mechanistic models to assess herpetofauna responses to climate change (Kearney and Porter 2004; Buckley 2008, 2010; Kearney et al. 2010; Sinervo et al. 2010; Buckley et al. 2010). Mechanistic models, such as biophysical models, capture hypothetical biological processes and derive parameters of species phenotypes to model distributions. Such models have not yet been widely applied in Europe (but see e.g. Ceia-Hasse et al. 2014), while an overwhelming amount of data are available for several species including *C. asper* (Colomer et al. 2014). The exploration of mechanistic approaches to support assessments of climate change vulnerability will provide new insights into species' responses, while the use of these approaches alongside correlative SDMs could provide a robust basis for predicting and managing extinctions risks under climate change and should receive further exploration. Following the contrasting results of the present study and previous work, conservation management actions such as assisted dispersal aimed at preserving *C. asper* should wait until the full range of available methods has been explored.

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# Supplementary information File S1

**Supplementary table S1.** The source of the distribution records used in the present study


Asociación Herpetológica Española. Base de datos de Anfibios y Reptiles de España. http://www.herpetologica.es/programas/base-de-datos-herpetologica

BazNat - base de données naturalistes partagée en Midi-Pyrénées. http://www.baznat.net

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**Supplementary table S2.** Explanation of MIGCLIM settings as provided by Engler et al. (2012)

| Parameter | Description |
|---|---|
| Species initial distribution [iniDist] | A layer of integer, binary, values indicating whether a given cell is initially hosting the species (1) or not (0). |
| Habitat suitability map(s) [hsMap] | One or more layers indicating the habitat suitability of a given cell. |
| Reclassification threshold [rcThreshold] | unsuitable' habitats. Cells with values ≥ threshold are reclassified as 100% suitable while cells with values < threshold are reclassified as 0% suitable. [rcThreshold] must be an integer number in the range [0:1000]. If rcThreshold = 0, then habitat suitability values are not reclassified but are instead considered as habitat invasibility values (probability of a cell to become colonized given its habitat = habitat suitability/1000). Habitat with higher suitability have more likelihood to become colonized), the invasibility of a cell (e.g. the presence of another species can act as a competitor or facilitator), or both. Note that the invasibility values are interpreted by the model as an absolute probability of presence conditional on the species dispersing to the cell (e.g. all other things being equal, a cell with habitat suitability of 600 is twice as likely to be colonized as a cell with habitat suitability of 300). |
| Environmental change step number [envChgSteps] | Number of times the habitat suitability layer should be updated within a simulation. This value must be equal to the number of habitat suitability layers available. Simulations without environmental change can be carried out by setting envChgSteps = 1. |
| Dispersal step number [dispSteps] | Number of times dispersal should be simulated within each environmental change step. The total number of dispersal steps in a simulation is thus equal to [dispSteps] X [envChgSteps]. |
| Dispersal kernel [dispKernel] | Vector of values indicating the probability of a source cell to disperse values that are non-integer numbers (e.g. diagonals) are rounded to their closest integer number and attributed to that distance class. |
| Propagule production potential [propaguleProd]; [iniMatAge] | The probability of a source cell to produce propagules as a function of time since the cell became colonized. This is specified via 2 parameters: initial |

| | |
|---|---|
| | production for each age between initial and full maturity [propaguleProd]. This parameter can be used as a proxy for population growth in the cell, or for instance to refl ect that a species might need several years before starting to produce propagules, and even more time to reach its full reproductive potential. The time unit is a dispersal step, which will usually represent one year. |
| Barriers to dispersal [barrier]; [barrierType] | Layer of integer, binary, values indicating whether a given cell is a barrier to dispersal (1) or not (0). 'Barrier' cells are considered as permanently unsuitable for the species, but unlike regular unsuitable cells, they also impede dispersal across them (see Engler et al. 2012) |
| Long distance dispersal [lddFreq]; [lddMinDist]; [lddMaxDist] | Long distance dispersal events are randomly generated with a user-defined frequency [lddFreq] within a used-defined distance range [lddMinDist, lddMaxDist]. The frequency of long distance dispersal events is also Long distance dispersal events aim at representing non-standard ways of propagule dispersal. |
| [replicateNb] | Number of times the dispersal simulation is to be replicated. |

**Supplementary table S3.** CORINE landcover classes used in the present study. Dispersal barriers indicate if the class has been used as a barrier in the present study.

| Class ID | Class | Description | Dispersal barrier |
|---|---|---|---|
| 1 | Artificial surfaces | Continuous urban fabric | Yes |
| 2 | Artificial surfaces | Discontinuous urban fabric | Yes |
| 3 | Artificial surfaces | Industrial or commercial units | Yes |
| 4 | Artificial surfaces | Road and rail networks and associated land | Yes |
| 5 | Artificial surfaces | Port areas | Yes |
| 6 | Artificial surfaces | Airports | Yes |
| 7 | Artificial surfaces | Mineral extraction sites | Yes |
| 8 | Artificial surfaces | Dump sites | Yes |
| 9 | Artificial surfaces | Construction sites | Yes |
| 10 | Artificial surfaces | Green urban areas | Yes |
| 11 | Artificial surfaces | Sport and leisure facilities | Yes |
| 12 | Agricultural areas | Non-irrigated arable land | Yes |
| 13 | Agricultural areas | Permanently irrigated land | Yes |
| 14 | Agricultural areas | Rice fields | Yes |
| 15 | Agricultural areas | Vineyards | Yes |
| 16 | Agricultural areas | Fruit trees and berry plantations | Yes |
| 17 | Agricultural areas | Olive groves | Yes |
| 18 | Agricultural areas | Pastures | No |
| 19 | Agricultural areas | Annual crops associated with permanent crops | No |
| 20 | Agricultural areas | Complex cultivation patterns | No |
| 21 | Agricultural areas | Land principally occupied by agriculture, with significant areas of natural vegetation | No |
| 22 | Agricultural areas | Agro-forestry areas | No |
| 23 | Forest and semi natural areas | Broad-leaved forest | No |
| 24 | Forest and semi natural areas | Coniferous forest | No |
| 25 | Forest and semi natural areas | Mixed forest | No |
| 26 | Forest and semi natural areas | Natural grasslands | No |
| 27 | Forest and semi natural areas | Moors and heathland | No |

| | | | |
|---|---|---|---|
| 28 | Forest and semi natural areas | Sclerophyllous vegetation | No |
| 29 | Forest and semi natural areas | Transitional woodland-shrub | No |
| 30 | Forest and semi natural areas | Beaches, dunes, sands | Yes |
| 31 | Forest and semi natural areas | Bare rocks | Yes |
| 32 | Forest and semi natural areas | Sparsely vegetated areas | No |
| 33 | Forest and semi natural areas | Burnt areas | Yes |
| 34 | Forest and semi natural areas | Glaciers and perpetual snow | Yes |
| 35 | Wetlands | Inland marshes | Yes |
| 36 | Wetlands | Peat bogs | Yes |
| 37 | Wetlands | Salt marshes | Yes |
| 38 | Wetlands | Salines | Yes |
| 39 | Wetlands | Intertidal flats | Yes |
| 40 | Water bodies | Water courses | Yes |
| 41 | Water bodies | Water bodies | No |

**Supplementary table S4.** The performance for the present and future SDMs according to the AUC and True Skill Statistic (TSS).

| Model | Training AUC | Test AUC | TSS |
|---|---|---|---|
| Present | 0.942 | 0.934 | 0.810 |
| 2020 | 0.940 | 0.932 | 0.863 |
| 2030 | 0.944 | 0.938 | 0.788 |
| 2040 | 0.941 | 0.934 | 0.891 |
| 2050 | 0.940 | 0.931 | 0.810 |
| 2060 | 0.942 | 0.934 | 0.812 |
| 2070 | 0.943 | 0.935 | 0.836 |
| 2080 | 0.942 | 0.934 | 0.782 |
| Average | 0.942 | 0.934 | 0.824 |

**Supplementary table S5*.** Percent contribution and permutation importance (%) of the climatic predictor variables for the species distribution models of *Calotriton asper* in the Pyrenees. Refer to the main text for the description of variables.

| Variable | Percent contribution | Permutation importance |
|---|---|---|
| BIO5 | 56.7 | 2.5 |
| BIO9 | 14.6 | 11.6 |
| BIO2 | 9.3 | 26.7 |
| BIO13 | 9.1 | 16.8 |
| BIO15 | 7.7 | 29.1 |
| BIO3** | 1.0 | 3.8 |
| BIO8 | 0.9 | 2.6 |
| BIO6 | 0.7 | 6.9 |

*The following table gives estimates of relative contributions of the environmental variables to the Maxent model. To determine the first estimate, in each iteration of the training algorithm, the increase in regularized gain is added to the contribution of the corresponding variable, or subtracted from it if the change to the absolute value of lambda is negative. For the second estimate, for each environmental variable in turn, the values of that variable on training presence and background data are randomly permuted. The model is reevaluated on the permuted data, and the resulting drop in training AUC is shown in the table, normalized to percentages. As with the variable jackknife, variable contributions should be interpreted with caution when the predictor variables are correlated. Values shown are averages over replicate runs.

** The most dissimilar variable in the study region (see Supplementary information Figure S3)

**Supplementary table S6**. Sample localities of *Calotriton asper* in the Pyrenees used for assessing loss of genetic diversity at the mtDNA level. Sequences of a single mitochondrial gene (cytochrome *b*) were obtained from GenBank.

| Locality | Country | Code | Nucleotide diversity | Haplotype | Number of samples |
|---|---|---|---|---|---|
| Auriac | France | AU | 0 | H[1] | 7 |
| Bernard | France | BD | 0 | H[1] | 10 |
| Berga | Spain | Ber | 0 | H[2] | 21 |
| Betharram | France | BM | 0 | H[1] | 10 |
| Bujaruelo | Spain | Buj | 0.00084 | H[1]H[3] | 36 |
| Cailla | France | CA | 0 | H[1] | 13 |
| Genie Longue | France | GL | 0 | H[1] | 10 |
| Irati | France | Ira | 0.00305 | H[1]H[2]H[5] | 24 |
| Isaba | France | Isf | 0.0003 | H[2]H[4] | 18 |
| Pto. Montrepos | Spain | Mon | 0.00045 | H[1]H[2] | 24 |
| Ordino | Andorra | Ord | 0.00115 | H[1]H[2] | 34 |
| Pas du Loup | France | PL | 0 | H[1] | 10 |
| Portalet | France | Pof | 0 | H[1] | 13 |
| Port du Rat | France | Por | 0 | H[1] | 12 |
| San Juan de la Peña | Spain | Saj | 0.00084 | H[1]H[6] | 22 |
| La Cerdanya | Spain | Top | 0 | H[2] | 19 |
| Vall Fosca | Spain | Vaf | 0 | H[7] | 21 |
| Valle de Pineta | Spain | Vap | 0.00126 | H[3]H[8] | 26 |
| Vidrà | Spain | Vid | 0 | H[2] | 15 |
| Vilanova de Meià | Spain | Vim | 0 | H[2] | 18 |
| Zuriza | Spain | Zur | 0.00128 | H[2]H[8] | 21 |

**Supplementary table S7**. Sample localities of *Calotriton asper* in the Pyrenees used for assessing loss of genetic diversity (mean expected heterozygosity, $H_e$) in populations sampled using AFLP loci. Sequences were obtained from Milá et al. (2010)

| Locality | Country | Code | $H_e$ | Number of samples |
|---|---|---|---|---|
| Arcouzan | France | AR | 0.104 | 9 |
| Auriac | France | AU | 0.006 | 7 |
| Bernard | France | BD | 0.053 | 26 |
| Betharram | France | BM | 0.026 | 30 |
| Cailla | France | CA | 0.032 | 23 |
| Courbiere | France | CO | 0.057 | 6 |
| Cass-Rats | France | CR | 0.039 | 19 |
| Font de Dotz | France | FD | 0.040 | 4 |
| Genie Longue | France | GL | 0.056 | 16 |
| Irati | France | IR | 0.053 | 15 |
| Labouiche | France | LA | 0.038 | 17 |
| Olhadoko | France | OL | 0.051 | 13 |
| Pas du Loup | France | PL | 0.019 | 18 |
| Ribaui | France | RI | 0.105 | 14 |
| Siech | France | SI | 0.051 | 7 |
| Valmanya | France | VA | 0.019 | 10 |
| Vicdessos | France | VI | 0.048 | 7 |

**Supplementary table S8.** Extractions of MIGCLIM model output (% of total cells) of each dispersal scenario in a buffer of seven kilometres (70 years times 100 m dispersal) surrounding populations with nucleotide diversity (>0) or unique haplotypes.

| *No dispersal barrier* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | **Nucleotide diversity** | **Stable** | **Potential suitable** | **2020** | **2030** | **2040** | **2050** | **2060** | **2070** | **2080** | **Dispersal** | **Lost** | **Unsuitable** | **Extinct** |
| Buj[2] | 0.00084 | 17.7 | 3.0 | 0 | 4.4 | 4.5 | 2.9 | 1.8 | 1.6 | 4.4 | 19.5 | 52.5 | 7.3 | *** |
| Ira[1] | 0.00305 | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80.4 | 19.6 | * |
| Isf[1] | 0.00030 | 23.0 | 0.2 | 0 | 0 | 0 | 0.6 | 0.4 | 0.1 | 1.0 | 2.2 | 62.3 | 12.3 | ** |
| Mon | 0.00045 | 0.0 | 0.6 | 0 | 0 | 0 | 0.0 | 0 | 0 | 0 | 0 | 55.9 | 43.4 | * |
| Ord | 0.00115 | 12.3 | 0.0 | 0 | 2.5 | 1.9 | 1.2 | 0.4 | 0.5 | 0.3 | 6.8 | 73.5 | 7.4 | ** |
| Saj[1] | 0.00084 | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 21.3 | 78.7 | * |
| Vaf[1] | 0 | 11.9 | 0.0 | 0 | 1.6 | 1.1 | 0.5 | 0.2 | 0.3 | 0.3 | 3.9 | 75.5 | 8.7 | *** |
| Vap[2] | 0.00126 | 19.7 | 3.5 | 0 | 3.7 | 2.6 | 0.9 | 0.6 | 0.4 | 4.0 | 12.2 | 52.1 | 12.5 | *** |
| Zur[2] | 0.00128 | 39.4 | 0.0 | 0 | 0.2 | 0 | 1.2 | 0 | 0 | 0.4 | 1.8 | 58.8 | 0.0 | ** |
| ***Strong dispersal barrier*** | | | | | | | | | | | | | | |
| Buj[2] | 0.00084 | 17.7 | 3.0 | 0 | 4.4 | 4.5 | 3.0 | 1.8 | 1.6 | 4.3 | 19.6 | 52.5 | 7.3 | *** |
| Ira[1] | 0.00305 | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 80.4 | 19.6 | * |
| Isf[1] | 0.00030 | 23.0 | 0.1 | 0 | 0 | 0 | 0.6 | 0.4 | 0.1 | 1.0 | 2.2 | 62.3 | 12.3 | ** |
| Mon | 0.00045 | 0.0 | 0.6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 55.9 | 43.5 | * |
| Ord | 0.00115 | 12.3 | 0.0 | 0 | 2.5 | 1.9 | 1.2 | 0.4 | 0.5 | 0.2 | 6.8 | 73.5 | 7.4 | ** |
| Saj[1] | 0.00084 | 0.0 | 0.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 21.3 | 78.7 | * |
| Vaf[1] | 0[1] | 11.9 | 0.0 | 0 | 1.6 | 1.1 | 0.5 | 0.1 | 0.3 | 0.3 | 3.9 | 75.5 | 8.7 | *** |
| Vap[2] | 0.00126 | 19.7 | 3.5 | 0 | 4.8 | 2.5 | 1.0 | 0.5 | 0.4 | 4.0 | 13.2 | 52.4 | 11.2 | *** |
| Zur[2] | 0.00128 | 39.4 | 0.0 | 0 | 0.2 | 0 | 1.2 | 0 | 0 | 0.4 | 1.8 | 58.8 | 0.0 | ** |

[1]Unique haplotype
[2]Haplotypes are only found in two populations
*Extinct under all scenarios
**No direct dispersal opportunities. Extinct under no or limited dispersal opportunities
***Stable

**Supplementary table S8.** Extractions of MIGCLIM model output (% of total cells) of each dispersal scenario in a buffer of seven kilometres (70 years times 100 m dispersal) surrounding populations with expected heterozygosity ($H_e$) of sampled populations.

| *No dispersal barrier* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | **He** | **Stable** | **Potential suitable** | **2020** | **2030** | **2040** | **2050** | **2060** | **2070** | **2080** | **Dispersal** | **Lost** | **Unsuitable** | **Extinct** |
| AR $^{K=4}$ | 0.104 | 47.5 | 0.0 | 0.0 | 4.9 | 2.9 | 2.8 | 0.8 | 0.0 | 0.0 | 8.5 | 41.1 | 0.0 | *** |
| AU $^{K=4}$ | 0.006 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.6 | 88.4 | * |
| BD $^{K=4}$ | 0.053 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 70.5 | 29.5 | * |
| BM $^{K=2}$ | 0.026 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.1 | 69.1 | ** |
| CA $^{K=4}$ | 0.032 | 8.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 71.2 | 20.8 | ** |
| CO $^{K=4}$ | 0.057 | 69.0 | 0.0 | 0.0 | 1.1 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 29.0 | 0.0 | *** |
| CR $^{K=4}$ | 0.039 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 48.0 | 52.0 | * |
| FD $^{K=4}$ | 0.040 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 51.4 | 48.6 | * |
| GL $^{K=2}$ | 0.056 | 13.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 57.2 | 29.4 | ** |
| IR $^{K=1}$ | 0.053 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 80.5 | 19.5 | * |
| LA $^{K=4}$ | 0.038 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 60.3 | 39.7 | * |
| OL $^{K=1}$ | 0.051 | 7.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 76.8 | 16.2 | ** |
| PL $^{K=3}$ | 0.019 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 52.9 | 47.1 | * |
| RI $^{K=4}$ | 0.105 | 57.9 | 0.0 | 0.0 | 4.5 | 1.9 | 1.7 | 0.3 | 0.0 | 0.0 | 6.6 | 33.6 | 0.0 | ** |
| SI $^{K=4}$ | 0.051 | 34.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 65.9 | ** |
| VA $^{K=4}$ | 0.019 | 9.4 | 0.0 | 2.5 | 1.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 3.5 | 0.0 | 86.7 | ** |
| VI $^{K=5}$ | 0.048 | 59.3 | 0.0 | 0.0 | 5.8 | 0.9 | 0.2 | 0.0 | 0.0 | 0.0 | 5.9 | 0.0 | 33.8 | ** |

See next page for results Strong dispersal barrier

*Extinct under all scenarios
**No direct dispersal opportunities. Extinct under no or limited dispersal opportunities
***Stable
$^{K}$ assignment to *K* = 5 cluster

| *Strong dispersal barrier* | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Population** | **He** | **Stable** | **Potential suitable** | **2020** | **2030** | **2040** | **2050** | **2060** | **2070** | **2080** | **Dispersal** | **Lost** | **Unsuitable** | **Extinct** |
| AR $^{K = 4}$ | 0.104 | 47.5 | 0.0 | 0.0 | 4.9 | 2.7 | 2.9 | 0.8 | 0.1 | 0.0 | 0.0 | 41.1 | 0.0 | *** |
| AU $^{K = 4}$ | 0.006 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.6 | 88.4 | * |
| BD $^{K = 4}$ | 0.053 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 70.3 | 29.7 | * |
| BM $^{K = 2}$ | 0.026 | 4.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.1 | 69.1 | ** |
| CA $^{K = 4}$ | 0.032 | 8.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 71.2 | 20.8 | ** |
| CO $^{K = 4}$ | 0.057 | 69.0 | 0.0 | 0.0 | 1.2 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 29.0 | 0.0 | *** |
| CR $^{K = 4}$ | 0.039 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 48.0 | 52.0 | * |
| FD $^{K = 4}$ | 0.040 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 51.4 | 48.6 | * |
| GL $^{K = 2}$ | 0.056 | 13.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 57.2 | 29.4 | ** |
| IR $^{K = 1}$ | 0.053 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 80.5 | 19.5 | * |
| LA $^{K = 4}$ | 0.038 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 60.0 | 40.0 | * |
| OL $^{K = 1}$ | 0.051 | 7.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 76.7 | 16.4 | * |
| PL $^{K = 3}$ | 0.019 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 52.4 | 47.6 | * |
| RI $^{K = 4}$ | 0.105 | 57.9 | 0.0 | 0.0 | 4.5 | 1.9 | 1.7 | 0.4 | 0.0 | 0.0 | 6.5 | 33.6 | 0.0 | ** |
| SI $^{K = 4}$ | 0.051 | 34.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 64.2 | 1.6 | ** |
| VA $^{K = 4}$ | 0.019 | 9.4 | 0.0 | 2.5 | 1.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 3.6 | 85.5 | 1.2 | ** |
| VI $^{K = 5}$ | 0.048 | 59.3 | 0.0 | 0.0 | 5.7 | 1.0 | 0.2 | 0.0 | 0.0 | 0.0 | 5.9 | 33.8 | 0.0 | ** |

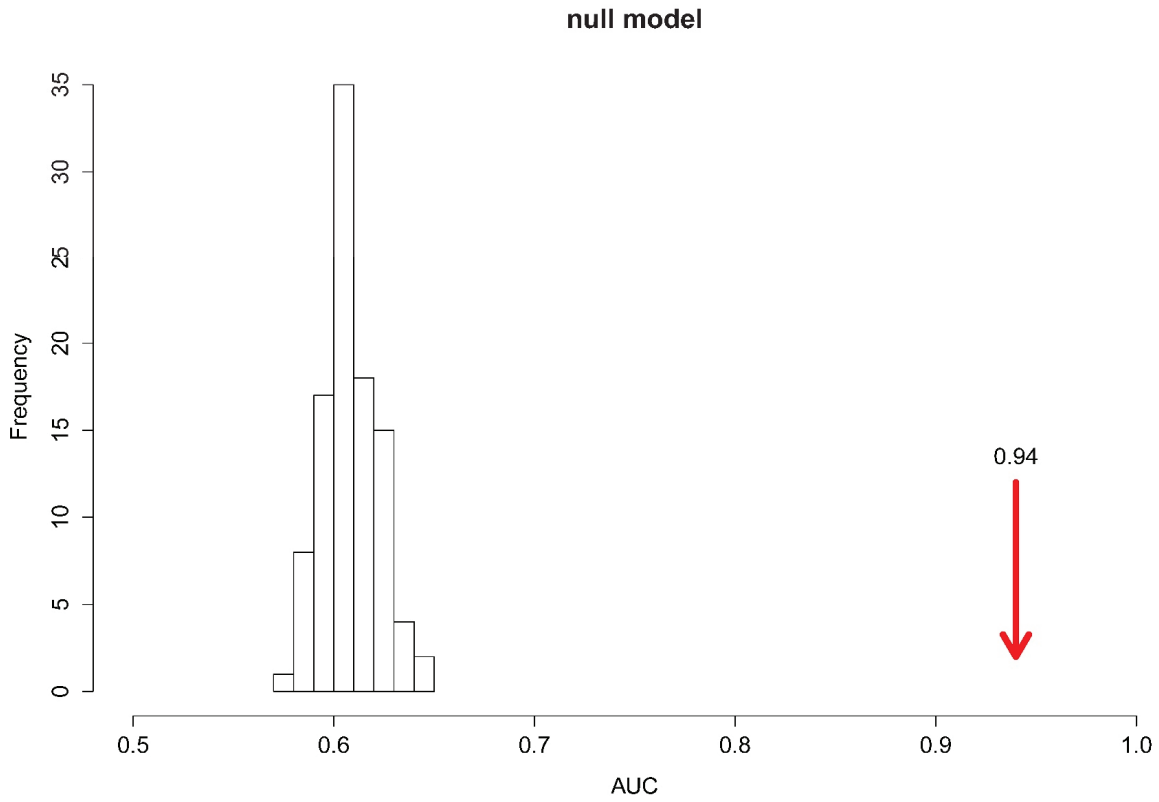


**Figure S1.** Null-model to test for significance of the SDM created using 100 null distributions of random points in the study area.

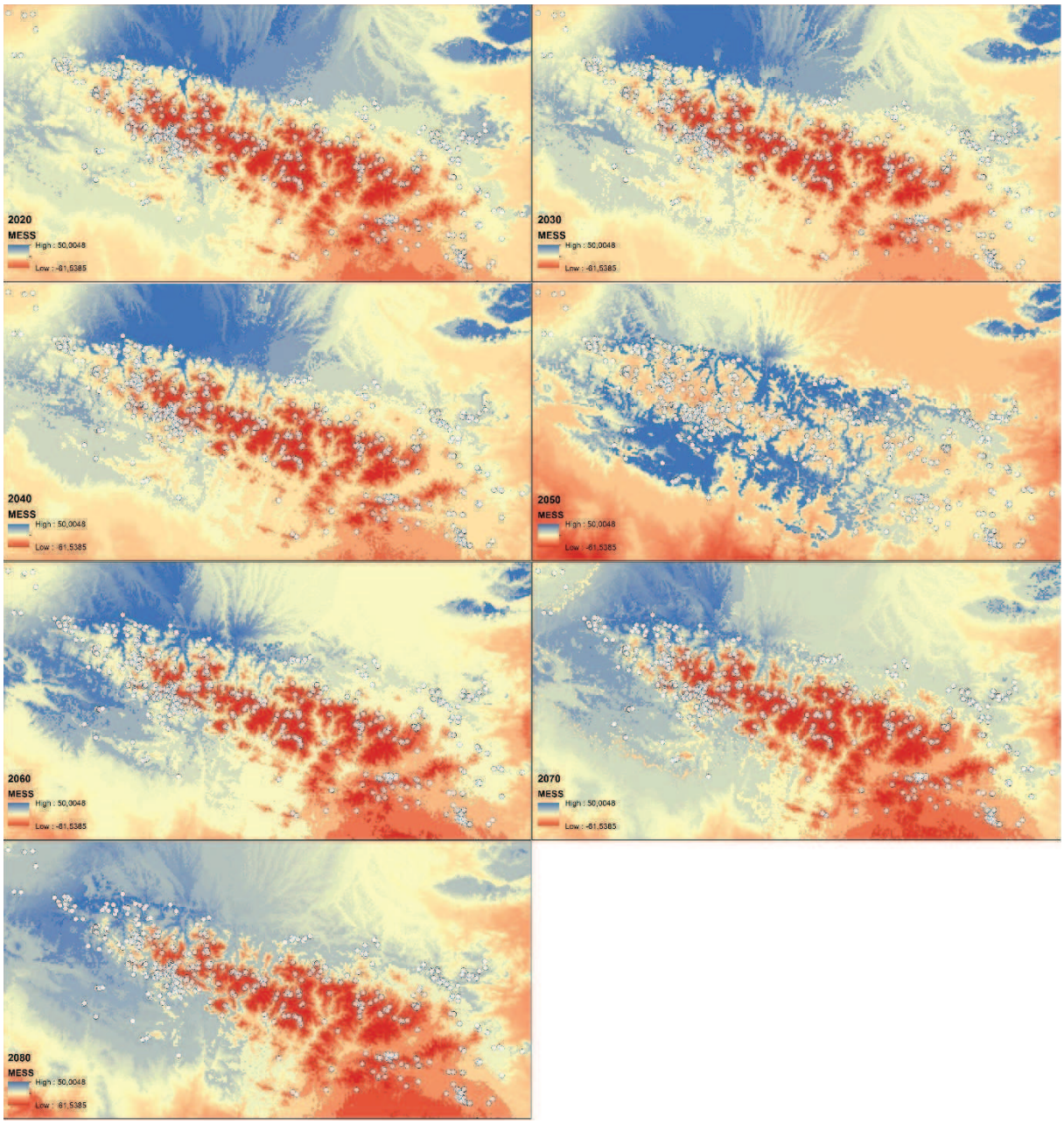


**Figure S2.** MESS pictures of the projected SDMs for the period 2020-2080. Red areas in red have one or more environmental variables outside the range present in the training data.

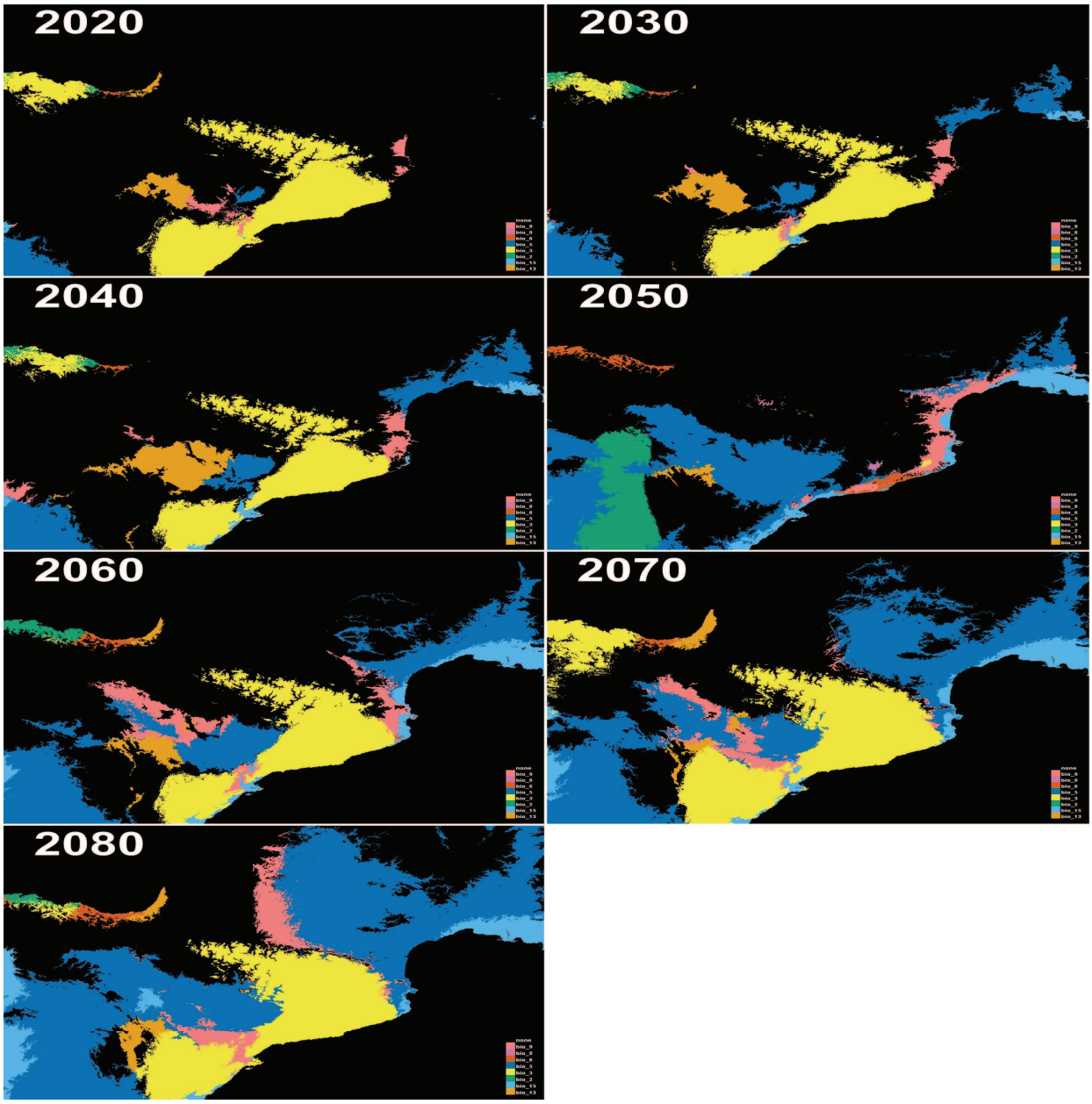


**Figure S3.** MoD pictures of the projected SDMs for the period 2020-2080 showing the most dissimilar variable, i.e., the one that is furthest outside its training range.

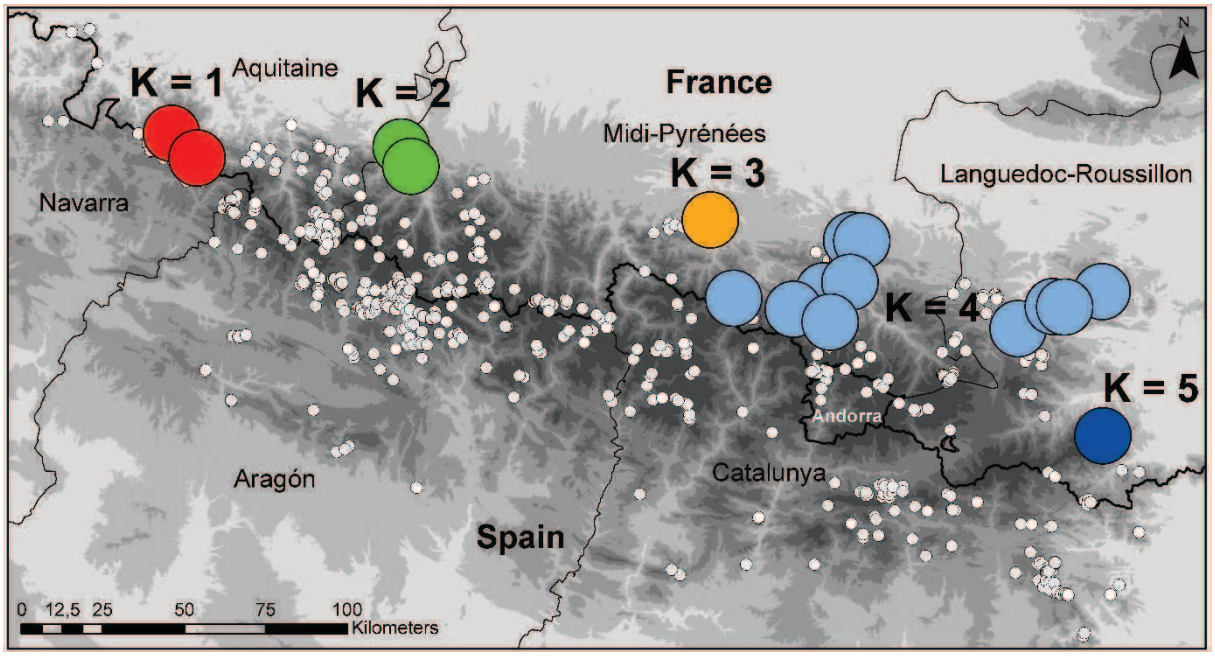


**Figure S4.** Distribution of *K* clusters used for assessment of climate change on evolutionary history

# General discussion

This thesis explored the integration of geospatial methods into evolutionary biology (biogeography, phylogeography and systematics) and conservation (climate change impact) through a series of case studies on Western Palearctic reptiles and amphibians. Despite that this topic has already seen an increasing number of appications and reviews in the literature there are still many understudied aspects and new methods are continuously being developed.

**Biogeographic patterns in the Western Mediterranean**

This thesis mainly focused on the biogeography and phylogeography of several reptiles and amphibians from the Western Palearctic. The first three chapters focused on the biogeography of *Alytes maurus*, *Discoglossus scovazzi* and *Natrix natrix* from North Africa, whereas Chapter 4 provided a review of the amphibian fauna of Morocco. These chapters expand our knowledge of these species and extend the already rich herpetological literature about this region. The available studies on the phylogenetics, biogeography and phylogeography of North African herpetofauna have so far focused on many genera: *Acanthodactylus* (Fonseca et al. 2009), *Agama* (Brown et al. 2002), *Amietophrynus* (Harris and Perera 2009), *Atlantolacerta* (Barata et al. 2012a), *Blanus* (Vasconcelos et al. 2006; Sampaio et al. 2015), *Bufo* (Garcia-Porta et al. 2012), *Bufotes* (Batista et al. 2006; Stöck et al. 2006), *Chalcides* (Carranza et al. 2008; Kornilios et al. 2010; Brown et al. 2012), *Chamaeleo* (Dimaki et al. 2008), *Coronella* (Santos et al. 2012), *Discoglossus* (Zangari et al. 2006), *Emys* (Stuckas et al. 2014), *Eumeces* (Perera et al. 2012), *Hemorrhois* (Carranza et al. 2006a), *Hyalosaurus* (de Pous et al. 2011), *Hyla* (Recuero et al. 2007; Stöck et al. 2008), *Macroprotodon* (Carranza et al. 2004), *Malpolon* (Carranza et al. 2006a), *Mauremys* (Fritz et al. 2006), *Mesalina* (Kapli et al. 2015), *Natrix* (Barata et al. 2008), *Pelophylax* (Nicolas et al. 2015), *Pleurodeles* (Carranza and Arnold 2003), *Podarcis* (Pinho et al. 2006; Lima et al. 2009), *Psammodromus* (Carranza et al. 2006b), *Ptyodactylus* (Perera and Harris 2010a; Metallinou et al. 2015), *Quedenfeldtia* (Barata et al. 2012b), *Salamandra* (Beukema et al. 2010), *Saurodactylus* (Rato and Harris 2008), *Scelarcis* (Harris et al. 2003a), *Stenodactylus* (Metallinou et al. 2012), *Tarentola* (Rato et al. 2012), *Testudo* (Fritz et al. 2009), *Timon* (Paulo et al. 2008; Perera and Harris 2010b),

*Trogonophis* (Mendonça and Harris 2007), *Uromastyx* (Harris et al. 2007) and *Vipera* (Velo-Antón et al. 2012). One of the clear patterns emerging from these studies is the exististence of high degrees of intra-specific genetic divergence between eastern and western populations of species occurring in the northern African Maghreb. The possible causes of this biogeographical pattern, however, have never been comprehensively investigated. Possible factors that have promoted this genetic divergence include range shifts and vicariance processes resulting from climatic fluctuations such as the Pliocene aridification and the Pleistocene glaciations (e.g. Krijgsman et al. 1999; Duggen et al. 2003; Beukema et al. 2010; Jimenez-Moreno et al. 2010). Furthermore, several studies have discussed the role of the Moulouya River Basin (MRB) as a possible barrier to gene flow (e.g. Arano et al. 1998; Álvarez et al. 2000; Zangari et al. 2006; Barata et al. 2008), but others have disputed this claim (Harris et al. 2003b,c; Paulo et al. 2008; de Pous et al. 2011; Santos et al. 2012). This thesis supports the hypothesis of the MRB as a biogeographic barrier for the genus *Discoglossus* and possibly the North African populations of *Natrix natrix*. Furthermore, the results of Chapter 3 show climatic stability throughout the Late Pleistocene in the Western and Eastern Maghreb. This finding can potentially explain the pattern of east vs. west genetic divergence of several of the more European related herpetofauna species that are restricted to the more humid areas (e.g. *Bufo bufo*, *Hyla meridionalis* and *Coronella girondica*). This biogeographic pattern is observed in a wide variety of North African species ranging from plants (Magri et al. 2007) and birds (Garcia et al. 2008) to mammals (e.g. Cosson et al. 2005) and future studies should focus on deciphering the drivers of this east vs. west genetic divergence in detail.

The collapse of the Gibraltar land bridge at the end of the Messinian Salinity Crisis has previously been proposed as a major event in shaping the biogeography of the Western Mediterranean, with numerous studies on groups ranging from plants to mammals. Although this has been the principal cause of vicariance events affecting some groups on each side of the Strait of Gibraltar (Steinfartz et al. 2000; García-París et al. 2003; Carranza and Wade 2004; Martínez-Solano et al. 2004; Zangari et al. 2006), the pattern for other genera has been less clear, with scenarios varying widely among species (e.g. Albert et al. 2007; Fritz et al. 2006; Pinho et al. 2006; Carranza et al. 2004, 2006a, 2006b, 2008; Paulo et al. 2008; Santos et al. 2012; Velo-Antón et al. 2012; Garcia-Porta et al. 2012; Stuckas et al. 2014). The results presented in Chapter 3 indicate a scenario of transmarine dispersal from the Iberian Peninsula

to Northern Africa. These result highlight again the complicated yet fascinating biogeographical history of this well studied region.

Chapter 5 focused on the biogeography, phylogeography and systematics of the Arabian species *Bunopus spatalurus*. Unlike the many studies on North Africa, the reptiles of the Arabian Peninsula have not been studied in detail using molecular analyses until very recently. For example, Carranza and Arnold (2012) provided an elaborate review of the *Hemidactylus* geckos of Oman and showed high inter-intraspecific genetic divergence within the members of this group. Likewise, Busais and Joger (2011), Šmíd et al. (2013a,b, 2015) and Vasconcelos and Carranza (2014) described additional species of *Hemidactylus*. Other recent studies have focused on the geckos of the genera *Asaccus* (Papenfuss et al. 2010), *Pristurus* (Papenfuss et al. 2008; Badiane et al. 2014), *Ptyodactylus* (Metallinou et al. 2015) and *Stenodactylus* (Metallinou et al. 2012; Metallinou and Carranza 2013). Altogether these studies have increased the number of species substantially and provided important new insights in the biogeography of this region. Chapter 5 builds upon this recent interest in the Arabian Peninsula and is the first study that combines molecular analyses and geospatial methods to explore the possible drivers of genetic divergence. The Hajar Mountains in Oman provide an excellent study region due the the high number of endemic species, relative small size and easy accessability. The observed pattern of deep intraspecific genetic divergence within the elevated species *Bunopus hajarensis* is not easily understood and the results presented in Chapter 5 do not find a clear hypothesis explaining this pattern. While Papenfuss et al. (2010) and Carranza and Arnold (2012) first identified high genetic divergence, work in progress using molecular phylogenies on other reptile groups inhabiting this massif indicate that this could be a common pattern. Future studies comparing the patterns of several endemic reptiles of the geologically complex Hajar Mountains would benefit from the use of an integrative approach that combines geospatial methods and statistical genetics (see e.g. examples in Chan et al. 2011) to obtain a better understanding of the patterns and processes that have shaped their unique diversity and distribution.

Chapter 6 focused on the application of geospatial methods in conservation biology by exploring the effects of climate change on the potential distribution and genetic variation of the endemic Pyrenean newt *Calotriton asper.* This chapter used a novel approach that combined species distribution modelling, high resolution gridded climate data with dispersal

modelling and data on both population genetic diversity and evolutionary history. This framework has potential implications for studying the conservation of other amphibians but is essentially applicable to any organism. The integration of geospatial methods and molecular data has been mainly applied in phylogeography studies while conservation biologists have not yet made it a consuetude to apply such integrative approaches. Despite that many authors have argued for the integration of genetics into conservation (e.g. Crandall et al. 2000; Fraser and Bernatchez 2001; Moritz 2002; Bálint et al. 2011; Anderson 2013; Franklin 2013; Pauls et al. 2013) and an increasing number of studies have explored methods to do so (e.g. Habel et al. 2011; Thomassen et al. 2011; Alsos et al. 2012; Jay et al. 2012; Pfenninger et al. 2012; D'Amen et al. 2013), there is still a major gap in both conceptual and metholodgical knowledge to fully benefit from the integration of different disciplines in conservation biology. Several reviews in both ecology and evolution have recently opted for the merging of disciplines into integrative research fields (e.g. Mouquet et al. 2012; Fitzpatrick and Keller 2015; Habel et al. 2015; Hand et al. 2015; Hoffmann et al. 2015). This is a major future research direction that, fuelled by the increasing availability and use of genomic data, will revolutionize ecological, evolutionary and conservation research.

**Limitations of used methods**

Among the most important issues in geospatial methods, and especially SDM approaches, are the many uncertainties associated with these methods. For example, Yackulic et al. (2013) reviewed a large number of SDM studies (Maxent) and concluded that the vast majority ingnored the most important aspects of presence only modelling. This is likely the result of the easy implementation of Maxent and most user lack understanding of what the software does, and why inputs and settings matter. There has been such an influx of conceptual and methodological advances that the umbrella term species distribution modelling should be considered an independent and very active research field that offers important applications in ecological, evolutionary and conservation research.

There have been many studies on the uncertainties of the modelling process: the statistical methods used for modelling species distributions, the input data, the global climate model (GCM) used to predict past and future ranges, the scale of the analysis and the specific dispersal abilities (Heikkinen et al. 2006; Araújo and New 2007; Beaumont et al. 2008; Seo et

al. 2009; Buisson et al. 2010). In addition to these uncertainties, correlative SDM approaches are subject to a wide range of problems arising from sampling bias, the choice of predictor variables, the quality of species distribution data, spatial autocorrelation, thresholds and the choice of the extent of the study region or background (see e.g. Franklin 2010; Peterson et al. 2011). The application of SDMs to model past or future distributions creates two additional problems: (1) species records no longer reflect stable relationships with environment, and (2) environmental combinations in future scenarios will not have been adequately sampled (Elith et al. 2010). Consequently, range-shifting species violate the equilibrium assumption and often require a certain degree of model extrapolation, making forecasting/hindcasting of species distributions extremely challenging (Elith et al. 2010). The present thesis aimed to minimize the effect of these uncertainties by a careful examination of the used data and model settings. For example, in Chapter 5 optimal model settings and complexity was obtained through the recently developed R package ENMeval (Muscarella et al. 2014). Furthermore, most of the chapters have carefully explored and reduced sampling bias in the distribution data. Chapter 5 used a recently developed spatial rarefying protocol as implemented in SDMtoolbox (Brown 2014) to remove spatially autocorrelated distribution records. Several studies have shown that the background used for model calibration (**M** in the **BAM** diagram of Soberón and Peterson 2005) has important implications in all aspects of SDM studies, including model parameterization, model validation and model comparisons (Barve et al. 2011). Although there is currently no framework or method to define **M** it is important the background region encompasses all known localities and includes areas that have been accessible to the species via dispersal over relevant time periods. Many studies have used very large backgrounds that often reflect political boundaries and this could lead to inflated test statistics, larger predicted distributions, less informative response variables and overfitting of SDMs (VanDerWal et al. 2009; Giovanelli et al. 2010; Anderson and Raza 2010; Stokland et al. 2011). The chapters in this thesis have all used carefully selected backgrounds and this has likely improved the quality of the models.

The chapters in this thesis mainly used mtDNA and nuclear DNA to address the specific objectives and questions. It is well known that the choice of molecular marker has major implications for studying spatio-temporal evolutionary processes. Chapter 1 for example failed to find geographic structure in the amphibian *Alytes maurus* from Morocco but this

would likely change when using faster evolving markers such as microsatellites. Hence, the use of mtDNA in this case does not provide information on the effects of contemporary geneflow between populations and possible genetic structuring as a result of recent habitat fragementation (e.g. deforestation). The results of Chapter 2 show discordance between mtDNA and nuclear DNA results in *Discoglossus* in the Iberian Peninsula. While the two subspecies of *D. galganoi* are clearly separated using mtDNA, the results of the nuclear DNA is less clear with specimens extensively sharing haplotypes. The results of Chapter 2 highlight the importance of considering and choosing the appropriate molecular markers to infer the evolutionary history of species. The use of additional markers such as microsatellites or SNPs would certainly add new insights into geneflow in the complex contact zone between these subspecies. Chapter 3 studied the biogeography of the elusive North African *Natrix natrix* populations. Like the previous studies (see Chapter 3), our work only used mtDNA to address the objectives while it is clear that this group has a rather conflicting taxonomy including a mismatch between morphology and genetics. As an example, the morphologically distinct *N. n. cetti* from Sardinia is genetically closely related to mainland Italy and the subspecies does not merit species status. The results presented in Chapter 3 would have benefitted from the inclusion of at least a nuclear gene in order to assess if the North African populations share nuclear haplotypes. Chapter 5 used a combination of mtDNA and nuclear DNA to study the phylogeography of *Bunopus spatalurus* and to assess the taxonomic status of the subspecies *B. s. hajarensis*. Following the use of a multilocus phylogeny and haplotype networks the latter was elevated to the species level. This shows the importance of using multilocus data in taxonomy. Chapter 6 used neutral mtDNA data and AFLPs to explore the effects of climate change on the endemic Pyrenean newt *Calotriton asper*. The method applied in this chapter somewhat arbitrary used the available genetic data as measures of evolutionary history and genetic diversity of populations. Although such data might be related to adaptive capacity in some cases, there is no clear evidence to support this. This chapter should ideally have used a combination of different molecular markers (e.g. mtDNA, SNPs or microsatellites) together with a complete sampling and statistical methodology to examine both the historical and adaptive axis of concern (Moritz 2002). Furthermore, the integration of other measure of intraspecific variation such as phenotypic or fundamental niche space should be incorporated to fully explore the effects of environmental change of species persistence.

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# Conclusions

1. Unexpectedly, our study shows that the endemic Moroccan amphibian *Alytes maurus* presents a low level of variability in the mitochondrial genes studied (12S, 16S and cytochrome *b*) with no clear geographical structuring. The low genetic variation in mtDNA can be explained by a much broader climatic suitability during the Last Glacial Maximum that allowed the connection among populations and subsequent homogenization as a consequence of gene flow.

2. A) The North African amphibian *Discoglossus scovazzi* shows, in general, a weak phylogeographic structure across Morocco on the basis of mitochondrial DNA sequences of the cytochrome *b* gene, with only populations centered in the Atlas Mountains characterized by the presence of slightly divergent haplotypes.

   B) In eastern Morocco, all populations east of the Moulouya River were clearly assignable to *D. pictus*. This species was also found along the Mediterranean coast west of the Moulouya, in the cities of Nador and Melilla, suggesting that not the river itself but the wide arid valley extending along much of the river (except close to the estuary) acts as a possible distributional barrier to these frogs.

   C) No sympatry of *D. scovazzi* with *D. pictus* was observed, and all specimens were concordantly assigned to either species by DNA sequences of cytochrome *b* and of the nuclear marker RAG1.

   D) Species distribution models of the two taxa show largely overlapping areas of suitable habitat, and the two species' niches are significantly more similar than would be expected given the underlying environmental differences between the regions in which they occur.

   E) The Iberian *D. galganoi galganoi* and *D. g. jeanneae* showed less clear-cut distributional borders, extensively shared RAG1 haplotypes, and had instances of

sympatric occurrence on the basis of cytochrome *b* haplotypes, in agreement with the hypothesis of a yet incomplete speciation. In this wide contact zone area we found mitochondrial sequences containing double peaks in electropherograms, suggesting nuclear pseudogenes or (less likely) heteroplasmy, possibly related to the ongoing admixture among the lineages.

3. A) According to the obtained genealogy, *Natrix natrix* specimens from North Africa and the Iberian Peninsula form a well-supported clade that includes three deep divergent lineages (Iberia, Morocco and Tunisia).

   B) The separation between the North African and the Iberian clade of *N. natrix* dates back to 2.5 Ma, and between the eastern and western North African populations to 2.2 Ma.

   C) *Natrix natrix* likely reached North Morocco through transmarine dispersal from southern Spain and rapidly expanded its range along the Mediterranean coast towards Tunisia. Quaternary climate stability in the eastern and western Maghreb and the presence of dispersal barriers in the form of the Moulouya and Chelif River basins that prevented gene flow likely contributed to the observed genetic diversity between the North African populations.

4. A) The amphibian fauna of the Kingdom of Morocco was traditionally regarded as poor and closely related to its European counterpart. However, an increase in research during the last decades revealed a considerable degree of endemism amongst Moroccan amphibians, as well as phenotypic and genotypic inter- and intraspecific divergence.

   B) Despite this increase in knowledge, a comprehensible overview is lacking while several systematic issues have remained unresolved. We herein present a contemporary overview of the distribution, taxonomy and biogeography of Moroccan amphibians. Fourteen fieldtrips were made by the authors and colleagues between 2000 and 2012, which produced a total of 292 new distribution records.

C) Furthermore, based on the results of the present work, we (i) review the systematics of the genus *Salamandra* in Morocco, including the description of a new subspecies from the Rif- and Middle Atlas Mountains, *Salamandra algira splendens* ssp. nov.; (ii) present data on intraspecific morphological variability of *Pelobates varaldii* and *Pleurodeles waltl* in Morocco; (iii) attempt to resolve the phylogenetic position of *Bufo brongersmai* and erect a new genus for this species, *Barbarophryne* gen. nov.; (iv) summarize and assess the availability of tadpole-specific characteristics and bioacoustical data, and (v) summarize natural history data.

5. A) According to the inferred topology recovered using concatenated and species tree methods, the genus Nacked toe gecko genus "*Bunopus*" is polyphyletic. *Bunopus tuberculatus* and *B. blanfordii* form a highly supported clade closely related to Crossobamon orientalis, while the two subspecies of "*Bunopus*" *spatalurus* branch together as an independent highly supported clade that diverged during the Miocene.

   B) Within *B. s. hajarensis*, three geographically structured clades can be recognized that diverged during the late Miocene to Pliocene. The paleodistribution models indicate climatic stability during the Late Pleistocene and the lineage occurrence and predicted contact zones obtained from phylogeographic interpolation therefore probably result from the older splits of the groups when these lineages originated in allopatry.

   C) As demonstrated by the results of the multilocus molecular phylogenetic analyses and the topological test carried out in the present study, the genus "*Bunopus*" is not monophyletic. To resolve this, we resurrect the genus *Trachydactylus* Haas and Battersby, 1959 for the species formerly referred to as *Bunopus spatalurus*.

   D) Considering the morphological differences, the high level of genetic differentiation in the 12S mitochondrial gene and the results of the phylogenetic and the *cmos* haplotype network analysis, we elevate *Trachydactylus spatalurus hajarensis* to the species level: *Trachydactylus hajarensis* (Arnold, 1980).

6. A) The present study indicates that climate change drastically reduces the potential distribution range of *C. asper*, and reveals dispersal possibilities to be minimal under the most realistic dispersal scenarios.

   B) Despite the major loss in suitable climate, the models highlight relative large stable areas throughout the species core distribution area indicating persistence of populations over time.

   C) The results, however, show a major loss of genetic diversity and evolutionary history.

   D) This highlights the importance of accounting for intraspecific genetic variation in climate change impact studies. Likewise, the integration of species' specific dispersal constraints into projections of species distribution models is an important step to fully explore the effects of climate change on species potential distributions.

This thesis specifically aimed at the integration of geospatial methods into evolutionary biology and conservation. The enormous development of both conceptual and methodological approaches has already revolutionised these fields but there is still potential for major improvements related to reducing uncertainties. This thesis explored a number of promising new geospatial methods in combination with more traditional molecular analyses. Such integrative approaches will ultimately allow us to better consider and examine the range of potential histories underlying both inter and intraspecific divergence patterns. Finally, as shown in Chapter 6, the combination of geospatial methods such as SDMs and dispersal models together with phylogeographic (or population genetic data) has major implications for conservation biology and should be further explored in during the next years.


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