

# Resilient arthropods: *Buthus* scorpions as a model to understand the role of past and future climatic changes on Iberian Biodiversity



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### Nota Prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do Decreto-Lei 74/2006, de 24 de Março, com a nova redação introduzida pelo Decreto-Lei 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada. A Faculdade de Ciências da Universidade do Porto foi a instituição de origem do candidato, tendo o trabalho sido realizado sob orientação do Doutor David James Harris, Professor Associado Convidado da Faculdade de Ciências da Universidade do Porto (FCUP) e Investigador Principal do Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBio/UP), e coorientação do Professor Doutor Miguel Arnedo, Professor Agregado do Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Unitat d'Artròpodes, Facultat de Biologia, Universitat de Barcelona e Investigador do Institut de Recerca de la Biodiversitat - Facultat de Biologia de la Universitat de Barcelona (IRBIO-UB). O trabalho laboratorial foi realizado no IRBIO-UB a no CIBIO-InBio/UP.

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#### Disclaimer

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Ao meu avô José Sousa. Por tudo o que me ensinou. Pela alegria de regressar à infância e ouvir um grilo a cantar debaixo da sua boina. [This page intentionally left blank]

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"We are like sailors who on the open sea must reconstruct their ship but are never able to start afresh from the bottom. Where a beam is taken away a new one must at once be put there, and for this the rest of the ship is used as support. In this way, by using the old beams and driftwood the ship can be shaped entirely anew, but only by gradual reconstruction."

Otto Neurath (1921)

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### Abstract

The Mediterranean region (Med) is one of Earth's largest Biodiversity hotspots, with a high number of plant endemics. This biological richness is a consequence of the Mediterranean complex geography, including several long Peninsulas and high Mountains, and complex geological history. The Med is the western remnant of an older Tethys Ocean entrapped between the African and Eurasian tectonic plates. The climate in the Mediterranean area remained mildly and subtropical, and mostly stable, during the Miocene. It changed around 3.2 Ma during the Upper Pliocene and assumed its present configuration, which ranges from temperate to arid, but even the former is characterized by strong annual fluctuations in temperature and precipitation (*i.e.* Mediterranean climate). The Pleistocene Glaciations did mark a departure from the Mediterranean climate. These climatic changes, together with the paleogeological changes, had a strong impact on the Mediterranean Biodiversity and left a lasting genetic footprint. At the end of the Pleistocene, in the Eastern Mediterranean area, started one of the earliest areas of human civilization, which slowly expanded throughout the Med. The prolonged human presence has had a strong negative impact on its Biodiversity, as much of the available land better suited for sustaining Biodiversity was converted to agricultural use. All these factors converge to make the Mediterranean an area in urgent need of conservation, even more so because its eastern portion includes yet another important Biodiversity hotspot, the Irano-Anatolian.

The Mediterranean region has also a high scorpion species' diversity, albeit markedly different in composition from North to South. The southern Med is richer in species and genera number but not in the number of families, which further highlights the complex paleo-history of the area. The genus *Buthus* is a specious example of this diversity that managed to colonise the Iberian Peninsula, but is an otherwise North African genus that also reaches the Middle East. In Africa, *Buthus* range extends from the Mediterranean shore as far south as the Sahel and as far east as the Horn of Africa.

Although Western Med *Buthus* genetic diversity was first studied more than a decade ago (in 2003), much remained unknown, even more so given the ten-fold increase in its species composition since the publication of the "Catalog of the scorpions of the world (1758-1998)". To tackle this fast pace of new species descriptions we present an update to the *Buthus* catalogue and gave some notes on the most important morphological characters used for *Buthus* species identification.

To advance the study of the genus phylogeny and phylogeography we first developed five Anonymous Nuclear Markers (ANM) that proved informative at the intra and interspecific level in *Buthus*. We also demonstrated for the first time that two ANM, together with two other markers developed for *Mesobuthus* scorpions, cross-amplify in the Buthidae, and therefore have the potential to reconstruct the phylogeny of this family, which includes almost half of the extant scorpion species.

To understand the phylogeography of the West Mediterranean *Buthus* we used a single mtDNA marker and a thorough sampling of the Maghreb. This was complemented with a smaller multilocus dataset that included samples from the entire Med to offer a near complete phylogeny of the genus. We did include a sample from Ethiopia, but the remainder Sub-Saharan diversity was not included. Both datasets recovered five main areas of *Buthus* diversification that were linked to five groups of species within the genus: *occitanus, tunetanus, mardochei, boumalenii* and *rochati* Our multilocus calibrated phylogeny inferred a Tortonian to Messinian (9.5 to 6.3 Ma) split for the five groups that we connected to the onset of aridification in North Africa (7 Ma). The dispersal into the Sub-Saharan region was found to post-date the Saharan onset, and thus must have happened during one of the cyclic Green Sahara phases. We were surprised to recover a dispersal over water for the colonisation of Cyprus given the prevalent view of scorpions as low dispersers. We further offered advice on the use of the widespread "*Mesobuthus* substitution rate" used for calibrations and its inference of older divergence times, which were unsupported by our data.

We used a thorough sampling of the Iberian Peninsula to reconstruct the phylogeography of the Iberian *Buthus* clade, again using the same matrilineal marker. We found a centre of diversification in the south of the Peninsula, a pattern common to many other taxa, and seven divergent lineages, all with complex patterns of phylogeography. We used a subset of samples to construct a multilocus dataset and apply a molecular' species delimitation approach. For the validation step, we used an implementation of the multispecies coalescent, which can infer the true species tree even when individual gene trees do not agree, applied to species delimitation. We recovered, with strong support, the existence of seven species in our dataset. Given the difficulties in the morphological identification of *Buthus* species, we used our sampling of the described species' type localities to pinpoint then to the delimited species. All four known species were supported, and we were able to remove from synonymy a fifth species. Nevertheless, two species remain unnamed pending a complete morphological reassessment of all Iberian species. We also constructed a calibrated phylogeny of the Iberian *Buthus* with the same multilocus subset of Iberian samples. We found that all

Iberian *Buthus* species originated during the Pliocene, and we suggested that tectonic and orogenic events were the main drivers of these speciation events.

Finally, we used Ecological Niche Modelling to understand if the Pleistocene Glaciations played a role in the observed phylogeography of the Iberian *Buthus* species. We modelled five of the seven delimited species. We discovered that Glaciations did make an impact on two species from the eastern area of the Iberian Peninsula. Nevertheless, for the remaining three species, no correlation was found between the predicted areas of persistence throughout the Last Inter-Glacial, Last Glacial Maximum, and into the Present.

Our work demonstrated that *Buthus* scorpions are a good model to understand Miocene to Pleistocene changes in the Mediterranean's Biodiversity distribution and composition. They should also prove useful to better understand the timings and stages of the Green Sahara Desert oscillations. *Buthus* should continue to be used as a tool to understand how past geological and climatic events shaped the current patterns of genetic diversity of the Mediterranean Biodiversity.

**Key-words:** Scorpiones; scorpion; *Buthus*; *Buthus* species; North Africa; Western Mediterranean; Eastern Mediterranean; Iberian Peninsula; Maghreb; Morocco; Ethiopia; systematics; taxonomy; phylogeny; phylogeography; Maximum likelihood; Bayesian inference; species discovery; species delimitation; Miocene; Messinian Salinity Crisis; Pliocene; Pleistocene; Climate; plate tectonics; vicariance; dispersal through water.

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### Resumo

A região mediterrânica é um dos maiores *hotspots* de biodiversidade do Planeta Terra, apresentando um elevado número de plantas endémicas. Esta riqueza biológica advém, não só da complexa geografia mediterrânica, que inclui várias penínsulas longas e elevadas cadeias montanhosas, mas também da sua complexa história geológica. O Mar Mediterrâneo é o remanescente ocidental do antigo Oceano Tétis enclausurado entre as placas tectónicas Africana e Euroasiática.

O clima da região mediterrânica manteve-se maioritariamente estável durante o Mioceno, podendo ser caracterizado como moderado e subtropical durante esse período. No entanto, há cerca de 3,2 Ma, durante o Plioceno Superior, o clima mudou e assumiu, com pontuais exceções, a sua configuração atual, variando de temperado a árido, mas até mesmo o primeiro é caracterizado por fortes flutuações anuais de temperatura e de precipitação (clima dito mediterrânico). As glaciações Plistocénicas representaram uma alteração a este padrão climático. Todas estas alterações climáticas, em conjunto com a paleogeologia do Mediterrâneo, tiveram um forte impacto na biodiversidade desta área, e deixaram nesta uma impressão genética duradoura

No final do Pleistoceno, na região do Mediterrâneo Oriental, surgiu um dos berços das primeiras civilizações humanas, que lentamente se expandiram por todo o Mediterrâneo. A presença humana tem tido um forte impacto negativo na biodiversidade mediterrânica, pois a maioria do território mais adequado para sustentar a biodiversidade que estava disponível foi convertido para práticas agrícolas ou para povoamento humano. Todos estes fatores concorreram para tornar a área mediterrânica numa área que urge conservar, ainda mais relevante porque a sua região oriental inclui o *hotspot* Irano-Anatolio de biodiversidade.

A região mediterrânica apresenta igualmente uma elevada diversidade específica de escorpiões, embora a sua composição seja marcadamente diferente de norte para sul. A região sul do Mediterrâneo é mais rica em número de espécies e géneros, mas não em número de famílias, o que reforça a evidência da complexa paleohistória da região. O género *Buthus* é um exemplo especioso desta diversidade, que apesar de ter conseguido colonizar a Península Ibérica, é um género Norte Africano, ainda que alcançando também o Oriente Médio. Em África, a sua distribuição estende-se desde a costa do Mediterrâneo, a norte, até ao Sahel a sul, e ao Corno de África, a leste.

Embora a diversidade genética de *Buthus* tenha sido estudada pela primeira vez há mais de uma década (em 2003), no Mediterrâneo Ocidental, muito permaneceu desconhecido, ainda mais dado o aumento de dez vezes no seu número de espécies desde a publicação do último catálogo taxonómico de escorpiões.

Para primeiro enquadrar toda esta diversidade específica, apresentamos uma atualização do catálogo taxonómico de *Buthus* publicado, e apresentamos algumas notas relevantes sobre os mais importantes caracteres morfológicos utilizados na identificação das espécies deste género.

Para avançar o estudo sobre a filogenia e filogeografia do género, desenvolvemos primeiro cinco Marcadores Nucleares Anónimos (ANM). Conseguimos demonstrar que estes ANM são informativos quer intra quer inter-especificamente em *Buthus*. Demonstramos igualmente, pela primeira vez, que dois destes ANM, conjuntamente com outros dois marcadores desenvolvidos para escorpiões *Mesobuthus*, amplificam outros géneros da Família *Buthidae*, pelo que têm potencial para ajudar a reconstruir a filogenia desta família, que inclui quase metade de todas as espécies extantes de escorpiões.

Para compreender a filogeografia dos Buthus do Mediterrâneo Ocidental, usamos um marcador de mtDNA e uma amostragem alargada do Magreb. Este estudo foi complementado com um conjunto de dados multilocus menor, que incluía amostras do Mediterrâneo, para obtermos uma filogenia quase completa do género. Neste conjunto de dados, incluímos uma amostra da Etiópia, mas a restante diversidade Saheliana não foi incluída. Recuperamos, com ambos os conjuntos de dados, cinco áreas principais de diversificação no género Buthus, que correspondemos a cinco grupos de espécies: occitanus (Norte de Marrocos e Europa), tunetanus (Tell-Atlas Oriental e Mediterrâneo Oriental), mardochei, (Bacia do rio Souss, Alto-Atlas Ocidental e Norte até Rabat), boumalenii (sudoeste do Alto-Atlas e noreste do Anti-Atlas), e rochati (Este e Sul do Anti-Atlas, pelo menos ate Tarfaya). A nossa filogenia multilocus calibrada permitiu inferir que os cinco grupos se separam entre o Tortoniano e o Messiniano (9,5 a 6,3 Ma), que ligamos ao início da aridificação do Norte de África (7 Ma). Concluímos igualmente que a dispersão para a área subsariana aconteceu após a formação do deserto do Saara e que, portanto, deve ter tido lugar durante alguma das fases verdes cíclicas do Saara. Surpreendentemente, apesar da visão prevalente que classifica os escorpiões como dispersores pouco capazes, inferimos uma dispersão sobre a água para explicar a colonização da ilha do Chipre. Oferecemos ainda alguns conselhos sobre a utilização da "taxa de substituição de Mesobuthus" em calibrações, pois infere tempos de divergência mais antigos, que no nosso caso não eram suportados pelos dados.

Usamos uma amostragem exaustiva da Península Ibérica para reconstruir a filogeografia dos *Buthus* ibéricos utilizando o mesmo marcador matrilineal. Encontramos um centro de diversificação no sul da Península, um padrão comum a muitos outros taxa, e sete linhagens divergentes, todas com complexos padrões fitogeográficos.

Utilizámos uma porção da amostragem para construir um subconjunto de dados multilocus que utilizámos numa abordagem molecular de delimitação de espécies. Para a etapa de validação utilizamos o coalescente multi-espécies, que pode inferir a árvore de espécies correta mesmo quando as árvores de genes individuais não são concordantes, aplicado à delimitação de espécies. Recuperamos sete espécies, muito bem suportadas, no nosso conjunto de dados. Dadas as dificuldades em identificar morfologicamente as espécies de *Buthus*, utilizámos a nossa amostragem das localidades tipo das espécies para determinar a sua correspondência com as espécies inferidas. Todas as quatro espécies descritas foram suportadas, e conseguimos ainda remover de sinonímia uma quinta espécie. No entanto, as duas espécies remanescentes permanecem sem nome até que um estudo morfológico completo possa ser feito que permita reavaliar todas as espécies.

Utilizamos o mesmo subconjunto de dados multilocus para construir uma filogenia calibrada dos *Buthus* ibéricos. Determinamos que todas as espécies ibéricas tiveram origem durante o Pleioceno, e sugerimos que foram eventos tectónicos e orogénicos que estiveram na origem desses eventos de especiação.

Finalmente, utilizámos a Modelação de Nicho Ecológico para compreender se as Glaciações Pleistocénicas tiveram influência na criação do padrão fitogeográfico dos *Buthus* ibéricos. Modelamos cinco das setes espécies e descobrimos que as Glaciações Pleistocénicas contribuíram para os padrões encontrados nas espécies orientais da Península Ibérica. No entanto, para as três restantes espécies, não encontramos qualquer correlação entre as áreas de persistência previstas desde o Último Interglacial, e passando pelo Último Máximo Glacial até chegarmos ao Presente.

O nosso trabalho demonstrou que os escorpiões *Buthus* são um bom modelo para entender mudanças ocorridas na distribuição e composição da Biodiversidade Mediterrânica, desde o Mioceno até ao Pleistoceno. Também devem ser úteis para inferir os tempos e modos das oscilações verdes do Saara. Os *Buthus* devem continuar a ser utilizados como ferramentas para melhor compreender como é que os eventos geológicos e climáticos passados moldaram a diversidade genética atual da Biodiversidade Mediterrânica. **Palavras-chave:** *Buthus*; Clima; Crise de Salinidade Messiniana; Delimitação de espécies; Dispersão sobre a água; Escorpião; Espécies de *Buthus*; Etiópia; Filogenia; Filogeografia; Inferência Bayesiana; Magrebe; Marrocos; Máxima Verossimilhança; Mediterrâneo Ocidental; Mediterrâneo Oriental; Mioceno; Norte de África; Península Ibérica; Placas tectónicas; Pleistoceno; Plioceno; Procura e Validação de espécies; Sistemática; Taxonomia; Vicariância; Scorpiones.

# **TABLE OF CONTENTS**

TABLE OF C	CONTENTS	. xvii
Chapter I	GENERAL INTRODUCTION	1
1 - Introdu	ction	3
1.1 Scorpi	ones, a resilient lineage of Arthropods	4
1.1.1 Sc	corpiones position in the Chelicerata tree of life	4
1.1.2 Or	rigins & paleo-history of Scorpiones	6
1.1.3 Sc	corpions overview	8
1.1.4 Di an	fficulties and recent breakthrough in Scorpiones extant family phylogen nd diversity overview	y, 9
1.1.5 Hu	umans and Scorpions	10
1.2 The M	editerranean region: an outline	12
1.2.1 Ge	eneral overview	12
1.2.2 Th 	ne complex geological history of the Mediterranean Sea since the Mioce	ene 16
1.2.3 CI	imatic changes in the Mediterranean area since the Miocene	22
1.2.4 Ph	hylogeography of the Mediterranean area since the Miocene	25
1.2.5 Sc	corpions species diversity in the Mediterranean area	28
1.3 The so	corpion genus <i>Buthus</i> , the model organism of this study	29
1.3.1 Ge	eneral overview	29
1.3.2 Bi	ology	31
1.3.3 Pł	nylogeography of Western Mediterranean Buthus	32
1.4 Gener	al objectives	36
1.5 Thema	atic Organization	36
1.6 Refere	ences	38
Chapter II	UPDATING BUTHUS TAXONOMY	51
PAPER 1:	Updated catalogue and taxonomical notes on the Old-World scorpion genus <i>Buthus</i> Leach, 1815 (Scorpiones, Buthidae)	53
Chapter III	DEVELOPMENT OF NEW MOLECULAR MARKERS	113
PAPER 2:	Development of Anonymous Nuclear Markers for <i>Buthus</i> scorpions (Scorpiones: Buthidae) using massive parallel sequencing, with an overview of nuclear markers used in Scorpions phylogenetics	115
Chapter IV	MEDITERRANEAN BUTHUS - PATTERNS AND PROCESSES	135

xviii | FCUP

PAPER 3:	Phylogeographic patterns of <i>Buthus</i> scorpions (Scorpiones: Buthid the Maghreb and South-Western Europe based on CO1 mtDNA sequences.	ae) in 137
PAPER 4:	Can a scorpion cross the sea? Biogeographical answers from a muphylogeny of Mediterranean <i>Buthus</i> (Buthidae, Scorpiones)	ultilocus 147
Chapter V	IBERIAN BUTHUS - DIVERSITY, DISTRIBUTION AND	
PHYLOGEO	GRAPHY	197
PAPER 5:	Pliocene tectonics and not Pleistocene glacial oscillations as the m driver of Iberian <i>Buthus</i> speciation (Buthidae, Scorpiones)	ain 199
Chapter VI	GENERAL DISCUSSION	247
6.1 New A	nonymous Nuclear Markers developed	249
6.2 Medite	erranean Biogeography and Phylogeography	249
6.3 Iberiar	Peninsula Phylogeography	252
6.4 Specie	es delimitation: a case of success in Iberian Buthus	253
6.5 Buthus	s taxonomy	254
6.6 Conclu	uding remarks and Future perspectives	255
6.7 Refere	ences	256
APPENDICE	ES	259
Appendix	A: General Introduction	261
Appendix	B: Paper 2	266
Appendix	C: Paper 3	271
Appendix	D: Paper 4	277
Appendix	E: Paper 5	282

# LIST OF TABLES

Table I-1. Higher level taxonomy of the Scorpiones
Table II-1. Current composition of the groups proposed by Sousa et al. (2012) andPedroso et al. (2013) based on cox1
Table II-2. List of the countries for which there are valid records of the occurrence of         Buthus species.       70
<b>Table III-1.</b> Primer sequences for seven anonymous nuclear loci developed from aNGS approach of Iberian <i>Buthus</i>
<b>Table III-2.</b> Summary diversity statistics for 12 nuclear sequence markers for fiveIberian Buthus lineages plus one Moroccan Buthus
<b>Table III-3.</b> Nuclear Loci used in 30 molecular phylogenetic or phylogeographic studiesof Scorpiones, ordered chronologically
<b>Table IV.I-1.</b> MtDNA Lineage and GenBank accession code of all the samples used in           the study.         139
<b>Table IV.I-2.</b> Pairwise net sequence divergence (Kimura 2-parameter) among the fourclades of <i>Buthus</i> sequences, and the within-clade sequence divergence values 143
<b>Table IV.I-3.</b> Net pairwise sequence divergence (Kimura 2-parameter) among the           subclades of <i>Buthus</i> .         143
Table IV.II-1. List of specimens sequenced in this study.         152
Table IV.II-2. Primer sequences and PCR annealing temperatures used with the           mtDNA markers         155
Table IV.II-3. Rates of mtDNA nucleotide substitutions used in this study
<b>Table IV.II-4.</b> Priors used for the three different Beast analyses that used the MessinianSalinity Crisis as a vicariant event.159
Table IV.II-5. Updated composition of the five species groups identified within Buthus           160
<b>Table IV.II-6.</b> Mean sequence divergence (uncorrected p-distances, values inpercentage) of <i>Buthus</i> species represented in mtDNA Dt3.163
Table IV.II-7.         Summary diversity statistics for the multilocus dataset, and for the           mtDNA dataset 1.         163
<b>Table IV.II-8.</b> Selected 10 nodes of the tree depicted in Fig. 7, with explanation on their placement within the tree
<b>Table IV.II-9.</b> Node age values obtained with the different Beast analyses using the           other 5 calibrations.         167
<b>Table IV.II-10.</b> Divergence time estimates of the 4 molecular markers found to have a relaxed molecular clock behaviour, for the 6 differently calibrated Beast analyses 169
Table V-1. List of specimens sequenced for the main dataset used
<b>Table V-2.</b> Range (minimum and maximum) and units of the bioclimatic variables used for modelling the distribution of the Iberian <i>Buthus</i> species in the study area
<b>Table V-3.</b> Genetic diversity and demographic measures calculated for the cox1mtDNA gene fragment for the Iberian lineages identified in Fig 2A.213

<b>Table V-4.</b> Mean <i>cox1</i> mtDNA sequence divergence (uncorrected p-distances, values in percentage) of the Iberian <i>Buthus</i> lineages identified in Fig V.2A.
Table V-5. Main dataset information.         216
Table V-6. Estimated divergence times according to the Beast analysis
<b>Table V-7.</b> Total number of presence points of each <i>Buthus</i> species used to train (NTrain), test (N Test), and validate (N Valid) the maximum entropy models.220
<b>Table V-8.</b> Measures of the contribution of the bioclimatic variables to the ENMs of the           Iberian Buthus species derived from the maximum entropy models.           220

# **LIST OF FIGURES**

Figure I-1. Extant Arachnida Orders cladogram
Figure II-2. Dorsal view of Proscorpio osborni
Figure I-3. Current most plausible fossil scorpions phylogeny
Figure I-4. Scorpions worldwide distribution
Figure I-5. Cladogram of all extant scorpion Families10
Figure I-6. World incidence and mortality of scorpionism
Figure I-7. Relief map of the Mediterranean Sea12
Figure I-8.Large-scale atmospheric circulation interactions over North Africa and theMediterranean area.13
<b>Figure I-9. A</b> - Map of the Mediterranean Area with the Köppen-Geiger climate classification represented14
Figure I-10. Map of the Mediterranean area with Hotspot areas highlighted15
Figure I-11. Map of the Mediterranean area featuring Tectonic Plates and major fault           lines.         16
Figure I-12. The Tethys Ocean during the last 250 Ma
Figure I-13. Mediterranean paleogeographical and paleotectonic reconstruction fromOligocene onwards.18
Figure I-14. Mediterranean paleogeographical and paleotectonic reconstruction in the           Miocene.         19
Figure I-15. Mediterranean paleogeographical and paleotectonic reconstruction in the           Late Miocene.         20
Figure I-16. Mediterranean paleogeographical and paleotectonic reconstruction from           the Miocene-Pliocene transition
Figure I-17. Global climate change from the Miocene to the present
Figure I-18. Distribution of the terrestrial Biomes as defined by Olson et al. (2001)23
Figure I-19. Miocene Past point distributions of the terrestrial Biomes, reconstructed from palaeobotanical data:
<b>Figure I-20.</b> Overview of faunal glacial refugia in the Mediterranean area, biogeographical sub-centres and hybrid zones of the south-western Palaearctic region 26
<b>Figure I-21.</b> Geographical distribution of 52 putative micro-refugia and of the 10 regional hotspots of plant biodiversity within the Mediterranean area
Figure I-22. Map of Mediterranean and North Africa species diversity organised by country
<b>Figure I-23.</b> General morphology of a <i>Buthus</i> scorpion, with tagmata and subtagmata differentiated by colour
Figure I-24. Buthus female displaying maternal care, with first instars on top of           mesosoma.         31

<b>Figure I-25</b> Sampling (A) and strict consensus MP tree of the combined 2 mtDNA
genes (B) of Gantenbein and Largiadèr (2003) work
<b>Figure I-26.</b> Phylogeographic patterns of <i>cox1</i> mtDNA found in the Atlas mountains regions of Morocco
Figure I-27. Sampling (A) and ML phylogram of the Iberian <i>Buthus</i> (B) of Sousa et al. (2010)
<b>Figure I-28.</b> A - Map with Iberian <i>Buthus</i> distribution according to Lourenço and Vachon (2004), and Teruel and Pérez-Bote (2005)35
Figure II-1. Buthus mariefranceae, from south of Morocco
Figure II-2. Map of <i>Buthus</i> species distribution, and the known number of species by country
Figure II-3. Original illustration of Scorpio occitanus (Amoreux 1789a)56
Figure II-4. The two key morphological diagnostic characters of the genus Buthus57
Figure II-5. Cumulative number of valid <i>Buthus</i> species
<b>Figure II-6.</b> Original drawings of the habits of <i>Scorpio europaeus</i> Linnaeus, 1758 (A) and <i>S. occitanus</i> (B), according to Amoreux (1789b)60
Figure II-7. Map representing the five phylogenetic <i>Buthus</i> cox1 groups in the Maghreb62
Figure II-8. Chart and boxplot summary of Buthus species maximum sizes
Figure II-9. Graphical representation of the variation in the number of rows in the movable finger of all <i>Buthus</i> species64
<b>Figure II-10.</b> Graphical representation of the variation in pectinal teeth number of <i>Buthus</i> species arranged by geographical areas to facilitate comparison
Figure II-11. Graphical representation of relation between female and male pedipalp chela aspect ratio in <i>Buthus</i> species
Figure II-12. Map of North Africa Maghrebian Buthus species' type localities
Figure II-13. Reproduction of C. L: Koch's 1839 B. halius figure 382 (plate CLXIII)77
Figure II-14. Photo of the syntypes of <i>B. nigrovesiculosus</i>
Figure II-15. Detailed morphology of the larger syntype of B. nigrovesiculosus
Figure II-16. Photo of a <i>B. parroti</i> female syntype87
Figure III-1. Phylogenetic neighbour-joining tree of the <i>cox1</i> 118
Figure IV.I-1. Phylogram depicting phylogenetic relationships estimated using Bayesian inference
Figure IV.I-2. Map showing overall distribution of the four Buthus clades retrieved 141
Figure IV.I-3. Map showing the distribution of <i>Buthus</i> clade D subclades as depicted in Figure 1
Figure IV.I-4. Detailed map of the High Atlas region of Morocco representing all <i>Buthus</i> subclades present in the area, as depicted in Figure 1
Figure IV.II-1. Map of Buthus sampled localities in the Western Mediterranean 150
Figure IV.II-2. Map of the known distribution of the five main mtDNA lineages within the genus <i>Buthus</i>
Figure IV.II-3. Histograms of Buthus sequences uncorrected p-distances

Figure IV.II-4. Marker rate variation (mean rate and ucld.mean) among the diferent Beast analises, when compared to the IP LogN Beast run
Figure IV.II-5. Marker rate variation (mean rate and ucld.mean) among the Beast analises, when compared to the 16S marker
Figure IV.II-6. Selected Node age estimates according to the six scenarios ran in Beast
Figure IV.II-7. Ultrametric Beast tree of the IP LogN calibration
Figure IV.II-8. Reconstructed scenario for the early phylogeny of the genus Buthus.175
Figure IV.II-9. Map of <i>Buthus</i> sampled localities in the Eastern Western Mediterranean
Figure IV.II-10. Relation between Pedipalp Chela length to width ratio and adult body size
Figure V-1. Map of Buthus sampled localities in the present study
Figure V-2. Results from the mtDNA phylogeny
Figure V-3. Population structure estimate with K = 8
Figure V-4. Iberian Buthus phylogenies base on the multilocus dataset
Figure V-5. Response curves (RC) for the two Bioclimatic variables
Figure V-6. Maps of the predicted persistence
Figure VI-1. Schematic illustration of the tempo of the tectonic phases and magmatic activity in Morocco

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### **ABBREVIATIONS**

- 16S 16S rDNA small subunit
- 28S 28S rDNA large subunit domain
- ABGD Automatic Barcode Gap Discovery
- AIC Akaike information criterion
- ANM Anonymous Nuclear Markers
- AUC Areas under the curve
- **BI Bayesian Inference**
- BIC Bayesian information criterion
- BIOV Bioclimatic variables
- BLAST Basic Local Alignment Search Tool
- bp Base pairs
- BS Bootstrap
- CCiTUB Centres Científics i Tecnològics de la Universitat de Barcelona
- CCR correct classification rates
- CIPRES Cyberinfrastructure for Phylogenetic Research
- CofV Coefficient of variation
- cox1 Cytochrome c oxidase subunit I
- CTMC Continuous-time Markov chains
- D4kD Defensin 4kD
- DNA Deoxyribonucleic acid
- ENM Ecological Niche Modelling (or Ecological niche-based models)
- EPIC Exon-primed Intron-crossing
- ESS Effective sample size
- FCT Fundação para a Ciência e Tecnologia
- GB GenBank
- GCM Global Climate Models
- GMYC General Mixed Yule Coalescent
- GTR General Time Reversible nucleotide substitution model
- H3 -Histone 3
- HKY Hasegawa-Kishino-Yano nucleotide substitution model
- ICZN International Code of Zoological Nomenclature
- ITS Internal Transcribed Spacer
- LGM Last Glacial Maximum

- LIG Last Inter-Glacial
- Lys-C Lysozyme precursor C
- Ma Million years ago (subjective, before the present)
- MCMC Markov chain Monte Carlo
- Med Mediterranean region
- MetT Methyl transferase
- ML Maximum Likelihood
- MSC Messinian Salinity Crisis
- mtDNA mitochondrial DNA
- MTP minimum training presence
- My Million years (a measure of time)
- NGS Next Generation Sequencing (= Massive Parallel Sequencing)
- NNI Nearest Neighbour Index
- NPCL Nuclear Protein Coding Loci
- NSRF National Strategic Reference Framework
- nuDNA nuclear DNA
- ON.2 North Portugal Regional Operational Program
- PCR Polymerase Chain reaction
- PK Protein kinase-like
- POPH-QREN Programa Operacional Potencial Humano Quadro de Referência
- Estratégico Nacional
- PP posterior probability (as in Bayesian posterior probability)
- PSRF Potential Scale Reduction Factor
- rDNA Ribosomal DNA
- RNA Ribonucleic acid
- ROC Receiver Operating Characteristic curve
- RRL Reduced Representation Library
- SD (or stdev) Standard Deviation
- SDM Species Delimitation Methods
- SNP Single Nucleotide Polymorphism
- SYM Symmetrical model of nucleotide substitution
- TMRCA Time to the most recent common ancestor
- ULC Uncorrelated relaxed clock

# Chapter I GENERAL INTRODUCTION

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#### 1 - Introduction

The study of the Earth's Biosphere (past, present, and future), is the goal of Biology, and one might argue, of humankind.

Biological diversity can be assessed at different levels, from molecular to organismal, and into the ecosystem. Several disciplines have emerged to study them. Many, like Systematics and Ecology, are well establish, while others, such as Phylogeography, are still discovering their role. Nonetheless, all were modified by the advances of Molecular Biology, especially the introduction of the Polymerase chain reaction (PCR) technique. PCR's and sequencing brought a wealth of information it in the last two decades that is testing and changing our understandings on the Evolution of Life. Massive parallel sequencing marked an exponential-fold increase in the amount of information generated, although its usage is still capped by a bioinformatics bottleneck.

Species are fundamental units of Biology. Yet defining them has long remained a seemingly intractable problem, prone to multiple and heated debates. The recent decoupling between the discussion on the theoretical definition of species and the operational criteria used to delimit them in nature has brought some calm to the debate. In this work we adopt the modern view of species as independently evolving metapopulations (de Queiroz, 2007; Mayden, 1997) that exist within the continuum of Earth's Life History.

Species delimitation is both fundamental and necessary for informing political decisions and for everyday communication, an essential human activity.

### 1.1 Scorpiones, a resilient lineage of Arthropods

### 1.1.1 Scorpiones position in the Chelicerata tree of life

The Chelicerata Heymons, 1901 subphylum are a very diverse lineage within the Arthropoda von Siebold, 1848, second only to the Hexapoda Latreille, 1825 in number of known species. They appeared early in the fossil record, which at least in part explains the lack of resolution in the ancestral relationships among chelicerates.

Xiphosura Latreille, 1802 are the first unequivocal extant Chelicerata, appearing in the fossil record in the Upper Ordovician (circa 445 Ma) (Rudkin, Young, and Nowlan 2008). Two other Orders, now extinct, also appear at this early stage in the fossil record: Chasmataspidida Caster & Brooks, 1956, at the Middle Ordovician (circa 460 Ma) (Dunlop, Anderson, and Braddy 2004) and Eurypterida Burmeister, 1843 (sea scorpions), at the Middle Ordovician (circa 455-460 Ma) (Størmer 1951). Eurypterida have long been considered the sister clade of Scorpiones C. L. Koch, 1850, or alternatively, the stem group from which Scorpiones evolved (Dunlop and Webster 1999). The two lineages share many similar morphological traits, including what appears to be a telson in the distal portion of the metasoma, but this trait only appears on specialised eurypterids. Recent cladistic analysis of Chelicerata, extinct and extant, do not recover the clade Eurypterida + Scorpiones (Garwood and Dunlop 2014; Shultz 2007). Their results support the clade Merostomata (Xiphosura (Eurypterida + Chasmatapspidida).

It is gaining acceptance that all other extant lineages of Chelicerata are included in the class Arachnida Lamarck, 1801. These include 12 extant Orders: Actinotrichida (= Acariformes), Anactinotrichida (= Parasitiformes s.l.), Amblypygi, Araneae, Opiliones, Palpigradi, Pseudoscorpiones, Ricinulei, Schizomida, Scorpiones, Solifugae and Uropygi (Thelyphonida), and four extinct orders: Haptopoda, Phalangiotarbi, Trigonotarbida, and Uraraneida. The monophyly of the Anactinotrichida is still an open debate, as are many of the relationships between the various extant orders (Sharma et al. 2014).



Figure I-1. Extant Arachnida Orders cladogram adapted from Sharma et al. (2014) phylogram (Fig. 11B) obtained from a ML analysis of 500 slowevolving genes. Outgroups were removed and Orders were collapsed. Schizomida and Palpigradi were not analysed.

The placement of Scorpiones, within the Arachnida has also changed drastically over the years (e.g. Jeram 1994b; Weygoldt and Paulus 1979). Scorpiones were for a long time considered the sister group of the remaining Arachnida (reviewed in Dunlop and Webster 1999). Very recently Sharma et al. (2014) reconstructed the phylogeny of Chelicerata using Phylogenomics (Fig. 1), with analyses that included 3,644 orthologous loci. However, possibly due to accelerated evolutionary rates in Acariformes, Parasitiformes, and Pseudoscorpiones the recovery of a monophyletic Chelicerata or Arachnida was not obtained, potentially due to long branch attraction. Only their analysis of 500 slow-evolving genes recovered those clades with strong support. Among the few relationships that were not recovered with high support was included the branch of the tree with the three above orders.

The robust placement of Scorpiones as a derived Arachnida, and the sister clade to the long accepted Tetrapulmonata (Araneae (Uropygi, Schizomida, ?Amblypygi)), proposed by Scholtz and Kamenz (2006), allows for the assumption that all Arachnida Orders were already diversifying at least since the Silurian, around 430 Ma (Dunlop 2010). If early scorpions were aquatic in origin, as is commonly accepted, this would imply multiple terrestrialization events within the chelicerates, although this assertion is far from proven (Dunlop, Tetlie, and Prendini 2008). The short internal branches of the reconstructed Arachnida tree with slow-evolving genes (Sharma et al. 2014) supports the theory of a rapid radiation early in the history of Arachnida (Dunlop 2010; Giribet et al. 2002; Regier et al. 2010).

### 1.1.2 Origins & paleo-history of Scorpiones

Scorpions are the oldest known arachnids. They first appear in the fossil record in the Silurian, around 430 Ma (Dunlop and Selden 2013), although probably at this time they still had an aquatic (or amphibian) lifestyle (but see Dunlop, Tetlie, and Prendini 2008). One of the earliest known scorpions is *Proscorpio osborni* (Whitfield, 1885) (Fig. 2). The first terrestrial scorpions are known from fragments from the Lower Devonian (circa 408-393 Ma, Shear *et al.* 1996), with several examples known from the Lower Carboniferous (e.g. Jeram 1990).

These scorpions' present book-lungs that appear to have evolved directly from the book-gills of the first scorpions. These changes are among the few observable differences in body plan from the early aquatic scorpions, and it is because of this conserved body plan that Scorpiones are considered among the "living fossils". Other important changes relate to modifications in the legs, that became progressively gracile and especially with the shortening of the tarsus when compared with the basitarsus, a modification for walking on land (e.g. Waddington *et al.* 2015). Other important changes include a reduction in chelicera size and other modifications in the prosoma to form a preoral cavity that allows liquid feeding, characteristic of all terrestrial arachnids (Jeram 2001). Other necessary changes, for example, behavioral or physiological, albeit have surely occurred, hardly ever leave a mark in the fossil record.



**Figure I-2.** Dorsal view of Proscorpio osborni (Whitfield, 1885) (Scorpiones) from the late Silurian (428 Ma) of New York, U.S.A (adapted from Dunlop 2010).



Figure I-3. Current most plausible fossil scorpions phylogeny (modified from Dunlop et al., 2007).

During the Carboniferous period, the diversity of body plans of Scorpiones species reached its peak (Dunlop et al. 2016). This may be explained in part by the concurrent existence of aquatic and terrestrial species, although some authors now believe that most Palaeozoic scorpions may have been terrestrial (see Jeram, 1994b). Throughout the Carboniferous period, three different suborders of scorpions persisted. The most plesiomorphic were the Palaeoscorpionina, and the most derived were the Neoscorpionina. In this thesis, the Scorpiones higher level taxonomy proposed by Stockwell (1989) and posteriorly emended by Jeram (1998, 1994a, 1994b) and Dunlop et al (2008) (Table 1) is followed. This cladistic classification is in stark contrast to the one proposed by Kjellesvig-Waering (1986), still heavily used today.

Table I-1. Higher level taxonomy of the Scorpiones. Ranks are ordered as Phylum, Subphylum, Class,Order, Suborder and Infraorder. † - depict extinct taxa.ARTHROPODA von Siebold, 1848

CHELICERATA Heymons, 1901 ARACHNIDA Lamarck, 1801 SCORPIONES C. L. Koch, 1850 Palaeoscorpionina† Stockwell, 1989 Mesoscorpionina† Stockwell, 1989 Neoscorpionina Thorell & Lindström, 1885 Palaeosterni† Stockwell, 1989 Orthosterni Pocock, 1911 The Orthosterni includes all 19 extant Families of Scorpiones (Fig. 2, Suppl. Table I.1), as well as other taxa that went extinct. Notwithstanding its Carboniferous origins, the monophyletic crown group with all extant scorpion has a possible post-Carboniferous last common ancestor, because thus far all fossil Carboniferous scorpions belong to the stem group (Jeram 1994a, 1998) (Fig. 3).

For the remaindering of this thesis, references to the extant Orthosteni Scorpiones will be simplified as Scorpiones or scorpions, depending on the context.

Modern scorpions share at least 11 morphological synapomorphies (Coddington *et al.* 2004 and references herein) including chelate pedipalps, pectines (ventral sensory comb-like sensory appendages in the mesosoma), a five-segmented metasoma (post-abdomen) that ends with a modified telson composed of a proximal vesicle with a pair of internal venom glands and a distal aculeus (analogous to a hypodermic needle) for venom delivery. Most of these traits are readily observed in Fig. 2 of *P. osborni*.

#### 1.1.3 Scorpions overview

Scorpions are opportunistic nocturnal predators that usually adopt a sit-and-wait strategy. Scorpions exist in all continents except Antarctica (Fig. 4), have colonised most terrestrial habitats except those found in boreal latitudes, although they do occur in alpine habitats (Ochoa et al. 2011), and can even be found in the intertidal zone (Polis 1990). Scorpion's maximum diversity is found in subtropical areas, especially in desert environments where the most diverse scorpion communities have been observed. Also in the deserts, scorpions can be the most abundant predators (in biomass), only supplanted by ants and termites (Polis 1990).



Figure I-4. Scorpions worldwide distribution (in red) (source vaejovidae.com/Distribution.htm).
Despite being considered a living fossil, with a non-changing *bauplan* over the last 430 My, by all measures scorpions are sturdy survivalists, having survived four mass extinction events. According to Polis (1990), their long-term success can be better explain by "ecology, behaviour, physiology, and life history" plasticity rather than morphological diversification.

More than 100,00 species of Arachnida have been described (Chapman 2009), the vast majority of which are either Acari *s.l.* or Araneae, that together represent around 87% of the total. Scorpions represent only a small portion of the arachnids, less than 2%, with around 2,320 described species (Rein 2016, Chapman 2009 listed 2,400). Of these, Buthidae represent nearly half ( $\approx$ 45%) (Rein 2016), making them the most species diverse scorpion family (Suppl. Table I.1).

# 1.1.4 Difficulties and recent breakthrough in Scorpiones extant family phylogeny, and diversity overview

Scorpiones systematics remained long unresolved, as can be readily observed by the changes that have occurred over the past 10 years. This was made abundantly clear by the two concurring classifications of Soleglad & Fet (2003) and Prendini & Wheeler (2005) (Suppl. Table I.2- I.4). Both classifications derive from the original cladistic work of Stockwell (1989, unpublished), and its original scoring of several morphological traits, amplified with more taxa and traits analysed. Although both Soleglad & Fet (2003) and Prendini & Wheeler (2005) presented data from a limited in scope molecular phylogenetic tree, they support their conclusions on the more robust morphological dataset. Their different classifications reflect above all the differences in the scoring of morphological traits, and thus the urgent need for a robust molecular approach, at least to test independently their different hypotheses.

It was only after the work of (Sharma et al. 2015) that a robust molecular phylogeny of the entire order was reconstructed using a phylogenomics approach. The authors tested several data matrixes, ranging from 136 to 5025 orthologous genes and all resolved the Scorpiones as a monophyletic clade nested within the Arachnida, again as the sister clade to the Tetrapulmonata. Furthermore, the Familial relationships could be resolved with very strong support and changed some long assumed relationships within the Scorpiones (for a complete overview of the newly proposed classification see Suppl. Table I.1). In their phylogeny, the Scorpiones are grouped in two clades, the Buthida Soleglad & Fet 2003 and the lurida Soleglad & Fet 2003, although these parvorders were substantially modified from their original description. These two clades split unevenly the 19 families accepted by Sharma et al. (2015) (Fig. 5), although interestingly each clade

comprises roughly half of the known extant scorpion species. Furthermore, the Vaejovidae, Hormuridae, and Chactidae were found to by polyphyletic.



The earliest known unambiguous records of modern scorpion families in the fossil record are from the Lower middle Cretaceous (Fig. 3), with representatives of the Chactidae and Hemiscorpiidae from around 110 Ma (Menon 2007) and of the Chaerilidae from 90-110 Ma (Santiago-Blay et al. 2004). A much early record of Protobuthidae, an extinct Buthida, from the Anisian age of the Triassic (around 240 Ma) (Lourenço and Gall 2004) has been published although Dunlop (2010) dispute the claim that it closely resembles any extant family.

### 1.1.5 Humans and Scorpions

Scorpions are one of the most easily recognisable groups of organisms on Earth. They are also among the most feared by humans. They have long been part of Human life and folklore, and this is best symbolised by their representation in Scorpius, one of the original Babylonian zodiac signs, opposite to Orion in the night sky, and who was killed by a scorpion while threatening to kill all animals on Earth. Scorpions are present on the early Human written history, including the Babylonian Epic of Gilgamesh, the ancient Egypt's Book of the Death, the Bible, both Old and New Testaments, etc. Scorpions were also referred in antiquity by the Chinese, the Maya, the Greeks, etc. Although most representations are negative, the scorpion god Serket had an important benign role in the Ancient Egyptian mythology.

Presently it is the fear of scorpionism that drives human-scorpion interactions. Scorpionism is an important medical condition in several areas of the World, especially in North Africa and the Middle East, South Asia and Central and South Americas (Fig. 6) (Chippaux 2012; Chippaux and Goyffon 2008). Modern purified anti-venoms are used in Brasil but have for example gone out of production in Morocco (Chippaux 2012), although in this country routine eradication protocols have been implemented.



**Figure I-6.** World incidence and mortality of scorpionism, highlighting high disparities in different World regions (modified from Chippaux 2012)

# 1.2 The Mediterranean region: an outline

## 1.2.1 General overview

The Mediterranean area has a complex topography (Fig. 7) with its Sea, several Peninsulas (the largest being the Anatolian, the Balkan, the Iberian and the Italian), a great number of Islands (the five largest are: Sicily, Sardinia, Cyprus, Corsica, Crete), several high mountains (mountains and other areas with peaks above 3,000m a.s.l.: Atlas, Mount Lebanon, Anti-Taurus, Taurus, Mount Etna, Alps, Pyrenees and the Betic Ranges) and depressions (the lowest is the Qattara in Egypt). These mountains systems were all originated by the Alpine-Himalayan orogeny belt (Rosenbaum and Lister 2002), albeit at different times, as this is an episodic chain of events that started in Late Mesozoic (perhaps 110 Ma) and continues to the present (Lister, Forster, and Rawling 2001). This processes can perhaps be approximated as a Geological analog to the Punctuated equilibrium theory (Eldredge and Gould 1972). In the Mediterranean area, severe bending of this belt (Fig. 7) started at the Miocene, 30 Ma (Rosenbaum 2014).



Figure I-7. Relief map of the Mediterranean Sea in which the Alpine-Mediterranean oroclines (bending orogenic belts) are highlighted (modified from Rosenbaum 2014).

The Mediterranean Sea is an almost landlocked sea, connected to the Atlantic Ocean and the Black Sea by two narrow Straits, respectively Gibraltar and Bosporus (Fig. 10). The artificial Suez Canal further connects it to the Red Sea (Fig. 10). Politically, 23 countries have at least a portion of coast along the Mediterranean Sea, although much more are influenced by it, which makes for a complex area to manage.

The Mediterranean climate, located at mid-latitude between 30 and 45°N (Fig. 8), is characterized as Temperate according to the Köppen-Geiger climate classification (Fig. 9), with dry summers and rainy winters (Fig. 9B), a consequence of its location between subtropical high-pressure systems to the west and south, and westerly wind belts to the north that oscillate from summer to winter (Fig. 8) (Harding, Palutikof, and Holt 2009). These cyclic conditions have created a landscape with forests, woodlands, and scrublands of sclerophyllous plants, extensively modified by Human activities, settlements, and agriculture. The Temperate Mediterranean climate can also be found in parts of Chile, California, South Africa and Australia.



**Figure I-8.** Large-scale atmospheric circulation interactions over North Africa and the Mediterranean area. (modified from Harding, Palutikof, and Holt 2009).

In the broader Mediterranean area, other types of climate exist. The two other most widespread are the Desert or Arid climate and the Steppe or Semi-arid climate (Fig. 9). The Sahara, the largest of the Hot Deserts, occupies a vast portion of North Africa where very little precipitation occurs and warm days exist all year round (Fig. 9B), which greatly reduces the vegetation present. Both Warm and Cold Steppe climates exist in the Mediterranean Area, usually bordering the Hot Desert and/or the Mediterranean climates, but also in the Anatolian Peninsula and the Levant. Although in this later areas total precipitation in the warmest six months of the year is still below potential evapotranspiration, it is above a 50% minimum while Deserts fall below that line, and thus more vegetation can be found in Steppes when compared with Deserts.

The Mediterranean Basin is one of Earth's 36 biodiversity hotspots (Fig. 10) (Mittermeier et al. 2004; Myers et al. 2000; Noss et al. 2015) ranking very high on Global Biodiversity conservation priorities. With 13.000 endemic vascular plant species (4.3% of the World total), and a residual 4.7% of primary vegetation remaining of a potential 2,085,292 km<sup>2</sup> it's the hotspot with the second largest potential area but also one of the most depleted, it clearly fits the definition set by Myers et al. (2000). Significantly, The

Mediterranean Basin is usually defined as the portion of land with a Mediterranean climate that surrounds the Mediterranean Sea (Fig. 9) the wider Mediterranean area includes in its Eastern portion the Irano-Anatolian biodiversity hotspot (899,773 km<sup>2</sup>) (Fig. 10). This topographically complex hotspot was first identified in Mittermeier et al. (2011).



**Figure I-9. A** - Map of the Mediterranean Area with the Köppen-Geiger climate classification represented. Mediterranean climate: Hot and Cold Dry-Summer; Desert climate: Hot and Cold Arid; Steppe climate Hot and Cold Semi-Arid. (modified from Peel, Finlayson, and McMahon 2007). **B** - Climate charts of Norther Hemisphere localities. Mediterranean – Malta (Malta); Warm Steppe – Murcia (Spain); Hot Desert – Sabha (Lybia). Steppe climate charts are more varied than those of the other Climates represented.

As already explained, human impact also played an important role in the creation of the complex landscape patterns that can be found in this area. Human modifications increased exponentially in historical time, within or after the Neolithic Revolution, and the transition to the stable environment of the Holocene (Zahid, Robinson, and Kelly 2016). This resulted from an exponential growth in Human populations that ensued from the beginning of sedentarism and the expansion of agriculture, although this is disputed (Aimé et al. 2013; but see Page et al. 2016). The Fertile Crescent, located in the Eastern Mediterranean area, was one of the earliest and most significant places of Agricultural beginnings. Although insignificant in geological time, Humans historical presence in the Mediterranean area has resulted in a marked decline, in the Mediterranean Basin, of the area presently occupied by primary vegetation (Myers et al. 2000), and explains why the Mediterranean is an area in great need of effective conservation measures, if what remains of its Biodiversity is to be preserved (Henne et al. 2015).



**Figure I-10.** Map of the Mediterranean area with Hotspot areas highlighted. Mediterranean Basin, red dashed area; Irano-Anatolian, yellow dashed area. Gateways: 1 – Strait of Gibraltar; 2 – Strait of the Bosporus, 3 – Suez Canal.

The complexity mix of topography, climate, and Human presence explain the very high levels of biological diversity that are presently found in the Mediterranean area. However, this diversity is rooted in the underlining extremely rich and complex paleoclimatic history of this area.

# 1.2.2 The complex geological history of the Mediterranean Sea since the Miocene

The Mediterranean Sea is located between Africa, Asia, and Europe, in a region with active Tectonic movement that continues to compress and reduce its size (Fig. 11).



**Figure I-11.** Map of the Mediterranean area featuring Tectonic Plates and major fault lines. (source: http://eurasiatectonics.weebly.com/anatolian-plate.html)

The Mediterranean Sea is the western remnant of the much older Western Tethys Ocean. The formation of this ancient Ocean is complex (Müller and Seton 2015; Stampfli 2000; Stampfli and Borel 2002). A Paleo-Tethys Ocean existed to the East of Pangea and started to close when Cimmeria split from Gondwana (around 250 Ma) and started moving northwards (Fig. 12A) while leaving behind the newly formed Meso-Tethys Ocean (Dèzes 1999; Stampfli, Borel, and Cavazza 2001).

In much the same area, at around 140-155 Ma, the Neo-Tethys (or Alpine-Tethys) Ocean started forming after the separation of present day Australia and India (Fig. 12B) from Gondwana (Müller and Seton 2015). The Eastern Neo-Tethys closed around 43 Ma when the Indian subplate collided with Eurasia creating in its place the Indian Ocean (Müller and Seton 2015). At the same time, the Western Neo-Tethys (Proto-Mediterranean) continued to shrink as Africa moved north, until splitting into the Eastern Proto-Mediterranean and the Para-Tethys Sea (Stampfli 2000) (Fig. 12C) due to Alpine orogeny. The latter would later split into the modern Black and Caspian Seas (Harzhauser and Piller 2007; Rögl 1998; Steininger and Wessely 1999). For comments on Tethys, nomenclature usage see Robertson and Mountrakis (2006).



Figure I-12. The Tethys Ocean during the last 250 Ma. A – Triassic; B – Cretaceous; C – Eocene. (A and B modified from Dèzes 1999; C modified from Popov et al. 2004).

The evolution of the Western and Eastern areas of the Mediterranean Sea, although obviously linked, exhibit differences that relate to their proximity to the Atlantic and Indian Oceans respectively. The Western Mediterranean Sea (West Med) has a much younger crust (Rosenbaum, Lister, and Duboz 2002), which was formed during the complex bending's of the Eurasian and African plates (Van Hinsbergen, Vissers, and Spakman 2014; Rosenbaum 2014). The paleogeography of the Eastern Mediterranean (East Med) is complicated, and many details remain difficult to adequately reconstruct, particularly in its northern connection with the Para-Tethys Sea and the motions of the Aegean and Anatolian plates. The connection between the Para-Tethys and the Mediterranean are beyond the scope of this summary, but in concordance with the rest of the Mediterranean paleo-history they were complex, involving at times multiple connections and disconnections (Orszag-Sperber 2006) until the Black Sea once again reconnected with the Mediterranean around 7,500 years ago (Schulz, Bechtel, and Sachsenhofer 2005), during the Holocene sea-level rise, in the area where the Aegean and Anatolian plates connect (Fig. 11).

A summary of the main events in the evolution of the Mediterranean Sea is described below, focusing on events up to the establishment of a land-bridge between the African and Eurasian plates during the Messinian:



**Figure I-13.** Mediterranean paleogeographical and paleotectonic reconstruction from Oligocene onwards. Red arrows depict directions and sense of shear in the exhumed rocks and direction of extension in the sedimentary basins. (modified from Jolivet et al. 2006).

#### Eocene-Oligocene boundary (35 – 30 Ma) (Fig. 13A)

During this period the African and Eurasian ocean crusts had already started to collide in the Western Proto-Mediterranean (Guiraud et al. 2005; Jolivet and Faccenna 2000) but in what was to become the East Med, a gateway persisted between the Eastern Proto-Mediterranean and the Indian Ocean (Popov et al. 2004; Rögl 1998). A further consequence of the African and Eurasian plates colliding was the separation of the Arabian plate from the main African plate around 30 Ma, which started above the Afar triangle plume (Jolivet and Faccenna 2000) extending East to the newly opening Gulf of Aden. Consequently, also, the Horn of Africa emerged. Around the same period (30 Ma), in the Western Proto-Mediterranean, Iberia was still composed of several terrains that were just starting to move away: the Betic-Rif Cordillera, the Balearic Islands, the Kabylies, Corsica, Sardinia, and Calabria (Van Hinsbergen et al. 2014).

#### Oligocene- Miocene boundary (~25 – 23 Ma) (Fig. 13B)

Iberia terrains of the Western Proto-Mediterranean continued to migrate eastwards (Jolivet et al. 2006; Rosenbaum et al. 2002). Although it had started rifting earlier, the Eritrean Red Sea, a continental sea at this time, became a marine realm around 24 Ma (Bosworth, Huchon, and McClay 2005), when it connected south with the Gulf of Aden and north with the Mediterranean Sea (Fig. 14C). These marine areas continued deepening until around 20 Ma.

#### Early Miocene: ~ 21 – 18 Ma (Fig. 14A)

In the early Burdigalian (21 Ma), the Balearic Islands separated from the Kabylies blocks and the Betic-Rif Cordillera started arching into its two components (Platt et al. 2013; Rosenbaum et al. 2002). Afterward, at around 18 Ma, Corsica, Sardinia and Calabria collided with the Apennines (Jolivet et al. 2006; Rosenbaum et al. 2002). In the

east Proto\_Med, at around 19 Ma (Cox 2000; Harzhauser et al. 2007) or 20 Ma (Okay, Zattin, and Cavazza 2010) the Arabian (African) and Anatolian (Eurasian) plates collided in the Bitlis-Zagros suture zone. This closed the Indo-Proto-Mediterranean gateway and originated the "*Gomphotherium* Landbridge" (Rögl 1998) which promoted the exchange of faunal elements between the two distinct plates. Some authors have advocated an earlier, short-lived land bridge, around 23-22 Ma (reviewed in Harzhauser et al. 2007).



**Figure I-14.** Mediterranean paleogeographical and paleotectonic reconstruction in the Miocene. Figures C to E zoom in the Arabian plate region. Red areas in the latter Figures represent volcanic extrusions. (A and B modified from Jolivet et al. 2006; A to C modified from Bosworth, Huchon, and McClay 2005).

#### Middle Miocene: ~ 15 Ma (Fig. 14B)

The Kabylies blocks continued moving south until they collided with Africa (around 15-16 Ma, Fig. 15C) (Van Hinsbergen et al. 2014; Rosenbaum et al. 2002). The Indo-Proto-Mediterranean gateway reopened during the "Langhian transgression" (16-14 Ma) (Hamon et al. 2013; Rögl 1998), although the exact duration of this period remains uncertain. The gateway was much shallower at this stage (Hamon et al. 2013), as this re-opening was most probably caused by sea-level rise. This gateway finally closed in the Early Serravallian (14 Ma) (Rögl 1998), although a later closing has been proposed at 12 Ma (Cox 2000) or 11 Ma (Hüsing et al. 2009). This closing finally isolated the Eastern Proto-Med from the Indian Ocean. At this time the Red Sea started evaporating (~14 Ma) (Bosworth et al. 2005).



**Figure I-15.** Mediterranean paleogeographical and paleotectonic reconstruction in the Late Miocene. Figure C focus on the West Mediterranean region, in which intermediate blue represents marine back-arc basin. Figures D and E zoom in the Strait of Gibraltar area. (E and F modified from Jolivet et al. 2006; D3 modified from Rosenbaum et al. 2002; E3 and F2 modified from Martín et al. 2009).

#### Middle-Late Miocene: 10 Ma (Fig. 15A)

The Betic-Rif Cordillera reached its current position during the Tortonian, at around 10 Ma (Rosenbaum et al. 2002), at which time the Mediterranean Sea acquired is present geography. The Betic-Rif Arch originally created two gateways between the Atlantic Ocean and the Mediterranean Sea, the "Betico-Rifian Portals" (Fig. 15D) (Blanc 2000). Also around 10 Ma the Bab al-Mandeb Strait completely closed, creating a land-connection between Arabia and Africa (Fig. 15D) (Bosworth et al. 2005).

#### Late Miocene: ~ 6 Ma (Fig. 15B)

The Messinian was a time of great turmoil in the Mediterranean area. In the summary of Roveri et al. (2014), the Betic gateway was the first to end, around 6.3 Ma (Fig. 15E), while the end of the Rifian gateway occurred sometime before 6.0 Ma (Martín et al. 2009) (but see Hüsing et al. 2010). The closing of the last connection between the Atlantic Ocean and the Mediterranean Sea and consequent termination of Atlantic water inflow eventually led to its desiccation because, in the Mediterranean Sea, the rate of water evaporation surpasses the amount of fresh water that arrives from rivers alone (Fig. 16A) (Blanc 2006). This extraordinary event was coined the Messinian Salinity Crisis (MSC) (Hsü, Montadert, and Bernoulli 1977). Three phases are now accepted to explain the desiccation of the Mediterranean, which at first was noticeable only in its shallow margins (5.8 Ma), but eventually propagated to the entire basin reaching a peak when a deep decline in sea water level occurred (5.6 Ma, Fig. 16A) (Popov et al. 2004) that left large

Evaporates in its lowest sub-basins. At 5.55 Ma Lago-Mare conditions started to prevail, a time in the Med when water exchange with other basins restarted, either with the Para-Tethys, the Atlantic or both, which could have been episodic or continuous, but details are still uncertain (Roveri et al. 2014). The MSC ended with the very rapid refilling of the Mediterranean Sea at the beginning of the Zanclean (Fig. 16B), either straight away, or in a two events model (Bache et al. 2012), at which time the Strait of Gibraltar was created (Krijgsman et al. 1999; Loget and Van Den Driessche 2006; Roveri et al. 2014).



**Figure I-16.** Mediterranean paleogeographical and paleotectonic reconstruction from the Miocene-Pliocene transition. A – The MSC at its shallowest point; B – The Med after the opening of the Strait of Gibraltar. Intermediate blue in the figures represents freshwater lakes. (modified from Bache et al. 2012).

Meanwhile, in the East Mediterranean, the Bab al-Mandeb Strait opened around 5 Ma (Fig. 14E), marking the end of the land connection between Arabia and Africa through this area (Bosworth et al. 2005; Fernandes, Rohling, and Siddall 2006). At this time, the Suez Isthmus was uplifted, ending the Mediterranean – Red Sea connection and becoming in the process the single land corridor between the African-Eurasian Continents (Bosworth et al. 2005). This land-bridge has only sporadically been broken since this time, during periods of very high sea-levels, until, of course, the opening of the artificial Suez Canal in 1869.

The MSC lasted only 600 Ky, having taken place between 5.971 and 5.33 Ma (Roveri et al. 2014) but left profound and lasting consequences in the Phylogeography of Western-Palearctic Biota. The MSC provided the last land bridge connection between Western Europe and North Africa, and possibly also between the Aegean and Anatolian Peninsulas (Poulakakis et al. 2015) and between the latter and the island of Cyprus (Poulakakis et al. 2013). Europe was not only connected to North Africa through the Betic-Rif Arch (presently Spain and Morocco) but also through the Calabrian Arch (presently Italy, Sicily and Tunisia) (Rosenbaum et al. 2002). The latter corridor, which reaches its deepest region in what is now the Sicilian Strait, has greater complexity with author's diverging over the character of this connection, if it was a real land-bridge or a stepping-stone system, especially in the later part of this period.

From the Pliocene onwards the Mediterranean area continued to change, even if less dramatic events were taking place, especially in the areas of the Italian Peninsula and the Aegean Sea. Furthermore, during the Glaciation cycles of the Pleistocene, Sea levels changed accordingly. Moreover, tectonic motion remains and has been promoting orogenic changes throughout the Med basin to the present (Fig. 7).

# 1.2.3 Climatic changes in the Mediterranean area since the Miocene

It has now become clear that a few drivers control the general Climate of the Planet. These include astronomical cycles (e.g. Milankovitch cycles), atmospheric composition (e.g.  $CO_2$  concentration) and plate tectonics (e.g. when a strait is closed changing a global ocean current) (Zachos et al. 2001).



**Figure I-17.** Global climate change from the Miocene to the present. Data points were recorded by the Deep Sea Drilling Project and Ocean Drilling Program sites based on deep-sea benthic foraminiferal oxygen isotope. Modified from Zachos, Dickens, and Zeebe (2008). MedCl – Mediterranean climate onset?; LMC – Late Miocene cooling; MMCT – Middle Miocene Climatic Transition; MCO – Miocene Climatic Optimum.

Paleo-Climate reconstruction, shaped by the modelling of these drivers plus revelations from ice-drilling and pollinic reconstruction, has revealed that from the end of the Oligocene through the middle Miocene (until the Serravallian Age, 11.62 MA) the climate remained warm, peaking at the Miocene Climatic Optimum (17 to 15 Ma, during the Burdigalian Age, Fig. 17) (Zachos et al. 2001). During this period a Humid Subtropical climate existed in the Mediterranean area, with mild, stable temperatures, and high

humidity that allowed the existence of subtropical laurophyllous forests, the "Tertiary-Tethyan vegetation" (Axelrod 1975). This type of vegetation also existed in the Iberian Peninsula nevertheless in the south shrublands and savannas had started to develop in the Langhian (Fig. 19), an arid trend that included drier woodlands in the Central portion of Iberia by the Serravallian (Fig. 19) (Pound et al. 2012). Although less evidence exists for North Africa, "Tertiary-Tethyan vegetation" was also prevalent at these times (Rodríguez-Sánchez and Arroyo 2011). Nevertheless, during the Langhian, the Middle Miocene Climatic Transition ( $\approx$  14.2 to 13.8 Ma, Fig. 19) (Hamon et al. 2013; Zachos et al. 2001) initiated a trend towards a cooler climate. From Tortonian to Messinian Ages (11.63 to 5.332 Ma) a great shift from an equable climate (Axelrod 1992), of roughly aseasonal equal temperatures throughout the world, to a modern climate type occurred, with its strong gradient of equator-to-pole temperature difference and strong seasonality at higher latitudes (Herbert et al. 2016).



**Figure I-18.** Distribution of the terrestrial Biomes as defined by Olson et al. (2001). **A** – present distribution; **B** – Pliocene reconstructed distribution from palaeobotanical data, modified from Carrión et al. (2010).

This was especially clear at middle and high latitudes during the late Miocene cooling (Messinian,  $\approx$ 7.2–5.4 Ma) (Herbert et al. 2016). In the Mediterranean this represented a shift towards a cooler and less humid climate (Tzanova, Herbert, and Peterson 2015), although that did not bring major changes on vegetation from the

previous Ages in the Iberian Peninsula (Pound et al. 2012), with a continuing aridification of the south-western Iberia (Jiménez-Moreno, Fauquette, and Suc 2010). By contrast in North Africa, since the Tortonian, the existence of savannas, grasslands, shrublands and sclerophyll woodland started to prevail (Fig. 19) (Pound et al. 2012) depicting an increase in aridity when compared with Europe. Furthermore, it was around the Messinian Age that the Sahara Desert originated, around 7 Ma (Schuster et al. 2006), although this has been challenged with a more recent onset (Kröpelin and Swezey 2006). Moreover, Zhang et al. (2014) have postulated an even earlier onset of desertification in Nort Africa, during the Tortonian. This aridification was cyclically interrupted by alternating periods of humid climate and riverine systems (Armitage et al. 2007; Feakins and deMenocal 2008; Trauth, Larrasoaña, and Mudelsee 2009).



**Figure I-19.** Miocene Past point distributions of the terrestrial Biomes, reconstructed from palaeobotanical data: Four different Ages are shown, modified from Pound et al. (2012). Colour scheme as in Figure 18.

During the Pliocene, a small reversal of this cooling trend occurred (Fig. 17) (Herbert et al. 2016; Zachos et al. 2001), with a climate somewhat more equable than at present (Haywood, Sellwood, and Valdes 2000). In the Iberian Peninsula the vegetation trend continued further, with the replacement of thermophilous with mesothermic plants, and a marked increase in steppes (Jiménez-Moreno et al. 2010), with a progressive vegetation North/South gradient similar to the present starting (Fig. 18B) (Feddi, Fauguette, and Suc 2011). In Morocco even further aridification is recorded (Feddi et al. 2011). This Temperate, stable climate, changed abruptly 3.2 to 3.4 Ma, during the Piacenzian Age, when a Mediterranean Climate, with its cyclic patterns of drier summers and humid winters, was established (Fig. 17) (Jiménez-Moreno et al. 2010; Suc 1984). This led to a drastic change in the vegetation, to a more drought resistant type, and probably also to a more diverse landscape, with forests, scrubland, etc, which can be found in the area today, characterised by a mosaic of habitats. Tzedakis (2007) challenge this view, pointing that such a Mediterranean climate could have existed intermittently for the entire Tertiary, and thus that sclerophyllous vegetation evolution is much older. These changes in the climate finally led to the almost complete eradication of the Laurisilva (Rodríguez-Sánchez and Arroyo 2011), and a marked increase in steppes (Tzedakis 2009). By the end of the Pliocene ice sheets became permanent also

in the Northern Hemisphere (Zachos et al. 2001), and the cyclic climate that would characterize the Pleistocene was starting (Haywood et al. 2009).

The Pleistocene, from 2.58 Ma to 11,700 BCE (Fig. 17), marked a period of severe climatic oscillations throughout the Planet, Mediterranean area included, with cycles of Glacial and Interglacial climate (EPICA Community Members 2004), which provoked a further decline of forests on the Northern Mediterranean (Feddi et al. 2011). With the beginning of the Calabrian Age, herbaceous vegetation further spread (Tzedakis 2009), although these would alternate with the extension of forest accompanying the alternating Glacial and Interglacial periods (Feddi et al. 2011). The Sahara accompanied these oscillations, contracting and expanding periodically (Trauth et al. 2009), although three long humid periods have also been reported for the Pleistocene (Trauth et al. 2005). By Middle Pleistocene, the vegetation in the Mediterranean area reached a composition similar to the present (Tzedakis 2009).

Thus, the onset of the Mediterranean climate occurred at least 3.4 Ma (Fig. 17), although ardification and steppe-like vegetation in portions of the Mediterranean area is clearly older, perhaps 16 Ma (Carrión et al. 2010) supporting the conclusions of Tzedakis (2007), which advocates an even earlier start.

#### 1.2.4 Phylogeography of the Mediterranean area since the Miocene

Phylogeography was first envisioned to study the "principles and processes governing the geographical distributions of genealogical lineages" (Avise 1998). Thus, it has been at the forefront of the study of speciation since its inception (Avise 2000; Avise et al. 1987; Hewitt 2001). The incorporation of the coalescent theory (Avise 2000; Hickerson et al. 2010) gave it a powerful framework with which to address these issues at the intersection between micro and macro-evolutionary processes (Avise 2000) to understand the spatial and historical structure of biodiversity (Hewitt 2004).

The Pleistocene left a strong mark in the genetic structure of European biodiversity (e.g. Hewitt 2000, 2011). Species ranges shrunk of suffered local extinctions during the Glacial periods when great portions of Northern Europe were occupied by permanent ice sheets or permafrost (Randi 2007) that would posteriorly be re-colonised during the range expansions of the Inter-Glacial periods. Such patterns have been identified in multiple studies (reviewed in Various 2007) and have led to establishing a hypothesis in which Southern Europe, and especially the Mediterranean peninsulae (Iberian, Italian and Balkan) (Fig. 20), acted as climate refugia (Gavin et al. 2014). Although these patterns have been found to be broadly accurate, the Caucasus (Hewitt 2000) and even

further east into Asia (Bilton et al. 1998; Hewitt 2011b; Randi 2007), have also acted as refugia for North European fauna.



**Figure I-20.** Overview of faunal glacial refugia in the Mediterranean area, biogeographical sub-centres and hybrid zones of the south-western Palaearctic region (modified from Husemann et al. 2014). H – hybrid zones R – refugia. Subcentres: 1 – Atlanto-Mediterranean; 2 – Tyrrhenian; 3 – Adriato-Mediterranean; 4 – Ponto-Mediterranean; 5 – Cyprian. 6 – Cretian; 7 – Cyrenian; 8 – Mauritanian; 9 – Canarian.

The climate refugia hypothesis has been further refined with the identification of multiple refugia within refugia (microrefugia) (Gómez and Lunt 2007) in those southern areas. Similar results have been found when plotting the centres of persistence for several plant species (Médail and Diadema 2009) (Fig. 21). Moreover, for species with greater tolerance to the cold, small suitable areas may have persisted further north of the Mediterranean peninsulae (Gavin et al. 2014; Salvi et al. 2013) but not that much north (Tzedakis, Emerson, and Hewitt 2013). Species differential cold tolerance (vulgarly termed mediterranean and continental) (Schmitt 2007) are also part of the explanation for the platitude of microrefugia that have been proposed more often not coincident between taxa, especially for this dichotomy.

These range contractions and expansions, together with the persistence of multiple microrefugia, left genetic imprints on Mediterranean (and European) species. These include species with shallow genetic diversity (at least in their northern range) and recent demographic expansion signals (e.g. Carranza, Arnold, and Pleguezuelos 2006; Miraldo et al. 2011; Veríssimo et al. 2016) and species with hybrid zones (e.g. Alves et al. 2006; Gonçalves et al. 2007; Sequeira et al. 2005), sometimes complex (e.g. Freitas et al. 2016; Martínez-Freiría et al. 2008), were genetically divergent populations from different microrefugia/refugia would re-contact after a glacial retreat (Fig. 20). Also important is the emergence of species complex (≈ cryptic species), which most often refers to genetically divergent populations/species with little or no morphological differentiation

that have thus been lumped together before the advent of molecular phylogenetic studies (e.g. Gómez-Zurita et al. 2012; Habel et al. 2008; Rato et al. 2016; Tamar et al. 2015). The consummate example of this complexity is probably the *Podarcis* Wagler, 1830 walllizard (e.g. Harris et al. 2002; Kaliontzopoulou et al. 2011; Lima et al. 2009; Pinho, Harris, and Ferrand 2007).



**Figure I-21.** Geographical distribution of 52 putative micro-refugia and of the 10 regional hotspots of plant biodiversity within the Mediterranean area (similar to those identified for the fauna in Fig. 20) (Médail and Diadema 2009).

However, despite the undisputed importance of the genetic imprints of the Quaternary glaciations in the Mediterranean biodiversity (Hewitt 2000), ever more examples have been presented with older speciation events. These older phylogeographic patterns have been determined to have started at the Pliocene (Gvoždík et al. 2015; Maia-Carvalho et al. 2014), the Miocene (Douady et al. 2003; Froufe et al. 2016; Kaliontzopoulou et al. 2011; Velo-Antón et al. 2012) and even the Oligocene for several invertebrate species (Bidegaray-Batista and Arnedo 2011; Mora et al. 2016; Opatova, Bond, and Arnedo 2016). These works have highlighted the importance of North Africa in the long term persistence of Western Palearctic fauna (Husemann et al. 2014). They have also demonstrated that the complex paleo-geo-climatic history of the Mediterranean area played an important role in shaping present Mediterranean biodiversity. Furthermore, these works have demonstrated a strong connectivity between North Africa and South Europe, with the former functioning as a centre of origin for the biodiversity that would later find a climate refugee in the Mediterranean peninsulae during the Quaternary glaciations.

Interestingly, these results further advance the need to reassess our understanding of the temporal inconsistencies of current taxonomic ranks (Avise and Liu 2011; Johns

and Avise 1998), especially the invertebrate/vertebrate discrepancies at least at the species level.

### 1.2.5 Scorpions species diversity in the Mediterranean area

The Mediterranean area has a rich assemblage of scorpion diversity, having been recorded seven of the 19 Families accepted families (Akravidae, Buthidae, Euscorpiidae, Hemiscorpiidae, Iuridae, Scorpionidae, Troglotayosicidae). They are unevenly distributed, Euscorpiidae is almost exclusively European (and comprises most of its scorpion fauna), Troglotayosicidae is found only in Iberia (and South America) and Akravidae, Hemiscorpiidae, Iuridae occur only in the Eastern Mediterranean. Of the 40 genera (20% of all known extant scorpion genera) recorded in this area, the three most species diverse are *Euscorpius* Thorell, 1876 (37), *Buthus* Leach, 1815 (35) and *Compsobuthus* Vachon, 1949 (23), that together represent more than 40% of the 229 recorded species in the area (10% of all known extant scorpion species in an area that occupies perhaps 2% of the total Earth surface). As can be seen in Fig. 22, the countries with higher species diversity in the Mediterranean area are Morocco (50), Argelia (33), Egypt (28), Greece (27) and Turkey (26).

If we include a broader area encompassing all of North Africa and most of the Middle East (Fig. 22), areas that have a commum paleo-history to the Mediterranean area as previously explained, the total number of genera (67) and species (432) almost doubles (31.8% and 18.6% respectively, of the extant scorpion diversity). The Hormuridae scorpion family also appears in the tropical African countries, although with only six recorded species. This broader area contains three other scorpion species-rich countries, Ethiopia (43), Yemen (37) and Saudi Arabia (29) (Fig. 22). Nevertheless, Sub-Saharan Africa is greatly under-sampled, even when compared to other under-sampled regions here represented, a problem common in many other animal groups (Harris and Froufe 2005).



Figure I-22. Map of Mediterranean and North Africa species diversity organised by country. Number of species according to data at http://scorpiones.pl/maps, corrected for the genus *Buthus* only.

# 1.3 The scorpion genus *Buthus*, the model organism of this study

#### 1.3.1 General overview

*Buthus* Leach, 1815 scorpions are an important component of the scorpion fauna of the Maghreb, and the only Buthidae genus that occurs in Western Europe. *Mesobuthus* Vachon, 1950 is the only other Buthidae to reach Europe, as its range extends to the southern area of the Balkan Peninsula.

*Buthus* is among the most venomous of all scorpion genera, although far less than many of the genera concurrently listed, which includes *Androctonus*, *Centruroides*, *Hottentotta, Leiurus, Mesobuthus, Parabuthus* and *Tityus*, all Buthidae, and *Hemiscorpius* (Hemiscorpiidae), as the most often quoted. There is a total of perhaps 40 know venomous species (Chippaux and Goyffon 2008). *Buthus* venomosity is considered lower in Europe than in North Africa, which can be empirically corroborated by the absence of serious recorded cases of scorpionism in Europe when compared to the wealth of cases reported for North African countries (Chippaux and Goyffon 2008).



#### An overview of the genus external morphology is given in Figure 23.

**Figure I-23.** General morphology of a *Buthus* scorpion, with tagmata and subtagmata differentiated by colour. Prosoma is covered dorsally by the Carapace and ventrally by the Sternum. Mesosoma and Metasoma segments are numbered. The first is covered dorsally by the Terguites (Ter.) and ventrally by the Sternites (Ste.). Legend: L. e. – Lateral eyes; M. e. – Median eyes; Co. – Coxa; Tr. – Trochanter; Fe. – Femur; Pa. – Patella; Ti. – Tibia (or *manus* in the Pedipalp); Ba. – Basitarsus; Ta. – Tarsus (or movable finger in the Pedipalp); Ap. – Apotele; Ge. op. – Genital operculum; Pe – Pectines; Spi. – Spiracle. (modified from Keegan 1980).

The genus *Buthus*, the type genus of the Buthidae family, was created by Leach, in 1815, with *Scorpio occitanus* Amoureux, 1789 from the Souvignargues, Languedoc-Roussillon, France, as the type species. Leach gave a pauper description and this resulted in a genus with a large number of species but lacking any internal coherence. In 1952, Vachon redefined and restricted the genus to only the species that were morphologically similar to its type species (Fig. 23). Unfortunately, he did so using a very conservative approach to its taxonomy, recognizing only four species, split several subspecies further split into several varieties. Lourenço (2003) marked a renewed interest in the genus taxonomy and diversity with the description of five new species. A detailed overview of the genus systematics and distribution are given in chapter tow of this thesis.

## 1.3.2 Biology

Very little details are known with certain about the biology of the genus, or of any one species. That lack of knowledge at the species level is exacerbated by the significant changes that occurred within the genus taxonomy (see Chapter Two), as for long, an in much of its range, almost all diversity was identified as the nominotypical *B. occitanus* species. As such, much of the information summarize here will be given for the genus as a whole, unless clear species identification could be ascertained. Much of the information presented in this section was summarised from Polis (1990) and all references herein.



Figure I-24. *Buthus* female displaying maternal care, with first instars on top of mesosoma.

(Photo from arachnoboards.com, from user Harashil).

Like all scorpions, *Buthus* are viviparous and give birth to live offspring (Fig. 24). After a gestation period of 10 months on average, a female will give birth to between 10-50 offspring. According to Levy and Amitai (1980) *B. israelis* parturition usually takes place between September and October; with about a dozen progeny. Iberian *Buthus* parturition occurs around June-July (personal observations). *Buthus* scorpions take an average of six moults to reach adulthood.

Laboratory research as demonstrated that *Buthus* have a peak of activity during the first hours of the night, although they can be found active outside of their burrows from dusk till dawn (personal observations). *Buthus* scorpions are usually described as sit and wait predators that will not venture far from the entrance of the burrow. Although this may be the case when considering non-starving adults, juveniles or starving individuals may active forage for prey and will only return to the burrow to consume it. Examples include the remarkable foraging of termites by *B. israelis*, which will sting several individuals before collecting then and carry then back to their burrow (Skutelsky 1995). *Buthus*, especially juveniles have also been found to prey by climbing to shrubs (e.g. Piñero et

al., 2013). This may allow for a wealth of otherwise inaccessible prey or may contribute to reducing intraspecific predation.

Buthus species occur in a wide range of habitats, although they can be generally considered to prefer mesic habitats. Those can range from coastal dunes to Mediterranean shrublands and low-intensity agricultural landscapes (personal observations). Some species have been recorded in could, mountainous areas such as *B. lienhardi* in the Moroccan High Atlas Mountains and *B. montanus* in the Spanish Sierra Nevada. There are also a few *Buthus* species recorded in desert environments, *B. saharicus* occurs in the Algerian Sahara (Sadine, Bissati, and Lourenço 2015), and *B. israelis* is also found in the Sinai and Palestinian deserts (Levy and Amitai 1980).

Thus far the no *Buthus* species has ever had its conservation status evaluated in a systematic process, like those used, for example, by I.U.C.N. No doubt most species would be labeled as Data Deficient, as they are only known from their type localities. Nevertheless, it is reasonable to assume that habitat destruction or degradation is a strong threat to species that occur in areas with intense Human usage. How much that can be exacerbated by the current trend of climate change is unknown, but *Buthus* species occur in Mediterranean habitats that are themselves threaten and might shrink by up to 25% (Barredo, Caudullo, and Dosio 2016). Klausmeyer and Shaw (2009) have predicted that Morocco would be especially affected, and Morocco is the main area of diversity for the genus

#### 1.3.3 Phylogeography of Western Mediterranean Buthus

*Buthus* species have a circum-Mediterranean distribution. This general pattern is oversimplified, however, in Europe *Buthus* can only be found in the Iberian Peninsula, the south of France and Sicily. Moreover, several species have been described south of the Saharan desert, well into the Sahel region. Nevertheless known *Buthus* species diversity reaches its maximum in the Maghreb countries, and Morocco is by far the most specious, with 17 species (see Chapter 1).

A few studies have analysed the phylogeny of Maghreb and southern Europe *B. occitanus* species complex, as was understood before Lourenço (2003) work. The first, and most comprehensive in geographical scope, number and genomic diversity of the markers analised (2 mtDNA and 1 nuDNA), was that of Gantenbein and Largiadèr (2003) (Fig. 25). They analysed *B. atlantis* and what was then four subspecies within *B. occitanus*, and found three highly divergent clades that corresponded to the Iberian Peninsula, Morocco, and Tunisia. Furthermore, they found tree clades within the Iberian Peninsula and several divergent groups within the *B. o. mardochei* samples. Their ML

analysis of the nuDNA fragment recovered a clade containing both Iberian and Tunisian samples. Gantenbein and Largiadèr (2003) only obtain the monophyly of the Iberian samples in the MP analyses of the mtDNA fragments combined (Fig. 25), thus supporting a single colonization event of Iberia that would fit well whit the onset of the MSC, according to the authors.



Figure I-25. Sampling (A) and strict consensus MP tree of the combined 2 mtDNA genes (B) of Gantenbein and Largiadèr (2003) work. Map colours according to the scheme used in the tree.

Two studies using allozymes were also made with *Buthus* samples. Othmen et al. (2004) analysed 18 loci from Tunisian samples, including islands. Gantenbein (2004) analysed 15 loci from a geographic sampling similar to the Fig. 25, with samples from both sides of the Strait of Gibraltar. Both studies found low genetic variation but strong genetic structuring at the population level. Similar to Gantenbein and Largiadèr (2003), Gantenbein (2004) also recovered a clade containing both lberian and Tunisian samples.

During the work of this thesis, further advances were made on the understanding of the mtDNA phylogeographic patterns of Moroccan *Buthus*. Habel et al. (2012) analysed four *Buthus* species (*B. elmoutaouakili*, *B. malhommei*, *B. albengai* and *B. draa*) from the Atlas Mountains region and found "12 distinct genetic groups, mostly in accordance with the orographic structure of the mountain systems" (Fig. 26A). Husemann et al. (2012) made a detailed study of *B. elmoutaouakili*, founding also "5 distinct genetic lineages" (Fig. 26B). Using a molecular clock approach, Husemann et al. (2012) found that this species' lineages had split at the Miocene/Pliocene transition, with further splits hypothesized to have occurred during Pleistocene climatic oscillations.



**Figure I-26.** Phylogeographic patterns of *cox1* mtDNA found in the Atlas mountains regions of Morocco. A – Complex pattern four *Buthus* species (Habel et al. 2012); B – *B. elmoutaouakili* detailed pattern (Husemann et al. 2012).

Sousa et al. (2010) analysed the Iberian Peninsula *Buthus* with a single mtDNA fragment (cox1), with a denser sampling of the western portion of the Peninsula (Fig. 27A). The authors found two new lineages (Fig. 27B) in addition to the three first found by Gantenbein and Largiadèr (2003). Again, relationships between lineages could not be resolved due to the lack of support. And the grouping of an Iberian sample with one from Tunisian made the Iberian *Buthus* paraphyletic, although that grouping had little support.



Figure I-27. Sampling (A) and ML phylogram of the Iberian Buthus (B) of Sousa et al. (2010).



**Figure I-28.** A - Map with Iberian *Buthus* distribution according to Lourenço and Vachon (2004), and Teruel and Pérez-Bote (2005). B - Map with Iberian *Buthus* distribution according to Rossi (2012). Colours according to Fig. 26.

The taxonomy of Iberian *Buthus* also changed in the last 15 years. First Lourenço and Vachon (2004) described two new endemic species from southern Spain, *B. ibericus* Lourenço & Vachon 2004 (Cadiz province) and *B. montanus* Lourenço & Vachon 2004 (Sierra Nevada) (Fig. 28A). The authors also analysed further material from Iberia and concluded that *B. occitanus* was widespread (Fig. 28A). Later Rossi (2012) described a new species, *B. elongatus* Rossi 2012, from the region of Marbella (Fig. 27B), and suggested it should correspond to lineage 3 from Sousa et al. (2010). Rossi only found *B. occitanus* on the Eastern half of the peninsula (Fig. 28B), including animals to the southwest of Sierra Nevada.

#### 1.4 General objectives

The main aim of the present study is to unravel the diversity of the genus *Buthus* in the Mediterranean basin, with special attention to the Iberian Peninsula, and to identify the main drivers of diversification. To tackle this goal, we propose the following specific objectives:

To update the taxonomic catalogue of the genus;

To conduct a molecular phylogenetic analysis of a thorough sampling of individuals from the Western Mediterranean (*i.e.*. Maghreb and Iberia) based on mtDNA to reveal genetic diversity and phylogeographic patterns;

To develop new nuclear markers for species delimitation and phylogeographic studies;

To delimit the main evolutionary lineages within the genus, with a focus on speciation in Iberian Peninsula, and to infer their phylogenetic relationships using a multilocus approach;

To estimate a timeframe for the diversification of the group using alternative calibrations in a Bayesian framework;

To use ecological niche modelling tools to infer the role Quaternary glaciations played in driving speciation and generating phylogeographic patterns in *Buthus*.

### 1.5 Thematic Organization

This thesis is organized in six chapters. It ends in Chapter 6 with a general discussion, and further appendices that contain all the supplementary information pertinent to the thesis.

In the current Chapter I, a general introduction, the objectives, and the structure of the thesis are provided.

Chapter II is composed of a manuscript that has been accepted for publication (pending a major revision, paper 1) and presents an updated Catalogue of the genus *Buthus* taxonomy, which has been changing at a fast pace since the publication of the last Catalogue in the year 2000. The work also provides an overview of the most important diagnostic characters used in the morphological identification of *Buthus* species. Several taxonomical changes were proposed to simplify and homogenise the genus taxonomy.

Chapter III is composed of a manuscript under preparation that presents five new Anonymous Nuclear Markers (ANM) developed for Iberian *Buthus* scorpions combining a reduced representation library with massive parallel sequencing. We proved that the new ANMs were variable at the intra and inter-specific levels, and that two could crossamplify in the broad Buthidae Family. We also provided an overview of all nuclear markers used in scorpions' molecular phylogenetic below the family level.

Chapter IV is composed of one published article and one article in preparation that reconstruct the phylogeny and phylogeography of the genus *Buthus* in the Mediterranean area. The first work (paper 2) focused on a thorough sampling of the Western Mediterranean, and, using mtDNA data, we were able to infer a phylogeographic structure with four well supported deep clades, three of which restricted to Morocco. The second work (paper 3) used a multilocus approach and a sampling that covered its entire Mediterranean range, to reconstruct a time-calibrated phylogeny of the genus *Buthus*. We contrasted several alternative calibrations: published mtDNA rates and the Messinian Salinity crisis as a vicariant event; with the known paleo-geological history of the Mediterranean to see which better fit the data.

We found five well supported groups within the genus, to from the North of the High-Atlas Mountains and three to the South of it, linked to the aridification of North Africa at 7 Ma. We also predict an Upper Pleistocene dispersal over water into the island of Cyprus.

Chapter V is composed of one article in preparation (paper 5). This work uses the same nuclear markers, but focus on a thorough sampling of the Iberian Peninsula to assess the Systematics of this monophyletic lineage of *Buthus*. Applying a species delimitation approach we found seven well supported species, two of which were unknown. We use the MSC to calibrate a phylogeny that found that the species in Iberia all diverged during the Pliocene. The recovered phylogeographic patterns are more recent, probably Pleistocene in age, although ecological niche modelling found low support for the Quaternary Glaciations as the drivers of these patterns. We also changed the status of a previously synonymised species.

Chapter VI provides a general discussion of the preceding four chapters. It focus on their key findings, and especially on how they can contribute to the understanding of the Natural History of the genus *Buthus*. *Buthus* taxonomy, evolutionary history and Iberian Peninsula speciation and phylogeographic patterns are highlighted. Finally, some remarks are given regarding future research topics that emerged from this thesis.

Research from this thesis has been published in one international peer-reviewed journals, such as Journal of Zoology (Sousa et al., 2012), and another has been accepted, pending a major revision (already submitted) (Sousa et al., Paper 1. Research from this thesis has also been presented in several International Congress and Meetings, namely in the XVI *Jornadas do Grupo Ibérico de Aracnología*, in Bragança, Portugal

(2016), with the invited talk "Os escorpiões do género Buthus na Península Ibérica. Diversidade, filogeografia e impacto da última glaciação inferidos com recurso a informação de AND multi-locus e modelação de nicho ecológico"; in the 28th European Congress of Arachnology, Turin, Italy (2014), with the talk "Improved Phylogeography of the scorpion genus *Buthus* and novel insights regarding Maghreb diversity through a cox1 Barcoding approach"; in the II Iberian Congress of Biological Systematics, Barcelona, Spain (2013), with the talk "Maghrebian scorpions: using molecular tools to improve knowledge on diversity and distribution"; and in the 1st Congress on Scorpion and Ophidian Envenomations, Marrakesh, Morocco (2013), with the talk "Maghrebian scorpions: Using molecular tools to improve knowledge on their diversity and distribution".

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# Chapter II UPDATING BUTHUS TAXONOMY

# Paper 1

Sousa, P. Arnedo, M. A. and D. James Harris (accepted<sup>\*</sup>). Updated catalogue and taxonomical notes on the Old-World scorpion genus *Buthus* Leach, 1815 (Scorpiones, Buthidae). ZooKeys.

\* pending a major revision

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PAPER 1: Updated catalogue and taxonomical notes on the Old-World scorpion genus *Buthus* Leach, 1815 (Scorpiones, Buthidae).

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# Abstract:

Since the publication of the ground-breaking "Catalogue of the scorpions of the world (1758-1998)" (Fet et al. 2000) the number of species in the scorpion genus Buthus Leach, 1815 has increased 10-fold, and this genus is now the fourth largest within the Buthidae, with 52 valid named species. Here we revise and update the available information regarding Buthus. A new combination is proposed: Buthus halius (C. L. Koch, 1839) n. comb. from Portugal and Spain. B. halius is removed from junior synonymy with Buthus occitanus (Amoreux, 1789), and proposed as a senior synonym of B. ibericus Lourenço and Vachon, 2004, new. syn. Moreover, following I.C.Z.N. article 23.9.2 we propose to maintain as valid B. ibericus (nomen protectum) and to consider the disued *B. halius* as a **nomen oblitum**. Buthus europaeus tridentatus Franganillo, 1918 is proposed as a junior synonym of *B. occitanus* (Amoreux, 1789) **n. syn.**. Buthus sabulicola Touloun, 2012 is proposed as a junior synonym of Buthus bonito Lourenço and Geniez, 2005 n. syn.. B. occitanus tunetanus neeli Gysin, 1969 is proposed as an informal senior synonym of Buthus tassili Lourenço, 2002 informal n. syn.. Two taxa are rised to species rank, Buthus nigrovesiculosus Hirst, 1925 n. stat. and Buthus parroti Vachon, 1949 n. stat.. We further confirm the restricted distribution of *B. occitanus* that is confined to southeastern France and northwestern Iberian Peninsula and does not occur in North Africa. Additionally, Androctonus barbouri (Werner, 1932) n. comb. from the Agadir region of Morocco, is hereby transferred to the genus Androctonus. We summarize and provide a critical appraisal of the diagnostic characters currently in use for the genus. The catalogue section considers the names for species, subspecies and varieties that have been used for Buthus scorpions. Information about types, including collection numbers and localities are included when available. Finally, an annotated listing of synonymies and an updated bibliography are given.

# Key words:

Taxonomy, new synonymy, new combination, new status, Geographic distribution, Africa, Asia, Europe, diagnostic characters

# Introduction

Members of the genus *Buthus* Leach, 1815 are medium-sized scorpions, usually yellowish in colour, with a robust metasoma that ends in a telson with a globular vesicle and a curved aculeus (Fig. 1). *Buthus* rest during the day in burrows under stones or shrubs and are active from dusk till dawn, although their activity typically peaks at the beginning of the night (Cloudsley-Thompson 1956). They are successful scorpions that, when present, tend to be the most abundant scorpion in their habitat, as for example in the Iberian Peninsula and Morocco. *Buthus* are usually described as sit-and-wait predators, although they can also actively search for prey (Skutelsky 1995, Piñero et al. 2013). *Buthus* is among the most venomous of all scorpion genera (Chippaux and Goyffon 2008). *Buthus* venom toxicity is considered much lower in Europe than in North Africa, which can be empirically corroborated by the few severe cases of scorpionism reported for Western Europe when compared to the North African countries (Chippaux and Goyffon 2008).



Figure II-1. Buthus mariefranceae, from south of Morocco. Photo by Arie van der Meijden.

*Buthus* exhibits a wide distribution range, spanning over two biogeographic realms, the Palearctic (Western) and the Afrotropical (Udvardy 1975, Olson et al. 2001). Interestingly, none of the chorotypes proposed by Vigna Taglianti et al. (1999) satisfactorily describes *Buthus* distribution. The genus extends from the temperate Mediterranean areas of south-western Europe to the tropical and sub-tropical grasslands south of the Sahel and into the Horn of Africa, including the semi-arid and arid regions of North Africa and the Middle East (Fig. 2). Although first considered of European origin (Vachon 1952a), current data support the hypothesis that the centre of origin of the genus is North Africa (Lourenço 2002). North Africa harbours a disproportionate number of species (Fig. 2) as well as four of the five main genetic clades found in *Buthus*, as defined by mitochondrial DNA sequence variation (Sousa et al. 2012, Pedroso et al. 2013) (Fig. 7).

*Buthus* species are known from 17 countries in Africa: Algeria, Cameroon, Central African Republic, Chad, Egypt, Eritrea, Ethiopia, Guinea, Libya, Mauritania, Morocco,

Niger, Senegal, Somalia, South Sudan, Sudan and Tunisia; five countries in Asia: Cyprus, Egypt (Sinai), Israel, Jordan and Yemen; and four European countries: France, Italy (Sicily), Portugal and Spain (Fig. 2). Unidentified Buthus species have also been reported from Burkina Faso, Djibouti, Gambia, Ghana, Guinea-Bissau, Ivory Coast, Nigeria, Iraq and Lebanon (Fig. 2). No records exist for Saudi Arabia or Syria, however the first might have been confused in the past with citations for the Arabian Peninsula (e.g. Vachon, 1952a), although the existence of Buthus in either of these countries cannot be excluded. The frequently cited occurrence of *Buthus* in Iraq is based on a single specimen, deposited in the Czech National Museum of Natural History (Táborský 1934, Kovařík 1992). As such the actual distribution of the genus remains poorly delimited. Old records from mainland Greece and Turkey are highly doubtful, as these are well prospected areas with no recent Buthus collections (Ersen Yağmur pers. commun. for Turkey) (Fig. 2). The former records most likely refer to the genus Mesobuthus Vachon, 1949. As for the record for Malta, it was considered dubious by Fet and Lowe (2000), although other Buthus reported on islands that were previously regarded as doubtful have turned out to be correct, namely B. kunti Yağmur, Koc and Lourenço, 2011, from Cyprus, described from freshly collected material and *B. trinacrius* Lourenço and Rossi, 2013, from Sicily, based on 130-year-old material. Other island records include B. occitanus in the Columbrete islands (Castilla and Pons 2007) and B. tunetanus (Herbst, 1800) in the Tunisian islands of Djerba, Kerkena and Zembra (Vachon, 1952a).



**Figure II-2.** Map of *Buthus* species distribution, and the known number of species by country. Also depicted are the species' type localities (numbers according to the species' Catalogue and Table 2) whenever known or the best possible approximation at present. Actual distribution within each country can be much smaller, but detailed distribution information is unknown for the majority of species.

*Buthus* is the type genus of the Buthidae C. L. Koch, 1837 (Koch 1837, 1850), the most diverse family within Scorpiones, with almost half of all known extant scorpion species (1101 of the 2311 known species) (Rein, 2016). The Buthidae also includes most of the species venomous to humans (Chippaux and Goyffon 2008). The genus *Buthus* is the second oldest valid genus of the order Scorpiones C. L. Koch, 1837, only surpassed by the single genus created by Linnaeus in 1758, *Scorpio*, to accommodate all the scorpion species he described.



**Figure II-3.** Original illustration of *Scorpio occitanus* (Amoreux 1789a). The work was retrieved from the Biodiversity Heritage Library and images were rearranged for compactness without re-scaling.

*Buthus* was first proposed by Leach (1815), with *Scorpio occitanus* Amoreux, 1789 as its type species (Fig. 3). The only diagnostic character for the genus proposed by Leach was the presence of eight eyes "*Oculi octo*." (Leach 1815 - page 391), apparently referring to the pair of central eyes and three pairs of lateral eyes. This character was mentioned as diagnostic for different scorpions early during Scorpiones taxonomy (De Geer 1778, Fabricius 1781). The same diagnostic character was used by Ehrenberg (in Hemprich and Ehrenberg 1828, 1829) for defining several genera and subgenera of scorpions with a varying number of eyes, ranging from six to 12. A more detailed explanation on the usage of the number of eyes in the classification of scorpions is given in Thorell (1876).

The poor description of Leach (1815) led to a rapid increase in the number of species included in the genus, which lacked any internal coherence. This taxonomical conundrum arose through the misidentification of the number of lateral eyes of *B. occitanus* (Amoreux, 1789), originally stated by Amoreux as three pairs. Several taxonomists of that century realised that there were actually four pairs of lateral eyes (*e.g.* Gervais 1844b; Simon 1879), but this information was not appreciated by some later authors. It has recently been shown that most Buthidae species (including *Buthus*) have five pairs of lateral eyes, although in many species two pairs of lateral eyes are much smaller in size and require extreme care and the help of UV light to be recognised (Yang et al. 2013; Loria & Prendini, 2014). Ehrenberg (in Hemprich and Ehrenberg,

1828) modified the original meaning of the genus to include the species that are now part of *Heterometrus* Ehrenberg in Hemprich and Ehrenberg, 1828 (Family Scorpionidae Latreille, 1802), all with five pairs of lateral eyes. Ehrenberg described several other genera that were soon synonymized with *Buthus*, at least by some taxonomists, which further exacerbated the taxonomical confusion within *Buthus*.

Because of the poor definition of the genus, many members (≈100 species) of the Buthidae family with no close relationship to the type species, were included in the genus Buthus up to the mid-20<sup>th</sup> century (Vachon 1952a, Levy and Amitai 1980, Lourenço 2002). Unfortunately, this obsolete taxonomy is still in use, for example in many toxicology and venom related papers on scorpions (e.g. Gopalakrishnakone et al. 2015). From 1948 to 1951, Vachon conducted a major taxonomic revision of the genus (compiled in Vachon 1952a), providing a more informative and exclusive definition, retaining only the species that were morphologically similar to the type species and hence restricting also the distribution range of the genus. He proposed two main morphological characters that in combination separate Buthus from all other known Buthidae genera: the central-lateral and posterior-median prosomal keels fused in a lyra shape (character shared with Cicileiurus Teruel, 2007, Leiurus Ehrenberg in Hemprich and Ehrenberg, 1828, Mesobuthus Vachon, 1950 and Odontobuthus Vachon, 1950, Fig. 4A), and the presence of only three granules on the tip of the movable finger (or tarsus) of the pedipalp chela (character shared with Androctonus Ehrenberg in Hemprich and Ehrenberg, 1828, Fig. 4B).



**Figure II-4.** The two key morphological diagnostic characters of the genus *Buthus*. A- Prosoma carapace with lyra-shaped keels (Hjelle 1990); B - Tip of pedipalp movable finger highlighting the three distal granules (distal denticle not included) (Lourenço 2002).

While studying specimens from northwest Africa, Vachon recognized that the genus included a large amount of undescribed diversity. Vachon took a very conservative approach to *Buthus* taxonomy, recognizing only four species, further split in 12 subspecies, ten of which under *B. occitanus*, and naming four different varieties, along with other forms with no formal rank, all within *B. occitanus*. This was partly justified by Vachon's view that *Buthus* species exhibited a large morphological plasticity, at least in the characters he used to diagnose the different taxa (Vachon, 1952a). Stahnke (1972), in his key to Buthidae genera, recognized 21 species and subspecies in *Buthus*, without further explanation, although this is probably an error resulting from an outdated interpretation of the genus taxonomy. In accordance with the ICZN article 45, none of Vachon's infra- subspecific varieties were included in the Catalogue of the Scorpions of the World (Fet et al. 2000). The *Buthus* Catalogue recognized as good five species and 12 subspecies, although the authors recognized that some taxa were probably not taxonomically good (Fet and Lowe 2000) Subsequently, Rossi (2015) transferred *Buthus insolitus* Borelli, 1925 to the recently erected genus *Gint* Kovařík et al., 2013. Lourenço

(2003) marked a renewed interest in the taxonomy and diversity of the genus, describing six new species, some of which corresponding to Vachon's infra-subspecific varieties.



Figure II-5. Cumulative number of valid named *Buthus* species. Only current valid species' names were plotted; in the year they were first described as species.

During the last 15 years, the rate of description of new *Buthus* species has increased exponentially (Fig. 5). At present, the genus is composed of 52 species, three of which were described in 2016, making it the fourth most diverse genus of Buthidae, only surpassed by the megadiverse scorpion genera *Tityus* C. L. Koch, 1836, *Centruroides* Marx, 1890 and *Ananteris* Thorell, 1891 (Rein, 2016). Thirty authors have been involved in the description of recent *Buthus* species, and most species (21) have been described in collaborative studies. Wilson Lourenço is by far the most prolific author, having authored or co-authored 29 *Buthus* species, 55% of the total.

# Material and methods

Nomenclature and measurements follow Stahnke (1970), except for trichobothriotaxy (Vachon 1974, Fet et al. 2005). All diagnostic morphological characters mentioned in the text refer to adults (or large sub adults) of both sexes, unless otherwise noted.

We confirmed most references prior to 1998 cited by Fet and Lowe (2000), but not all original literature could be obtained, and made some corrections following comparisons with additional sources (Vachon 1952a, Lamoral 1979, Polis 1990, Hendrixson 2006, Dupré 2013). We broadly followed the criteria applied by Fet and Lowe (2000) citing both taxonomical and faunistic works. To the best of our knowledge we cited all works that follow these criteria up to November 2016. Fet and Lowe (2000) cited around 180 articles pertaining to the genus *Buthus*, we added approximately 80 new articles, 10 of which were published before 1998.

Whenever possible, we provide coordinates for the type localities, using information available in articles or, if not available, finding approximate coordinates with the help of Google Maps (maps.google.com) and the GEOnet Names Server (geonames.nga.mil/gns/html). All coordinates are in WGS 1984 datum, in Latitude/Longitude format, in decimal degrees.

Collections abbreviation codes are listed below. Abbreviation codes follow Sabaj (2016), except for those marked with an asterisk that are not present there.

ARPC\* = Andrea Rossi Private Collection, Massa, Italy

CBGP\* = Centre de Biologie pour la Gestion des Populations (UMR INRA, Cirad, IRD, Montpellier SupAgro), Montferrier-sur-Lez France, France

FKPC\* = František Kovařík Private Collection, Prague, Czech Republic

MCSNB = Museo Civico di Scienze Naturali "Enrico Caffi", Bergamo, Italy (formerly MSNB)

CRBA = Centre de Recursos de Biodiversitat Animal of the Universitat de Barcelona, Barcelona, Spain

MCVR = Museo Civico di Storia Naturale di Verona, Verona, Italy

MCZ = Museum of Comparative Zoology, Harvard University, Cambridge, U.S.A.

MHNG = Muséum d'histoire naturelle de Genève, Geneva, Switzerland

MNCN = Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain

MNHN = Muséum national d'Histoire naturelle, Paris, France

MRSN = Museo Regionale di Scienze Naturali di Torino, Turin, Italy

MTAS\* = Museum of the Turkish Arachnology Society, Ankara, Turkey

MZUF = Museo di Storia naturale dell'Università di Firenze, sezione di Zoologia "La Specola", Florence, Italy.

NHMUK = Natural History Museum, London; England, UK (formerly BMNH, British Museum of Natural History).

UCAM\* = Université Cadi Ayyad, Faculte des Sciences Semlalia, "Laboratoire Ecologie et Environnement", Marrakech, Morocco (formerly Universite Cadi Ayyad, Faculte des Sciences, Semlalia, Depart. Biol., Lab. Ecol. Anim. Terrestre, Marrakech, Marocco) UGA\* = University of Ghardaïa, Ghardaïa, Algeria.

ZIN = Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (formerly ZISP).

ZMB = Museum für Naturkunde - Leibniz-Institut für Evolutions und Biodiversitätsforschung, Berlin, Germany (formerly ZMBH)

ZMH = Biozentrum Grindel und Zoologisches Museum, Hamburg, Germany

Additional abbreviations used in the text:

a.s.l. – above sea level

ICZN – International Code of Zoological Nomenclature

IOS – incorrect original spelling

ISS – incorrect subsequent spelling

juv. – juvenile or juveniles

M – male

F – female

MIS – misidentification

# On the type species of the genus Buthus

It is worth mentioning here the taxonomical confusing that surrounded the first *Buthus* species.

Leach first named the genus with Scorpio occitanus Amoreux, 1789 as the type species (Fig. 3; 6B). Amoreux (Amoreux 1789b) described this species to accommodate a scorpion from Souvignargues, Occitanie, France. Amoreux also called the same species Scorpio rufus, although he, as the first reviewer, chose the name S. occitanus to be the correct name for the newly described species (Amoreux 1789a). Amoreux also referred to this species as Malpertius' scorpion and, in his second paper where he gave a full description of the species, included drawings from this author (Maupertuis 1731) (Fig. 6B). It is clear from observing Fig. 6A that Amoreux was well aware of the differences between Scorpio occitanus and S. europaeus Linnaeus, 1758, which he considered a member of the genus Euscorpius Thorell, 1876. Unfortunately, the name S. europaeus Linnaeus, 1758 was subsequently used to refer to three different taxa: (1) S. maculatus De Geer, 1778 (now part of the genus Isometrus Ehrenberg, 1828) (Lönnberg 1898), (2) S. occitanus Amoreux, 1789 (Thorell 1876b), and (3) a Euscorpius species (Fet & Sissom, 2000). This taxonomical confusion was solved by ICZN decision 60 (ICZN 1957), article 1b that suppressed the name europaeus, Linnaeus, 1758 when used in combination with Scorpio, and article 4, that placed Scorpio europaeus Linnaeus, 1758 on the Official Index of Rejected and Invalid Specific Names in Zoology with the number 381. As such, the first author to use the name Buthus europaeus was Thorell in 1876, now a junior synonym of B. occitanus (Amoreux, 1789) (for further details see Braunwalder 1997, Fet et al. 2002).



**Figure II-6.** Original drawings of the habits of *Scorpio europaeus* Linnaeus, 1758 (A) and *S. occitanus* (B), according to Amoreux (1789b) reproduced from plate I of that work. These images are unfortunately rarely cited, as they are very informative regarding the reasoning of Amoreux while describing the new species.

Finally, although *Buthus* is considered the nominal genus of Buthidae, Koch (1837) used for the typification of the family, the species *Buthus spinnifer* Ehrenberg, 1828, which is currently the type species of the genus *Heterometrus* Ehrenberg, 1828 (Scorpionidae Latreille, 1802), and as such according to ICZN Article 65.2.1. ["type genus was misidentified (that is, interpreted in a sense other than that defined by its type species)] when the family-group name was established") we will submit to the ICZN a petition to fix the type species of the Buthidae. This was not done by Fet et al. (2000) as the authors probably interpret it as ICZN Article 65.2.3. ("type genus was, when established, based

on a type species then misidentified"), which does not necessarily require a ruling by the Commission.

# **Buthus taxonomy**

We follow the revised classification of Sharma et al. (2015) based on the first phylogenomics study on extant scorpions, which resolved most relationships between scorpion families.

Class **Arachnida** Lamarck, 1801 Order **Scorpiones** C. L. Koch, 1850 Suborder **Neoscorpiones** Thorell et Lindström, 1885 Infraorder **Orthosterni** Pocock, 1911 Parvoder **Buthida** Soleglad and Fet 2003 Superfamily **Buthoidea** C. L. Koch, 1837 Family **Buthidae** C. L. Koch, 1837

There are no subfamilies in use within the Buthidae, although many have been proposed and rejected (Fet et al. 2000, 2005). They defined six groups within the Buthidae, and placed *Buthus* in the *Buthus* group along with 38 additional genera. The phylogenomic study of Sharma et al. (2015) provided strong support for most groups (although generic level sampling was limited), including for the *Buthus* group, which was found to be the sister clade to the remaining Buthidae.

Vachon (1952a) considered *Androctonus* to be the sister taxa to *Buthus*. However, the only molecular phylogenetic study addressing the relationships between Buthidae genera that includes both genera (Fet et al. 2003), recovered *Buthus* as the sister taxa to a clade formed by *Androctonus* and *Leiurus*, albeit with low support.

There are no taxonomically distinct groups within the genus Buthus, although two "species complexes" are generally recognised. Vachon (1952a, p. 251) suggested that "la «lignée» atlantis se sépare avec facilité de l'ensemble des autres Buthus par la forme élancée des appendices de la queue, de la vésicule et divers autres caractères que nos tableaux de détermination préciseront.". This distinction was retained by Lourenço (2002, 2003) who referred to B. occitanus as a "«complexe de forms»", and subsequently also adopted in most subsequent taxonomical works describing new Buthus species. According to Lourenco and Geniez (2005), the two complexes are distinguished by the level of keel development, weaker in the atlantis group. However, Lourenço (2005a) subsequently wrote that B. occitanus from Europe has a weak keel development in contradiction with the previous morphological definition. This statement has been used by subsequent authors. For example, Rossi (2012) described *B. elongatus* Rossi, 2012 as belonging to the occitanus complex, but if the author had applied Vachon's definition it should have included it in the atlantis complex because of the slender metasoma (at least its fifth segment) when compared to B. occitanus. None of the published molecular phylogenies of Buthus supports the existence of the atlantis complex. The results of Gantenbein and Largiadèr (2003) grouped *B. atlantis* within the species included in the occitanus complex. Although Lourenço and Vachon (2004) acknowledged the work of Gantenbein and Largiader (2003), they rejected the phylogenetic position of B. atlantis presented in this study.

Recently, based on the information provided by a *cox1* mtDNA tree, Sousa et al. (2012) and Pedroso et al. (2013) have defined a series of groups, (see Table 1). These groups have been renamed to facilitate communication and have been expanded to include all available molecular data with reliable species identifications.

**Table II-1.** Current composition of the groups proposed by Sousa et al. (2012) and Pedroso et al. (2013) based on *cox1*. To date, only 19 out of the 52 valid named *Buthus* species (37%) have been analysed. \*, assignment based on Sousa (2017).

group	species	group	species	group	species
boumalenii	B. boumaleniii		B. atlantis B. elongatus	rochati	B. bonito B. draa
	B. elmoutaouakili B. lienhardi	occitanus	B. ibericus B. malhommei	rocnau	B. mariefranceae B. rochati*
mardochei	B. mardochei B. parroti		<i>B. maroccanus B. montanus B. occitanus</i>	tunetanus	B. chambiensis* B. pusillus* B. tunetatus

In Fig. 7 we present the current distribution of these five groups in the Maghreb and the Iberian Peninsula and southern France, based exclusively on specimens with available molecular data. The group assignment does not necessarily correspond to the species assignment in the original publications.



**Figure II-7.** Map representing the five phylogenetic *Buthus* cox1 groups in the Maghreb. Groups as defined by Sousa et al. (2012) and Pedroso et al. (2013), also including cox1 sequences from Gantenbein and Largiadèr (2003), Habel et al. (2012) and Husemann et al. (2012) (redrawing of Figure 1 from Pedroso et al. 2013).

# Diagnostic characters used in Buthus taxonomy

Several morphological traits have been used by recent authors as diagnostic characters (in the sense of Winston 1999) for *Buthus* species.

Colour is of limited utility in *Buthus* taxonomy, as the underlying colour varies in tones of yellow, orange, reddish or light brown within and between species. Only one species has a fully dark body, *Buthus maroccanus* Birula, 1903, in some cases even black. Other species also have the mesosoma of a darker colour than the rest of the body. Of greater taxonomical use are colour patterns, such as darker marks, over a lighter background colour, that can be present on the carapace, the mesosoma or the metasoma; the latter being the more informative.



**Figure II-8.** Chart and boxplot summary of *Buthus* species maximum sizes. Only the known maximum size per species is represented. Size information is only available for males from 44 species and females from 43 species. Some individual data might correspond to subadult specimens, since this information is not always explicit in species descriptions.

Adult size may also be diagnostic (Fig. 8). *Buthus* adult body sizes range from 38 to 90 mm (telson included) (the maximum size of 110 mm reported by Vachon (1952a) is presumably a mistake). Most species have a maximum size between 60 and 70 mm in females, and 55 to 70 mm for males (Fig. 8). On the 5% percentiles we have the smaller species of *Buthus* (less than 45 mm long), and the larger species of *Buthus* (more than 85 mm for females and 80 mm long for males).

Two additional meristic traits have been used as diagnostic characters, namely the number of rows of granules on the cutting edge of the movable finger of the pedipalp chela, and the number of pectinal teeth, a sexual dimorphic trait. Variation in the number of **rows of granules** is not very informative because species show an incremental overlap in the numbers of rows, which range from 8 to 14 (Fig. 9). **Pectinal teeth** number, although carrying a potentially greater amount of information as they have a wider range to vary from, is actually of limited usefulness because of the interspecific overlap

(Fig. 10); female counts range from 18–34 and males from 24–37. Pectinal teeth number is also of limited use due to the lack of any information for several species and the unknown range of variability for many other *Buthus* species (Fig. 10). It should be noted, however, that *Buthus elizabethae* Lourenço, 2005 is unique in having male pectines that do not overlap in their proximal portion (Lourenço 2005a).

species	range	08 0	<u>) 10</u>	11	12 13 14		14	species	range	<b>0</b> 8	09	10	11	12	13	14
B. mariefranceae	8- <b>9</b>						1	B. albengai	11-12							
B. amri	9-10						1	B. aures	11-12							
B. brignolii	9-10						1	B. awashensis	11-12							
B. adrianae	10						1	B. centroafricanus	11-12							
B. egyptiensis	10						1	B. chambiensis	11-12							
B. intumescens	10*						1	B. draa	11-12							
B. orientalis	10						1	B. elhennawyi	11-12							
B. trinacrius	10						1	B. hassanini	11-12							
B. yemenensis	10						1	B. lienhardi	11-12							
B. bonito	10-11						1	B. prudenti	11-12							
B. elmoutaouakili	10-11						1	B. pusillus	11-12							
B. jianxinae	10-11						1	B. tunetatus	11-12							
B. mardochei	10-11						1	B. haliusª	11-13							
B. parroti	10-11						1	B. israelis	11-13							
B. rochati	10-11						1	B. parisª	11-13							
B. tassili	10-11						1	B. barcaeus	12							
B. atlantis	10-12						1	3. boumalenii	12							
B. malhommei	10-12						1	B. confluens	12							
B. tunetatusª	10-12			_			1	B. dunlopi	12							
B. berberensis	11*						1	B. elizabethae	12							
B. duprei	11						1	B. elongatus	12							
B. intermedius	11*						1	B. kunti	12							
B. karoraensis	11						1	B. labuschagnei	12							
B. lourencoi	11						1	B. occitanus	12							
B. montanus	11						1	B. haliusª	12-13							
B. nigrovesiculosus	11*						1	B. maroccanus	12-13							
B. occidentalis	11						1	B. parisª	12-14							
B. saharicus	11						1	B. tunetatus <sup>a</sup>	12-14							
species	range	08 0	9 10	11	12	13	14	species	range	08	09	10	11	12	13	14

**Figure II-9.** Graphical representation of the variation in the number of rows in the movable finger of all *Buthus* species. <sup>a</sup> Species for which the bibliographic ranges are conflicting. \* Number of rows in species identified by us from images of the type specimens may be underestimated.

Trichobothria number and position are not useful for *Buthus* species diagnosis, as their location shows little variation and have as much intraspecific as interspecific variability (P. Sousa pers. obs.). Conversely, body **chaetotaxy** (other than trichobothria) is very useful for taxonomy. Vachon (1952a) defined three, albeit diffuse, states in *Buthus* body chaetotaxy: low ("*oligotriche*"), high ("*polytriche*") and medium ("*mésotriche*"), and used the number of setae on the fifth segment of the metasoma as example of the ranges: low has 3 or less setae, high more than 5-6 setae and medium 4 setae, although this latter category was fluid. Confusion can further arise from the fact that these categories apply to the metasoma and the pedipalp, and in the same species these two body parts can have different ranges of chaetotaxy. Nevertheless, this is a useful trait, and one that needs to be explicitly stated in species descriptions to avoid misinterpretations. The chaetotaxy of the leg tarsi and mesosoma terguites is also useful.



**Figure II-10.** Graphical representation of the variation in pectinal teeth number of *Buthus* species arranged by geographical areas to facilitate comparison. All known *Buthus* species are represented, although female and male are ordered independently, from smaller to largest. <sup>a</sup> Species for which the bibliographic ranges are conflicting.

Most other diagnostic traits in use for *Buthus* species are found in the metasoma and the pedipalp chela.

The length/width ratio of the **first metasomal** segment, which is typically square in most species but can be elongated or sturdy in certain species, is informative. This ratio is also applied to the **fifth metasomal** segment, and Vachon (1952a) further compared the ratios of the first and second segment, and sometimes even the third segment. The number of **keel rows** in the metasoma segments is also useful, with special attention paid to the presence, and in some cases the relative length (Vachon 1952a), of the Median-lateral keel in the second, third and fourth segments. Both the degree of development of the inferior median keels of the five segments (except perhaps the forth), and the existence of larger granules may also be used for taxonomical purposes. The number of lateral lobes in the **anal arch**, either two or three (the latter only in *B. atlantis* Pocock, 1889 and *B. lourencoi* Rossi, Tropea and Yagmur, 2013) may be misleading because in some species, or even specimens (Vachon 1952a), a third smaller lobe may be present between the two larger lobes, which has been interpreted as a third state (e.g. Sadine et al. 2015). For instance, Lourenço and Qi (2006) state that in *Buthus* 

*mariefranceae* Lourenço, 2003 the anal arch may sometimes have 3 lobes but this is mentioned neither in the original description nor in Vachon's descriptions. The relationship between the length of the aculeus and the length of the vesicle that form the **Telson** are also used in *Buthus* taxonomy. In most species, the aculeus is shorter than the vesicle, or as long as the vesicle at most. For a few species the aculeus is clearly shorter than the vesicle and for another handful of species, the aculeus is clearly longer. We here define the states using a 10% difference threshold, but other authors have used a 5% difference. This ratio is correlated with the shape of the aculeus, also in use, which can be more or less curved.



**Figure II-11.** Graphical representation of relation between female and male pedipalp chela aspect ratio in *Buthus* species. Only those species with available data from both sexes were plotted. The grey area represents species without sexual dimorphism. Males above that area have slender pedipalp chela than females, while males plotted below have more robust pedipalp chela than females.

The shape of the **pedipalp chelae** in *Buthus* taxonomy has gained increased usage in recent years. The shape can be approximated by using the length to width ratio of the chela, which reflects its specific robustness or slenderness. However, in many Buthus species the chela shape is sexually dimorphic, a trait that was first used in a species key by Kovařík (2006), although its use goes back at least to Vachon (1952a). As a measure of sexual dimorphism the pedipalp chelae has three possible states: 1) no sexual dimorphism (male = female); 2) slender chela in male (male > female); 3) chela of male more robust (male < female). There is data available for 29 species (56% of the known species), and from these we can estimate that 38% do not have sexual dimorphism (+/-10% threshold as a cut-off point), 52% of species have males with slender chelae and only 3 species (10%) show males with more robust chelae (Fig. 11). Interestingly, although chelae play a role in mating, defense, and as a sensory organ (van der Meijden et al. 2012), their prime importance in prey capture and handling (Polis 1990) may limit the slendering of female chelae, as these may be more prone to breakage (van der Meijden et al. 2012). For three of the 39 species, the available chelae data was contradictory, and they are further discussed below. Another useful pedipalp trait is the interrupted dorso-median keel of the patella in Buthus rochati Lourenço, 2003 (Vachon 1952a).

Several partial keys have been published over the years to assist *Buthus* species identification. However, due to the high rate of new species description (Fig. 5), they have become incomplete and even outdated in their taxonomy (Birula 1903, Vachon 1952a, Lourenço 2003, Lourenço and Vachon 2004, Kovařík 2006, Rossi 2012, Rossi et al. 2013, Teruel and Melic 2015). There are keys available for Morocco and the Maghreb by Birula (1903, only four species), Vachon (1952a, 10 species in the Moroccan *Buthus* key), Lourenço (2003, 10+1 species), and Kovařík (2006, Tunisia, 4 species); for North Africa (excluding Morocco) by Rossi et al. (2013, 13 species), and for the Iberian Peninsula by Lourenço and Vachon (2004), Rossi (2012), and Teruel and Melic (2015).

Unfortunately, the identification of the majority of *Buthus* species remains difficult, in part because of the limited number of diagnostic characters and the incomplete knowledge regarding their intraspecific variation. Sexual dimorphism of pedipalp chelae is a promising trait, but for many species the male or female is still undescribed, which limits its applicability. We urge authors in future *Buthus* species descriptions to mention the variation on all the traits mentioned here (see Rossi et al. 2013 for a nice example).

Confirming the fast pace of new *Buthus* species descriptions, a new species, *Buthus danyii* has been published by Rossi (2017) from Ghana, while this work was under revision. As such we were unable to include this species in the present update, however more information can be found in the original description.

We hope that the present catalogue will facilitate a more precise, informative and comparative description of future species. *Buthus* are an important component of the scorpions' fauna of North Africa and Western Europe, but it is only now becoming apparent that they are also diverse in the southern Sahara Desert, an area that should be prioritized in future surveys of *Buthus* scorpions.

# Catalogue

#### Genus BUTHUS Leach, 1815

*Buthus*: Leach 1815: 391; Latreille 1817: 310; Gervais 1844b: 203; Peters 1861 (part): 513; Thorell 1876a (part): 82; Thorell 1876b: 7; Simon 1879: 95-96; Karsch 1886 (part): 77; Pocock 1890 (part): 122; Karsch 1891: 18; Kraepelin 1891 (part): 35-42; Pocock 1893 (part): 312; Kraepelin 1895 (part): 79-80; Laurie 1896b: 131; Lönnberg 1897b (part): 194; Kraepelin 1899 (part): 9; Pocock 1900a (part): 13; Simon 1910: 67-68; Birula 1917a (part): 20-24, 164; Birula 1917b (part): 55; Pavlovsky 1924 (part): 77; Kastner 1941 (part): 230; Vachon 1948a: 206-208; Vachon 1949a: 155-162; Vachon 1952a: 155, 241-246, fig. 579; Vachon 1963b: 164, fig. 10; Bücherl 1964: 57; Stahnke 1972: 132, fig. 20; Vachon 1974: 906; Levy and Amitai 1980: 14-15; Francke 1985: 6, 15; Sissom 1990: 101; Nenilin and Fet 1992: 17; Kovařík 1998: 106; Fet and Lowe 2000: 91; Lourenço 2016b: 3-4.

**Type species** (by original designation): *Scorpio occitanus* Amoreux, 1789 [=*Buthus occitanus* (Amoreux, 1789)].

**Etymology:** Leach did not provide an explanation for his selection of the genus name. A search on the original usage of the word may shed some light on the intended meaning. Buthus is the Latin form of the Greek name βοῦθος (Bouthos), an unusual name of a winning athlete of the ancient Pythian Games, mentioned by Hesychius and Aristotle (Müller 1848, Christesen 2007). The name was more familiar in antiquity when used in the adage "Βοῦθος περιφοιτῷ", translated to the Latin as "Buthus obambulat", which translates into "Buthus who wanders", which apparently was applied to stupid and simple people (Müller 1848, Christesen 2007). In Hofmann et al. (1698) the entry for Buthus reads "athleta nobilis, qui bovem integrum unô die devorare solebat; unde natum proverbium in edaces, Buthus obambulat" which roughly translates to "a noble athlete, who used to devour a great ox in a day, and who gave rise to the proverb, Buthus obambulat". Noël (1824) entry for Buthus also refers to an athlete that devoured an ox in a single day, and that this voracity was the origin of the proverb "Buthus obambulat", which according to the author refers to gluttony. Interestingly Noël also states that Buthus, in combination with " $\beta \tilde{\mu} \varsigma$ ,  $\theta \dot{\mu} \epsilon v$  (thuein)", also refers to sacrifice. This opinion shares roots with the meaning of two other words with similar etymology, būthysia (used by Nero) that translates to "sacrifice of an ox" and būthytes (used by Pliny the Elder), that translates to a "sacrificed ox", according to the Gaffiot Latin-French dictionary (Various 2016). Recently Dupré (2016) reached a similar conclusion, although he states that Buthus originates from the composition of the Greek word "Gr. bous, ox; - thouéin [greek suffix?], killer". Potentially therefore, Buthus refers to a stupid or voracious animal, an ox killer or to a sacrifice of an ox, from the latter two we can interpret it as a powerful and dangerous animal. In our opinion the later makes more sense and agrees well with what was known at the time about the potent venom of Buthus scorpions. As such, it is our opinion that *Buthus* is a singular masculine Latin word (of Greek origin), which Leach intended as homage to an ancient hero (a trend at that time), and that refers to an animal so venomous that it could kill an ox.

**Distribution:** AFRICA: Algeria, Cameroon, Chad, Egypt, Eritrea, Ethiopia, Guinea, Ivory Coast, Libya, Mali, Mauritania, Morocco (including Western Sahara), Niger, Senegal, Somalia, South Sudan, Sudan, Tunisia. ?Guinea-Bissau, Nigeria, Burkina Faso, ?Gambia, Ghana, ?Djibouti. ASIA: Cyprus, Israel, Jordan, Yemen. ?Iraq, ?Lebanon, ?Saudi Arabia, ?Turkey. EUROPE: France, Italy (Sicily), Spain, Portugal. ?Malta, ?Greece (Corfu, Thessaly). All currently valid records of *Buthus* species per country are presented in Table 2. Figure 10 offers an additional zoom to the most diverse region of *Buthus* species diversity, the Maghreb.



**Figure II-12.** Map of North Africa Maghrebian *Buthus* species' type localities (numbers according to the Catalogue and Table 2) whenever known or the best possible approximation at present.

**Remarks:** There are several old records of *Buthus*, marked with a question mark above, which have never been found again (independently of the material in which they were based being lost or not). As such, many have not been checked since the genus was reduced in scope by Vachon (1949), or those localities remain doubtful because no *Buthus* has been collected there since. This is of special significance in countries like Greece and Turkey that have been in recent years reasonably well prospected. Type specimens for several *Buthus* species described early on were not designated or have since become lost, but this does not necessarily represent a taxonomic problem. For example *B. occitanus* has no type specimen (Fet and Lowe 2000), but its type locality is well established and no other *Buthus* species occurs nearby. In this case the designation of a neotype is not justified under the ICZN (article 75.2). However, this is not the case for other species that have neither type specimens nor localities, and that we will further discuss below.

**Table II-2.** List of the countries for which there are valid records of the occurrence of *Buthus* species. The ID corresponds to the numbers used in Figures 1 and 10, and on the Catalogue. C.A.R. is the abbreviation of the Central African Republic.

ID	Таха	lt	aly	Sp	pain		Cameroo	n Chad	Egy	pt	Ethiopia	Lybia	Moro	cco	Senegal	S. Suda	in Tunisia	a Israe	el Yemer	Total
	IdXd	France	I	Portugal		Algeria	С	.A.R.	Djibouti	Eritrea	G	Guinea	Mauritania	Niger	Somali	a s	Sudan	Cyprus	Jordan	(by taxa)
01	B. adrianae								х											1
02	B. albengai												Х							1
03	B. amri																		х	1
04	B. atlantis												х							1
05	B. aures					х														1
06	B. awashensis										х									1
07	B. barcaeus											х								1
08	B. berberensis								х	х	х				х					4
09	B. bonito												х							1
10	B. boumalenii												х							1
11	B. brignolii																х			1
12	B. centroafricanus							х												1
13	B. chambiensis																х			1
14	B. confluens												Х							1
15	B. draa												х							1
16	B. dunlopi																х			1
17	B. duprei																х			1
18	B. egyptiensis								х											1
19	B. elhennawyi													х	х					2
20	B. elizabethae											Х			х					2
21	B. elmoutaouakili												1							1
22	B. elongatus				x															1
23	B. hassanini							х												1
24	B. ibericus			х	x															2
25	B. intermedius																		х	1
26	B. intumescens								х											1
27	B. israelis								х	-> Sin	ai							X		2
28	B. jianxinae															х				1
29	B. karoraensis									х										1
30	B. kunti																	х		1
31	B. labuschagnei							х												1
32	B. lienhardi												х							1
33	B. lourencoi											х								1
34	B. malhommei												х							1
35	B. mardochei												Х							1
36	B. mariefranceae												х							1
37	B. maroccanus												х							1
38	B. montanus				Х															1
39	B. nigrovesiculosus												х	-> We	estern Sahara					1
40	B. occidentalis												х							1
41	B. occitanus	х			х															2
42	B. orientalis								х											1
43	B. paris					х							Х				х			3
44	B. parroti												х							1
45	B. prudenti						х													1
46	B. pusillus					х														1
47	B. rochati												Х							1
48	B. saharicus					х														1
49	B. tassili					х						х								2
50	B. trinacrius		X	-> Sicily																1
51	B. tunetatus					х						х	х				х			4
52	B. yemenensis																		х	1
ID	Taxa	lt Eronoo	aly	Sp Dortugo'	pain	Algori-	Cameroo	n Chad	Egy	pt Eritra-	Ethiopia	Lybia	Moro Mouritoni-	CCO	Senegal Sorrel	S. Suda	in Tunisia Rudon	a Israe	I Yemer	Total
	L	riance		r urrugal		мидена		.A.K.	JIDOULI	Entrea			wauntania	rviger	Somali	a 3	Ilbuud	Cyprus	JUIDAN	(by taxa)
	iotai (by country)	1	1	1	4	6	1	1 2	1 5	1	2	1 4	1 17	1	2 1	1	2 4	1 1	1 2	

#### 1. Buthus adrianae Rossi, 2013

*Buthus adrianae*: Rossi 2013: 188-191, fig. 1-2; Rossi, Tropea and Yağmur 2013: 3; 5, 8.

**Type material:** 1 adult M holotype (MCSNB N<sup>o</sup> 14011), EI-Hamam (30.8300<sup>o</sup>, 29.3150<sup>o</sup>), Alexandria, Egypt. Paratypes: 1 adult M and 1 adult F (ARPC), same locality. **Distribution:** known only from the type locality.

#### 2. Buthus albengai Lourenço, 2003

*Buthus albengai*: Lourenço 2003: 902-904, fig. 70-74; Lourenço and Geniez 2005: 5; Aboumaâd et al. 2014: 6; Touloun et al. 2014: 76; Lourenço 2016b: fig. 3.

**Type material:** 1 F holotype (MHNG), Ito Plateau (approx. 33.51°, -5.3°), Ifrane, Morocco. Paratypes: 3 F, same locality; 2 juv., Ifrane (Cedars woods); 1 M, 1 F juv., region north of Kenifra (all in MHNG).

**Distribution:** known from an area in Morocco that extends from ifrane to Kenifra. **Remarks:** It is one of the largest known *Buthus* species. Records by Habel et al. (2012) south of the High-Atlas are most likely misidentifications.

#### 3. Buthus amri Lourenço, Yağmur and Duhem, 2010

*Buthus amri*: Lourenço, Yağmur and Duhem 2010: 96-99, fig. 1-5; Lourenço 2013: 65; Lourenço and Rossi 2013: 9; Amr 2015: 186.

**Type material:** 1 M holotype (MNHN), Wadi Rum Desert (29.5363°, 35.4136°), Aqaba, Jordan. Paratypes: 1 adult F (MNHN), 2 adult F, 1 subadult F, 2 subadult M, 3 juv. (MTAS), all from the same locality.

**Distribution:** known only from the type locality.

#### 4. Buthus atlantis Pocock, 1889

*= Tityus tenuimanus* Banks 1910: 189 (synonymized by Lourenço and Francke 1984: 428). 1 F holotype (MCZ), Buena Vista Lake, California, USA (incorrect locality).

*Buthus atlantis*: Pocock 1889b: 340-341, pl. XV, fig. 4; Birula 1896: 244; Kraepelin 1891: 197, 199; Birula 1903: 107-108; Werner 1932: 300-305; Vachon 1949a: 162-169, fig. 345, 347, 349, 351, 354, 355, 357-362; Vachon 1952a: 254-255, fig. 345, 347, 349, 351, 354, 355, 357-362; Malhomme 1954: 25; Bücherl 1964: 57; Pérez 1974: 22; Levy and Amitai 1980: 15; El-Hennawy 1992: 98, 119; Kovařík 1998: 106; Fet and Lowe 2000: 91; Lourenço 2005a: 233-234; Lourenço and Geniez 2005: 5; Stockmann and Ythier 2010: 360-361; Stockmann 2015: fig. 5.

Buthus Atlantis (sic): Aboumaâd et al. 2014: 5.

*Buthus atlantis*: Vachon 1949a: 166-168; Vachon 1952a: 252, 254; Le Corroller 1967: 63; Kovařík 1995: 20; Fet and Lowe 2000: 91; Lourenço 2003: 883-885, fig. 18-22; Touloun et al. 2001: 2; Gantenbein and Largiadèr 2003: 120, 122; Touloun 2012: 43, fig. 9A.

*Buthus occitanus atlantis*: Kraepelin 1899: 26-27; Werner 1934b: 86-87, fig. 5; Schenkel 1949: 186-187.

Buthus (Buthus) atlantis: Birula 1910: 145; Birula 1917a: 213, 223.

*Tityus tenuimanus*: Cox 1921: 12; Ewing 1928: 22; Mello-Leitão 1931: 121, 140; Mello-Leitão 1939: 60, 64, 71; Comstock 1940: 27; Mello-Leitão 1945: 308; Gertsch and Soleglad 1966: 2; Hjelle 1972: 28; Lourenço and Francke 1984: 427, fig. 10-12.

**Type material:** 1 F holotype (NHMUK), Essaouira (formerly Mogador) (approx. 31.49°, -9.76°), Morocco.

**Distribution:** known to occur only in sandy dune habitats close to the Atlantic Ocean in Morocco, between Essaouira and Agadir.

**Remarks:** It is the largest known *Buthus* species.

#### 5. Buthus aures Lourenço and Sadine, 2016

Buthus aures Lourenço and Sadine 2016: 14-17, fig. 4-13.

**Type material:** 1 M holotype (MNHN), Batna region (35.5319°, 5.9194°), Aurès Mountains, Algeria. 1 M paratype (UGA), same locality. **Distribution:** known only from the type locality.

#### 6. Buthus awashensis Kovařík, 2011

*Buthus awashensis*: Kovařík 2011: 1-3, 5-8, fig. 5-16. *Buthus occitanus* (MIS): Kovařík and Whitman 2005 (part): 106. **Type material:** 1 M holotype (FKCP), Metahara (approx. 8.900°, 39.900°), Oromia, Ethiopia. Paratypes: 34 M, 34 F, 36 juv. (FKCP), all from the same locality; 1 M (FKCP), Dire Dawa, Ethiopia.

Distribution: know only from two Ethiopian localities, more than 200 km apart.

**Remarks:** The pedipalp chela length to width ratio given by the author for the type material suggest that some animals exhibit sexual dimorphism while others do not. If this is true, the utility of this ratio as a diagnostic character in *Buthus* would be compromised. Alternatively, it may be due simply to the use of immature specimens.

# 7. Buthus barcaeus Birula, 1909

Buthus occitanus barcaeus: Birula 1909: 508-511. fig. A, C; Borelli 1914a: 155-156; Borelli 1924: 5-7; Borelli 1928: 351; Caporiacco 1932: 395; Borelli 1934: 169; Caporiacco 1937: 345; Pérez 1974: 23; Levy and Amitai 1980: 16; El-Hennawy 1992: 98, 120; Kovařík 1998: 106; Fet and Lowe 2000: 95; Kovařík 2002: 5.

Buthus (Buthus) occitanus barcaeus: Birula 1910: 156; Birula 1917a: 223.

*Buthus barcaeus*: Kovařík 2006: 3, fig. 6; Kaltsas et al. 2008: 215; Lourenço and Cloudsley-Thompson 2012: 15; Lourenço and Simon 2012: 11; Rossi, Tropea and Yağmur 2013: 3-5, 7.

**Type material:** 4 M, 1 F juv., syntypes (ZIN), Barca (approx. 32.48°, 20.83°), 5 km E from Benghazi (Cyrenaica), Libya.

**Distribution:** know from several localities along the Mediterranean coast of Libya.

**Remarks:** The specimens present in the MNHN (F n<sup>o</sup> 4896), captured in Barca and identified by Vachon in 1974, have no intermediary keel on the fourth metasomal segment, which casts doubts about the use of the character in the diagnosis of *B. barcaeus*.

#### 8. Buthus berberensis Pocock, 1900

*= Buthus occitanus zeylensis* Pocock 1900b: 56-57 (synonymized by Levy and Amitai 1980: 16). 1 F holotype (NHMUK), Zeyla (northwestern Somaliland), Somalia.

*Buthus occitanus berberensis*: Birula 1903: 106-107; Birula 1909: 510; Birula 1910: 118; Kraepelin 1903: 558; Borelli 1904: 2-3; Giltay 1929: 196; Moriggi 1941: 84; Lamoral and Reynders 1975: 505; Levy and Amitai 1980: 16; EI-Hennawy: 1992: 98, 120; Kovařík 1998: 106; Fet and Lowe 2000: 95; Kovařík 2003 (part): 138.

*Buthus occitanus zeylensis*: Kraepelin 1903: 558-559; Borelli 1919: 363; Borelli 1931: 218; Caporiacco 1936: 137; Moriggi 1941: 84; Lamoral and Reynders 1975: 505-506; EI-Hennawy 1992: 98, 121-122; Kovařík 1998: 106.

Buthus berberensis: Lourenço 2008: 46; Kovařík 2011: 4-6.

Buthus (Buthus) occitanus berberensis: Birula 1917a: 123.

**Type material:** 1 M holotype (NHMUK), Somaliland, Somalia.

**Distribution:** know from Djibouti, Eritrea, Ethiopia and Somalia, although Birula (1903) recorded toponyms that are old and difficult to map.

**Remarks:** Lourenço (2008) stated that *B. o. zeylensis* might be a distinct species from *B. berberensis*, but that further material was required to confirm this possibility. Kovařík (2011) considered *B. o. zeylensis* a colour morph of *B. berberensis* present in juveniles and some males.

# 9. Buthus bonito Lourenço and Geniez, 2005

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8669 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8670 *= Buthus sabulicola* Touloun 2012: 46, 48-58, fig.10, 13, 14 (**new synonymy**). 1 F holotype (MNHN), Khnifiss lagoon, Tan-Tan Province, Morocco.

*Buthus bonito*: Lourenço and Geniez 2005: 1-5, fig. 1-8, 10; Touloun et al. 2008: 3-4, fig.1; Stockmann and Ythier 2010: 362-363; Pedroso et al. 2013: 300; Aboumaâd et al. 2014: 6; Touloun et al. 2016: 880, fig. 2D.

**Type material:** 1 M holotype (MNHN N° RS8669), Khnifiss Iagoon (approx. 27.93°, -12.34°), Tarfaya, Morocco. Paratypes: 2 F (MNHN N° RS8670), from the same locality. **Distribution:** known from the Atlantic coast of Morocco south of Tan-Tan extending almost to Dakhla in the Western Sahara (Touloun et al. 2016).

**Remarks:** Although the type material of *B*. *sabulicola* was collected in 2002 by Touloun, Stockmann and Slimani, the species was not formally described until the publication of the PhD thesis of Oulaid Touloun in 2012. The type specimens of *B*. *bonito* and *B*. *sabulicola* are from the exact same locality, the Khnifiss lagoon, and both descriptions are almost identical. Touloun et al. (2016), probably by mistake, indicated that the fifth metasomal segment and telson are darkened in *B*. *bonito*, but the trait does not appear in the accompanying figure.

## 10. Buthus boumalenii Touloun and Boumezzough, 2011

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8891

*Buthus boumalenii*: Touloun and Boumezzough 2011a: 183-186, fig. 2-7; Pedroso et al. 2013: 300; Aboumaâd et al. 2014: 6; El Hidan et al. 2016: 4.

**Type material:** 1 F holotype (UCAM), Tineghir (approx. 31.366°, -5.905°), Boumalene, Morocco. Paratypes: 1 M (UCAM), 1 M, 1 (MNHN, N° RS8891), all from the same locality. **Distribution:** known only from the Boumalne region of Morocco (El Hidan et al. 2016). **Remarks:** This species is the only known representative of a phylogenetic lineage present east of the High Atlas Mountains of Morocco. Because of its phylogenetic uniqueness, the conservation of this species should have top priority.

#### 11. Buthus brignolii Lourenço, 2003

Buthus brignolii: Lourenço 2003: 905-907, fig. 75-79; Rossi and Tropea, 2016a: 4.

**Type material:** 1 F holotype (MHNG), Djebel Meidob (approx. 15.21°, 26.44°), Darfur, Sudan.

**Distribution:** known only from the type locality.

**Remarks:** it is one of the four known "inland island" species of *Buthus* that have been found in the Mountainous regions in the heart of the Sahara Desert.

# 12. Buthus centroafricanus Lourenço, 2016

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs9069

Buthus centroafricanus: Lourenço 2016a: 73-77, fig. 1-11.

**Type material:** 1 M holotype (MNHN), Between Bria and Yalinga (as Jalinga) (approx. 6.52°, 22.62°), Province Haute-Kotto, Central African Republic. **Distribution:** known only from the type locality.

## 13. Buthus chambiensis Kovařík, 2006

Buthus chambiensis: Kovařík 2006: 1-3, fig. 2-5; Rossi, Tropea and Yağmur 2013: 3, 7.

**Type material:** 1 M holotype (FKCP), Djebel Chambi Mountain (approx. 35.17°, 8.56°), Kasserine Province, Tunisia. Paratypes: 1 M juv., 2 F, 1 juv., all from the same locality. **Distribution:** known only from the type locality.

#### 14. *Buthus confluens* Lourenço, Touloun and Boumezzough, 2012

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8919 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8920

*Buthus confluens*: Lourenço, Touloun and Boumezzough 2012: 22-24, fig. 1-11; Touloun et al. 2014: 76-77.

**Type material:** 1 M holotype (MNHN N° RS8919), Alhamra (35.39529°, -05.37181°), Tétouan, Morocco. Paratypes: 1 F (MNHN N° RS8920), 1 M (UCAM), all from the same locality.

**Distribution:** known from several localities in the Tingitana Pensinsula of Morocco, but also further to the south.

**Remarks:** Based on the colour pattern and pigmentation, the original authors suggested that *B. confluens* was the closest phylogenetic relative in Morocco to *B. ibericus*, from the Iberian Peninsula. However, because the presence of three dark bands on the metasoma is shared among several *Buthus* species, this claim should be further confirmed with additional data.

#### 15. *Buthus draa* Lourenço and Slimani, 2004

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8694

*Buthus draa*: Lourenço and Slimani 2004: 166-169, fig. 1-7; Lourenço, Sun and Zhu 2009: p. 72, fig. 2-6; Stockmann and Ythier 2010: 362-363; Touloun and Boumezzough 2011b: 186; Habel et al. 2012: 2, 4; Sousa et al. 2012: 68-69; Pedroso et al. 2013: 300; Yang et al. 2013: 2; Aboumaâd et al. 2014: 6; El Hidan et al. 2016: 4. *Buthus occitanus tunetanus neeli* (MIS): Touloun et al. 1999: 1-2; *Buthus tassili* (MIS): Touloun 2012: 37, 40-41, fig.7;

**Type material:** 1 M holotype (UCAM), Taznakht (30.51853°, -7.02595°), Ouarzazate, Morocco. Paratypes: 1 M, 2 F (UCAM), 1 M, 1 F (ZMH), 2 M, 1 F (MNHN), all from the same locality; 1 M (ZMH), Aït Bassou; 2 F juv. (ZMH), Aït Ounzar Oulad Aissa; 1 M (ZMH), near Agdez; 1 M (ZMH), Oulad HIal. The ZMH accession number for all paratypes is A7/03.

**Distribution:** *B. draa* can be found in the upper part of the Draa River, probably at elevations below 1500 m a.s.l.

**Remarks:** *B. draa* shares with *B. tassili* and *B. nigrovesiculosus* the presence of a darkened fifth metasoma segment and telson.

#### 16. Buthus dunlopi Kovařík, 2006

Buthus dunlopi: Kovařík 2006: 2-3, 6, fig. 7-8; Rossi, Tropea and Yağmur 2013: 5, 7.

**Type material:** 1 M holotype (FKCP), Remada (approx. 32.31°, 10.39°), Tataouine, Tunisia. Paratypes: 1 M, 3 F (FKCP), same locality. **Distribution:** known only from the type locality.

## 17. Buthus duprei Rossi and Tropea, 2016

http://zoobank.org/86EDFE2D-B287-4DCD-BD37-8B99FC58915C

Buthus duprei Rossi and Tropea 2016b: 25-28, fig. 1-12.

**Type material:** 1 M holotype (MCVR), Port Sudan (approx. 19.59°, 37.19°), Sudan. Paratype: 1 M juv. (ARPC N° 0809), same locality. **Distribution:** known only from the type locality.

#### 18. Buthus egyptiensis Lourenço, 2012

*Buthus egyptiensis*: Lourenço and Cloudsley-Thompson 2012: 12-16, fig. 1-7; Lourenço and Simon 2012: 12; Rossi 2013: 191-192; Rossi, Tropea and Yağmur 2013: 4, 7.

**Type material:** 1 F holotype (ZMH N° A20/12), Siwa (approx. 29.17°, 25.46°), Egypt. **Distribution:** known only from the type locality.

**Remarks:** One of the four known "inland island" species of *Buthus* that have been found within the Sahara Desert, although in this case from an Oasis. It is also one of the largest known *Buthus* species.

#### 19. Buthus elhennawyi Lourenço, 2005

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8637

Buthus elhennawyi: Lourenço 2005b: 246-249, fig. 1-7; Lourenço and Leguin 2012: 8.

**Type material:** 1 M holotype (ZMH N° A42/05), Fété-Olé (as Félé-Olé) (16.233°, -15.099°), Ferlo, Senegal. Paratype: 1 M (MNHN N° RS8637), Rosi (as Rossi), Niger. **Distribution:** this species is known from Niger and Senegal, from a single locality in each country, which are almost 2,000 Km apart.

**Remarks:** We used the location of Fété-Olé given in Vincke et al. (2010), a locality that has been part of long term ecological studies, to map this locality, instead of the original spelling "Félé-Olé".

#### 20. Buthus elizabethae Lourenço, 2005

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8638

Buthus elizabethae: Lourenço 2005a: 230-235, fig. 1-12, Lourenço 2005b: 249.

**Type material:** 1 M holotype (ZMH N° A36/05), S.W. of Gaoual (approx. 11.71°, -13.22°), Boké, Guinea. Paratypes: 1 F (ZMH N° A37/05), same locality; 1 M, 1 F (MNHN), Niokolo-Koba National Park, Senegal.

Distribution: this species is known from Guinea and Senegal,

**Remarks:** Given the geographical proximity, it is possible that the *Buthus* material reported to have been found in Guinea-Bissau might very well correspond to this species. Unfortunately the Guinea-Bissau material was lost in a fire, and hence only newly collected material could confirm this possibility.

## 21. Buthus elmoutaouakili Lourenço and Qi, 2006

*Buthus elmoutaouakili*: Lourenço and Qi 2006: 288-291, fig. 1-11; Habel et al. 2012 (part): 2, 3; Husemann et al. 2012 (part): 2, 4-5; Touloun and Boumezzough 2011b: 11-12, fig. 2C; Aboumaâd et al. 2014: 6.

*Buthus occitanus mardochei alluaudi*: Vachon 1949c: 363-367, fig. 409-416; Vachon 1952a: 291-295, fig. 409-416; Le Corroller 1967: 63; Pérez 1974: 23; Touloun 2012: 39, 57.

**Type material:** 1 M holotype (ZMH N° A18/06), Ait Baha (approx. 30.07°, -9.15°), Chtouka Aït Baha, Morocco.

**Distribution:** this species seems to be widely distributed across the western portion of the Anti-Atlas, although some misidentifications with *Buthus parroti* cannot be excluded. **Remarks:** According to ICZN article 45.5, Vachon's (1949) infrasubspecific name is unavailable. Although the name was published before 1961, it was only used as infrasubspecific by all subsequent authors.

#### 22. Buthus elongatus Rossi, 2012

Buthus elongatus: Rossi 2012: 273-278, fig. 1-6; Teruel and Melic 2015: 5-9.

**Type material:** 1 adult M holotype (MZUF N° 1432), Sierra Blanca (36.533°, -4.900°), Marbella, Malaga Province, Spain. Paratypes: 1 adult F (ARPC), same locality; 1 M, 1 F (MZUF N° 875), Playa del Alicate (36.499°, -4.818), Marbella, Malaga Province, Spain. **Distribution:** this species is known from the southern Iberian Mediterranean coast, close to Marbella.

**Remarks:** The second locality given by Rossi as Alicante (sic), had a typographic error, as the coordinates given by the author, together with their map in Fig. 7, provide sufficient evidence for the correct mapping of this locality. Both localities are under severe anthropomorphic pressure.

# 23. *Buthus hassanini* Lourenço, Duhem and Cloudsley-Thompson, 2012

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8927

*Buthus hassanini*: Lourenço, Duhem and Cloudsley-Thompson 2012: 319-321, 323, fig. 35-42.

**Type material:** 1 F (MNHN N° RS8927), Biti Tehëc (approx. 17.187°, 22.288°), Ennedi Plateau, Chad.

Distribution: known only from the type locality.

**Remarks:** Another of the four known "inland island" species of *Buthus* that have been found in a Mountainous region in the heart of the Sahara Desert. The type locality was pinpointed following the map provided by the authors (fig. 90).

# 24. Buthus ibericus Lourenço and Vachon, 2004 (nomen protectum)

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8605

*= Buthus halius* (C. L. Koch, 1839) (*nomen oblitum*) (new combination, new synonymy). Holotype lost according to Fet and Lowe (2000), Portugal.

*Buthus ibericus* Lourenço and Vachon, 2004: 88-91, fig. 31-42, Fernández 2004: 222; Teruel and Pérez-Bote 2005: 273-276, fig. 1; Armas and González-Moliné 2009: 553-

554; Fet 2010: 4; Sousa et al. 2010: 207; Rossi 2012: 274-275, 277-278; Pedroso et al. 2013: 300; Teruel and Melic 2015: 6-9.

*Androctonus halius* C. L. Koch 1839a: 69-70, pl.CLXIII, fig. 383; Gervais 1844a: 43; C. L. Koch 1850: 90; Simon 1879: 96.

Buthus occitanus (MIS): Berejano and Pérez-Bote 2002: 59.

**Type material:** 1 M holotype (MNHN N° RS8605), San José del Valle (36.6247°, - 5.6646°), Cádiz, Spain. Paratypes: 2 F (MNHN, N° RS8654), 1 M, 2 F (CRBA, N° CRBA-21826), and 2 F (MNCN N° 20.02/14857), all from the same locality.

**Distribution:** this species seems to have a wide distribution range in the western part of the Iberian Peninsula, although the limits of its distribution remain poorly defined.



**Figure II-13.** Reproduction of C. L: Koch's 1839 *B. halius* figure 382 (plate CLXIII). Right pedipalp chelae detail from three *Buthus* species published in the same work: A - *B. paris* (as Androctonus clytoneus), fig. 384 (same plate); B – *B. halius* with an arrow pinpointing the basal lobe; C – B. paris, fig. 352 (pl. CLI). All images were taken as provided by the pdf copy available in the BHL, which was made available by the Ernst Mayr Library of the Museum of Comparative Zoology, Harvard University.

**Remarks:** B. ibericus was first described from Spain and subsequently reported for Portugal (e.g. Sousa et al. 2010, Rossi 2012). Simon (1879) synonymized Androctonus halius with B. occitanus based on its type locality, which was wrongly stated to be Spain (page 98), because Simon considered B. occitanus (as B. europaeus) to be the only good species in Spain. Koch's original description is not by itself enough to synonymize both species. However, Koch's Fig. 383 illustration of Androctonus halius includes a basal lobe in the movable finger (Fig. 13 and 13B), which is the key diagnostic character for *B. ibericus* (Lourenço and Vachon 2004, Rossi 2012), together with its type locality (Portugal), supports this synonymy. Nonetheless, according to the I.C.Z.N. article 23.9, the junior synonym can remain valid to maintain taxonomical stability. To our knowledge the name *B. halius* has not been used since 1879 (article 23.9.1.1), and more than 25 works have been published in the past 12 years by more than 10 authors using the name B. ibericus (article 23.9.1.2). Not all works are cited here because they are neither taxonomical nor faunistic. As such we propose to maintain as valid the junior synonym B. ibericus (nomen protectum) according to prevailing usage (article 23.9.1), and to consider the senior synonym *B. halius* a nomen oblitum. Rossi, 2012 (page 278), for *B.* ibericus, states erroneously "Sexual dimorphism is not noticeable in the chela manus", which is in contrast to the original descriptions of both Koch (1839) and Lourenço and Vachon (2004) and to the complementary description in Teruel and Pérez-Bote (2005). In both works it can be observed that the male manus is more bulbous than the female's, which results in the males having a smaller length to width ratio. The type locality of B.

*ibericus* was precisely located following the indications of Iñigo Sánchez, the original collector. According to Lourenço and Vachon (2004), two paratypes were deposited in the University of Barcelona ("déposés à l'Université de Barcelona"), however, we were unable to determine at which of two possible Barcelona University institutions' were they intrusted.

# 25. Buthus intermedius (Ehrenberg in Hemprich and Ehrenberg, 1829)

http://www.systax.org/en/details/spm/88132

*Androctonus (Leirus) tunetanus intermedius*: Ehrenberg in Hemprich and Ehrenberg 1829: 354; Braunwalder and Fet 1998: 33-34.

Androctonus (Leiurus) tunetanus intumescens (MIS): Kovařík 2006: 10. Androctonus (Liurus) tunetanus intermedius: Ehrenberg in Hemprich and Ehrenberg 1831: (pages not numbered).

Androctonus occitanus intermedius: Gervais 1844a: 42.

Buthus (Buthus) occitanus intermedius: Birula 1917a: 228.

Buthus occitanus intermedius: Peréz 1974: 23.

Buthus intermedius (Ehrenberg): Lourenço 2008: 46-47.

**Type material:** 1 F (in bad conditions) (ZMB N<sup>o</sup> 146), Al Luhayyah (as Lohaie), Yemen. **Distribution:** Known only from the type locality.

**Remarks:** Fet and Lowe (2000) considered the locality as probably wrong since at that time no other *Buthus* had been collected again in Yemen. However, Lourenço's (2008) *Buthus yemenensis* revalidated Ehrenberg species' by providing concrete proof for the existence of *Buthus* species in this country. Doubst about *B. intermedius* type locality were the only evidence given by Kovařík (2006) for its synonimization with *Buthus intumescens* (Ehrenberg in Hemprich and Ehrenberg, 1829).

# 26. *Buthus intumescens* (Ehrenberg in Hemprich and Ehrenberg, 1829)

http://www.systax.org/en/details/spm/88133

*Androctonus (Leiurus) tunetanus intumescens*: Ehrenberg in Hemprich and Ehrenberg 1829: 354; Braunwalder and Fet 1998: 33.

*Androctonus (Liurus) tunetanus intumescens*: Ehrenberg in Hemprich and Ehrenberg 1831 (pages not numbered); Moritz and Fischer 1980: 316.

Androctonus occitanus intumescens: Gervais 1844a: 42.

*Buthus intumescens*: Kovařík 2006 (part): 10-11, 15, fig. 20; Kaltsas et al. 2008 (part): 215; Rossi 2013: 191-192; Rossi, Tropea and Yağmur 2013: 3, 6-8.

**Type material:** 1 (sex unknown) (in bad conditions) (ZMB N° 145), Egypt. **Remarks:** known only from a single specimen.

# 27. Buthus israelis Shulov and Amitai, 1959

Buthus occitanus mardochei israelis: Shulov and Amitai 1959: 219-225, fig. 1-3.

*Buthus occitanus israelis*: Pérez 1974: 23; Vachon and Kinzelbach 1987: 101; Fet and Lowe 2000: 95; Skutelsky 1995: 46; Skutelsky 1996: 50

*Buthus occitanus israelis* (Shulov and Amitai, 1959): Levy and Amitai 1980: 16-21, fig. 25-29; EI-Hennawy 1992: 101, 120; Kovařík 2006: 10; Lourenço, Yağmur and Duhem 2010: 96.

Buthus intumescens (MIS): Kovařík 2006 (part): 10-11.

Buthus israelis: Lourenço, Yağmur and Duhem 2010: 96-97; Yağmur, Koç and Lourenço 2011: 29.

Buthus israelis (Shulov and Amitai, 1959): Rossi 2013: 191-192; Rossi, Tropea and Yağmur 2013: 2-3, 6-7.

*Buthus occitanus mardochei israelis*: Vachon 1966: 211; Kovařík 2006: 10; Lourenço, Yağmur and Duhem 2010: 95.

Buthus occitanus typicus: Bodenheimer 1937: 235.

**Type material:** holotype lost (sex unknown), Mash'abbe Sade (as Mashavei Sadé) (approx. 31°, 34.78°), Negev desert, Israel.

**Distribution:** This species has been recorded in Egypt (the Sinai Peninsula) and Israel. Notwithstanding, Kovařík (2006) considered this species to be a junior synonym of *B. intumescens*.

**Remarks:** Levy and Amitai (1980) did not designate any neotype when they re-described the species. In contrast to the several infrasubspecific taxa described by Vachon that are unavailable according to the ICZN, *B. o. israelis* is an available name according to the ICZN article 45.6.4.1 ("a name that is infrasubspecific under Article 45.6.4 is nevertheless deemed to be subspecific from its original publication if, before 1985, it was either adopted as the valid name of a species or subspecies or was treated as a senior homonym"), which is the present case as Levy and Amitai re-described this taxon before 1985, and thus articles 45.5 and **45.5.1** do not apply. Several authors wrongly report the original authors of the species in parenthesis. The use of parenthesis is only to be made when a species is changed from one genus to another (ICZN article 51.3), which is clearly not the case with *B. israelis*.

## 28. Buthus jianxinae Lourenço, 2005

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8175

Buthus jianxinae: Lourenço 2005c: 22-23, fig. 1-12.

**Type material:** 1 M holotype (MNHN N° RS8175), Loka (approx. 4.21°, 30.91°), Equatoria, South Sudan.

**Distribution:** known only from the type locality.

#### 29. Buthus karoraensis Rossi and Tropea, 2016

http://zoobank.org/9EA2BC5A-9E0B-4457-8E32-EA3C3FAA0A74

*Buthus karoraensis*: Rossi and Tropea, 2016a: 4-7, fig. 1-13; Rossi and Tropea, 2016b: 25.

*Buthus occitanus berberensis* (MIS): Kovařík 2003 (part): 138. *Buthus occitanus* (MIS): Kovařík and Whitman 2005 (part): 106.

**Type material:** 1 M holotype (MZUF N° 610), Karora (17.703°, 38.365°) (small enclave in the Eritrean-Sudan border), Eritrea. Paratypes: 1 M, 4 F (MZUF N° 610); 1 M (MCSNB: N° 12749), 1 F (MCSNB: N° 12748), all from the same locality. **Distribution:** known only from the type locality.

#### 30. Buthus kunti Yağmur, Koç and Lourenço, 2011

http://zoobank.org/96DA8302-0891-4EF8-8D5B-DA8275325908 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8892

*Buthus kunti*: Yağmur, Koç and Lourenço 2011: 29-33, fig. 1-12. *Buthus europaeus* (MIS): Simon 1879: 97. *Buthus occitanus* (MIS): Kraepelin 1891 (part): 199. Buthus sp.: Levy and Amitai 1980: 21.

**Type material:** 1 F holotype (MTAS), Rizokarpaso (Dipkarpaz) (35.58472°, 34.42306°), Karpaz Region, Cyprus. Paratypes: 1 M juv. (MTAS), Zafer. 1 M juv. (MNHN N° RS8892), Güzelyurt.

**Distribution:** the species is only known from the northern portion of Cyprus.

**Remarks:** according to Yağmur, Koç and Lourenço (2011), this species is rare in the island.

## 31. Buthus labuschagnei Lourenço, 2015

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8992

Buthus labuschagnei Lourenço 2015: 22-24, fig. 13-22.

**Type material:** 1 F holotype (MNHN N° RS8992), Zakouma (Zakouma National Park) (approx. 10.89°, 19.82°), Salamat Region, Chad. **Distribution:** known only from the type locality.

#### 32. Buthus lienhardi Lourenço, 2003

*Buthus lienhardi*: Lourenço 2003: 899-902, fig. 62-69; Stockmann and Ythier 2010: 362-363; Touloun and Boumezzough 2011a: 186; Touloun 2012: 37, fig.5C; Aboumaâd et al. 2014: 6.

*Buthus occitanus tunetatus Lepineyi:* Vachon 1949: 353-359, fig. 393-400; Vachon 1952a: 281-286, fig. 393-400;

Buthus occitanus tunetatus lepineyi: Malhomme 1954: 29-30; Le Corroller 1967: 63; Peréz 1974: 22; Touloun et al. 1999: 1; Touloun et al. 2001: 2; Touloun 2012: 37. Buthus occitanus tunetatus (MIS): Touloun 2012: 104, 108.

**Type material:** 1 M holotype (MHNG), Oukaimeden (approx. 31.201°, -7.861°), Marrakech, Morocco. Paratypes: 1 F, 2 juv. (MHNG), same locality.

**Distribution:** the species is known from a wide range across the High-Atlas Mountains. **Remarks:** Vachon (1949) infrasubspecific name is not available as explained previously.

# 33. Buthus lourencoi Rossi, Tropea and Yağmur, 2013

http://zoobank.org/82B4235D-820E-4FE2-8AFC-6E0B4E28334D

*Buthus lourencoi*: Rossi, Tropea and Yağmur 2013: 2-3, fig. 3-10. *Buthus occitanus* (MIS): Kovařík and Whitman 2005 (part): 106.

**Type material:** 1 adult F holotype (MZUF N° 783), Mellaha (approx. 32.896°, 13.285°), Tripoli, Libya

**Distribution:** known only from the type locality.

**Remarks:** The type locality is now part of the large city of Tripoli. It is fairly unlikely that the species still occur within the boundaries of the city given the level of urban development. However, Mellaha, which was originally a military airport, is now the Mitiga International Airport, where large patches of unconstructed ground that may be suitable fot the species still exist.

#### 34. Buthus malhommei Vachon, 1949

*Buthus occitanus malhommei* Vachon 1949: 376; Vachon 1952a: 304-308, fig. 433-444; Fet and Lowe 2000: 95; Touloun et al. 2001: 2; Touloun 2012: 35, 104, 108, fig. 5A;.
*Buthus malhommei*: Lourenço 2003: 887-889, fig. 33-38; Stockmann and Ythier 2010: 364-365 (MIS); Sousa et al. 2012: 68-69; Aboumaâd et al. 2014: 5.

**Type material:** 3 M, 3 F, 7 juv., syntypes (MNHN), Mechra ben Abbou (approx. 32.646°, -7.800°), Settat, Morocco.

**Distribution:** Toulon (2012) greatly expanded the known distribution of this species along the basin of the Oum er Rbia River.

#### 35. Buthus mardochei Simon, 1878

*Buthus mardoche* (IOS): Simon 1878: 159-160; Simon 1879: 100; Kraepelin 1891: 199; Birula, 1896: 244

*Buthus (Buthus) mardoche* (IOS): Birula 1910: 145-146; Birula 1917a: 223 ("dubious species"); Werner 1932: 300-305.

Buthus occitanus mardochei: Vachon 1949c (part): 358-363, fig. 400-408; Vachon 1952a (part): 286-295, fig. 401-408; Malhomme 1954: 28-29; Pérez 1974: 22; Levy and Amitai 1980: 16; El-Hennawy 1992: 98, 120; Kovařík 1995: 20; Gantenbein et al. 1998a: 51; Gantenbein et al. 1998b: 33-39; Kovařík 1998: 106; Fet and Lowe 2000: 96; Touloun et al. 2001: 2; Gantenbein and Largiadèr 2003 (part): 120, 122.

*Buthus occitanus mardochei mardochei*: Le Corroller, 1967. 63; Touloun 2012: 39, 104, 108, fig.5D.

*Buthus mardochei*: Lourenço 2003: 889, fig. 39; Stockmann and Ythier 2010: 364-365; Aboumaâd et al. 2014: 5.

**Type material:** 1 F (MNHN N° RS1771, damaged), southern Morocco.

**Distribution:** this species appears to have a distribution parallel to that of *B. atlantis*, between Essaouira and Agadir, but is found further inland and away from the Atlantic coast.

**Remarks:** Vachon (1949d: 358) corrected what he considered Simon's incorrect original spelling of "mardoche" to "mardochei", since the form was named as a patronym after its collector, Rabbi Mardoché. Nevertheless, it is our understanding that this was an unjustified emendation, because the ICZN article 31.1 admit the use of a noun in apposition as was the case with "mardoche", however the ICZN article 33.2.3.1 admits the prevalence of this emendation as it continues to be attributed to "the original author and date" and is "in prevailing usage" and as such we refrain from any change to the name. Vachon (1949, 1952) also established that the species occurs roughly between Essaouira and Agadir, but not near the coast where it is replaced by *B. atlantis*.

#### 36. Buthus mariefranceae Lourenço, 2003

*Buthus mariefranceae*: Lourenço 2003: 889-893, fig. 40-46; Lourenço and Qi 2006: 291; Stockmann and Ythier 2010: 364-365; Sousa et al. 2012: 68-69; Touloun 2012: 40; Pedroso et al. 2013: 300; Aboumaâd et al. 2014: 6.

*Buthus occitanus mardochei mimeuri*: Vachon 1949c: 367-373, fig. 417-425; Vachon 1952a: 295-301, fig. 417-425; Le Corroller 1967: 63; Pérez 1974: 23; Touloun 2012: 40, 57.

**Type material:** 1F holotype (MHNG), Tan-Tan (approx. 28.43°, -11.1°), Guelmim Region, Morocco. Paratypes: 5 M, 4 F, 2 F juv. (MHNG), Goulimine.

**Distribution:** this species has a large distribution in Morocco, east and south of the Anti-Atlas Mountain.

**Remarks:** Vachon (1949) infrasubspecific name is not available as explained above.

#### 37. Buthus maroccanus Birula, 1903

*= Prionurus tingitanus*: Pallary 1928a: 350-351, fig. 4 (synonymized by Vachon 1949b: 281). Syntype, sex unknown (MNHN), Rabat, Morocco (Vachon, 1949, 1952).

Buthus occitanus maroccanus: Birula 1903: 106.

Buthus europaeus (MIS): Hirst 1925 (part): 416.

Buthus (Buthus) occitanus maroccanus: Birula 1910: 145; Birula 1917a: 223.

Buthus occitanus maroccanus: Giltay 1929: 196; Werner 1929: 31-32.

*Buthus maroccanus*: Werner 1932: 299; Werner 1934b: 84; Vachon 1949b: 281-287, fig. 364-371; Vachon 1952a: 255-261, fig. 364-371; Foley 1951: 33; Bücherl 1964: 57; Pérez 1974: 22: I.evy and Amitai 1980: 15; El-Hennawy 1992: 98, 119; Kovařík 1995: 20; Kovařík 1998: 106; Fet and Lowe 2000: 92; Sousa et al. 2012: 68-69; Stockmann and Ythier 2010: 366-367; Aboumaâd et al. 2014: 6..

Buthus marocanus (ISS): Le Corroller 1967: 63.

**Type material:** 3 M, F, syntypes (ZIN), Morocco; 1 specimen syntype (ZIN), locality unknown (Fet and Lowe, 2000).

**Distribution:** all known specimens have been captured in the Rabat Region (approx. 28.43°, -11.10°).

**Remarks:** it remains the only known *Buthus* species with a uniformly darkened body.

#### 38. Buthus montanus Lourenço and Vachon, 2004

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8604 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8653

*Buthus montanus* Lourenço and Vachon 2004:84, 86-87, 91, fig. 16-30; Fernández 2004: 222; Fet 2010: 4; Rossi 2012: 274, 277-278; Teruel and Melic 2015: 5-9.

**Type material:** 1 M holotype (MNHN N° RS8604), Sierra Nevada (between Puerto de la Ragua and Cerro Pelado) (approx. 37.11°, -3.14°), Granada Region, Spain. Paratypes: 1 M, 3 F (MNHN N° RS8653), same locality.

**Distribution:** known only from the type locality.

#### 39. Buthus nigrovesiculosus Hirst, 1925 (new status)

*Buthus europaeus nigrovesiculosus*: Hirst 1925: 416. *Buthus occitanus nigrovesiculosus*: Pérez 1974: 22; Fet and Lowe 2000: 96.

**Type material:** 1 M (adult?), 1 juv., syntypes (NHMUK), Boste (approx. 23.79°, -15.68°), Rio de Oro (Western Sahara), now Morocco. **Distribution:** known only from the type locality.

**Remarks:** Hirst identified this North African species as a subspecies of *B. occitanus*, but as currently circumscribed, *B. occitanus* does not occur in North Africa (Gantenbein and Largiadèr 2003, Sousa et al. 2012). The original description of *B. nigrovesiculosus* suggests morphological similarities to *B. draa* and *B. tassili* Lourenço, 2002. These three species have a dark, almost black, fifth segment of the metasoma (Fig. 14 and 15A, less clear in the male type, obvious in the juvenile, syntypes in the NHMUK). Males of these three species also show slender pedipalp chelae. The type series of *B. nigrovesiculosus* includes only two animals, and more material is necessary to correctly evaluate the relationship between these three species. Nevertheless the males of *B. nigrovesiculosus* can be distinguished from males of the other two species by a higher pectinal tooth count

(Fig. 10 and 15B, 36 versus <32 in the other two species), and from *B. tassili* by having a squared first metasomal segment.



Figure II-14. Photo of the syntypes of *B. nigrovesiculosus* (NHMUK). Photo by Sérgio Henriques.



**Figure II-15.** Detailed morphology of the larger syntype of B. nigrovesiculosus (NHMUK). A – Lateral view of the telson and two terminal segments of the metasoma; B – Ventral view of the mesosoma, with pectines clearly visible; C – External lateral view of the terminal half of the right pedipalp chela. All photos by Sérgio Henriques.

#### 40. Buthus occidentalis Lourenço, Sun and Zhu, 2009

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8844 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8845

Buthus occidentalis: Lourenço, Sun and Zhu 2009: 72-74, fig. 7-19; Yang et al. 2013: 2.

**Type material:** 1 F holotype (MNHN N° RS8844), Dakhlet Nouadhibou Region, in the coastal area (approx.20.28°, -16.24°), Mauritania. Paratypes: 1 M, 1 F juv. (MNHN N° 8845); 1 F, 1 M juv. (MHBU), all from the same locality. **Distribution:** known only from the type locality.

#### 41. Buthus occitanus (Amoreux, 1789) (restricted distribution)

*= Scorpio rufus*: Amoreux 1789a: 42-43 (synonymized by Amoreux 1789b, as the first revisor, ICZN article 24.2.2).

*= Androctonus ajax*: C. L. Koch 1839b: 53, pl. CXCIII, fig. 467 (synonymized by Simon 1879: 96). Type lost: Spain.

*= Androctonus eurialus*: C. L. Koch 1839b: 25-27, pl. CLXXXVII (not pl. CXXXVII, as listed in the text), fig. 448 (synonymized by Simon 1879: 96). Type lost, France.

*= Androctonus eurilochus*: C. L. Koch 1839b: 27-28, pl. CLXXXVII, fig. 449 (synonymized by Kraepelin 1891: 196). Type lost, locality unknown.

*= Buthus europaeus*: Thorell 1876b: 7. Type is the lost Linnaeus (1748, 1754), specimen, purportedly from Italy (see the above "type species of *Buthus*" section and Fet et al. 2002).

*= Buthus europaeus tridentatus*: Franganillo 1918: 122-123 (**new synonymy**). Type unknown, Janvier, Navarre, Spain.

*Scorpio occitanus*: Amoreux 1789a: 42-43, pl. I, fig. 2; Amoreux 1789b: 10-16, pl. I. fig. 3; Herbst 1800: 73-82: Latreille 1804: 122; Latreille 1806: 132; Maccary 1810: 5-48; Audouin 1826: 172-173, pl. VIII, fig. 1; Audouin 1827: 410-411, pl. VIII, fig. 1; Dufour 1856 (part): 570.

Androctonus ajax: C. L. Koch 1850: 90.

Androctonus euryalus (ISS): C. L. Koch 1850: 90.

Androctonus eurylochus (ISS): C. L. Koch 1850: 90; Kraepelin 1891: 196.

Androctonus (Prionurus) occitanus: Lankester 1885: 380.

*Buthus* europaeus: Karsch 1879a: 18; Simon 1879: 96-98; Pavesi 1880: 312-313; Simon 1880c: 29; Pavesi 1884: 450; Pavesi 1885: 197, 199; Simon 1885: 51; Pocock 1889a: 116; Thorell 1893: 358-359; Birula 1896: 241-243; Birula 1900c: 9; Hirst 1925 (part): 415-416; Gadeau de Kerville 1926: 71; Bacelar 1928: 191; Hugues 1933: 487-488.

Buthus occitanicus (ISS): Dalla Torre 1905: 3; Táborský 1934: 40.

*Buthus occitanus*: Leach 1815: 391; Risso 1826: 156-157; Peters 1861b: 513; Karsch 1881a: 89; Kraepelin 1891 (part): 196-199 (part), pl. l, fig. 5, pll., fig. 18; Kraepelin 1895: 80; Kraepelin 1899: 26; Kraepelin 1901a (part): 266; Werner 1902 (part): 598; Birula 1910: 118-120; Masi 1912: 101; Borelli 1914b: 460; Lampe 1917: 191; Pavlovsky 1924: 77; Pavlovsky 1925: 140; Werner 1925: 209; Werner 1936: 173; Schenkel 1938: 4; Feytaud 1940: 38-39; Vachon 1940: 242-247, 254-258, fig. 1-9, 29, 33, 61-64; Käsmer 1941: 231; Denis 1948: 155-156; Vachon 1948c: 61, fig. 5; Vachon 1949a: 156-160, fig. 331-344, 348, 372-380; Vachon 1950b: fig. 591; Vachon 1951a: fig. 641, 657, 663, 679, 687, 696; Vachon 1951b: 621-623; Vachon 1952a: 264; Vachon 1952b: 274-279; Vachon 1961: 31-32; Bücherl 1964: 57; Pérez 1974: 22; Vachon 1974: 873; Goulliart 1979: 2; Levy and Amitai 1980 (part): 15-16; Kinzelbach 1982: 53; Prost 1982: 5; Mari et al. 1987; Sissom 1990: 92, fig. 3.17C, L; Kovařík 1992a (part): 183; Reichholf and Steinbach 1992: 33, fig. 4-5; Crucitti 1993: 51; Crucitti et al. 1994: 57-66; Vincent 1994: 6; Crucitti and Chinè 1995: 15-26; Braunwalder 1997b: 3; Crucitti and Chinè 1997: 195-

200; Cloudsley-Thompson and Lourenço 1998: 1-2; Kovařík 1998: 106; Kovařík 1999 (part): 39, 42, fig. 3; Fet and Lowe 2000 (part): 92-94; Lourenço 2003 (part): 884, 886-887, fig. 27-32; Lourenço and Vachon 2004: 83-85, fig. 1-15; Kovařík and Whitman 2005 (part): 106; Teruel and Pérez-Bote 2005: 276; Castilla and Pons 2007: 258; Dupré et al. 2008: (pages unnumbered); Sousa et al. 2010: 207; Colombo 2011: 1; Rossi 2012: 274-278; Pedroso et al. 2013: 300; Rossi, Tropea and Yağmur 2013: 3; Martin-Eauclaire et al. 2014: 56; Teruel and Melic 2015: 6-9; Lourenço 2016b: fig. 2.

*Buthus occitanus occitanus*: Birula 1910: 118; Hadži 1929: 31; Vachon 1949a: 156-160, fig. 331-344; Vachon 1949c: 336; Vachon 1952a (part): 264; Le Corroller 1967: 63; Fet and Lowe 2000 (part): 94-95; Gantenbein and Largiadèr 2003 (part): 120, 122.

Buthus occitanus tridentatus: Fet and Lowe 2000: 97; Kovařík 2001: 79;

*Buthus (Buthus) occitanus*: Birula 1909b: 507; Birula 1910: 143; Birula 1914b: 644-664; Birula 1917a: 22, 38-39, 199, 213.

Buthus (Buthus) occitanus occitanus: Roewer 1943: 206.

Buthus cf. occitanus: Piñero et al. 2013: 88.

Scorpio australis (MIS): Asso 1784: 146, Tab. I, fig. 2.

Scorpio (Androctonus) occitanus: Gervais 1844a: 42-44; pl. XXIII, fig. 4.

Scorpio occitanicus (ISS): Serres 1822: 65.

Scorpion Occitanus (ISS): Latreille 1817: 105-106.

Type material: type unknown, Souvignargues, Occitanie Region, France.

**Distribution:** Traditionally, the distribution of *B. occitanus* was considered to span from the Moroccan Atlantic shores in North Africa to the Middle East in Asia and to Southern-Western Europe. However, following the description of new species in the genus, the present distribution of *B. occitanus* has been restricted to NE Spain and SW France. Several molecular phylogenetic studies have demonstrated that the species range does not extend beyond Western Europe (Gantenbein and Largiadèr 2003, Sousa et al. 2010, 2012, Pedroso et al. 2013). Lourenço and Vachon (2004) and Rossi (2012) include redescriptions of *B. occitanus* that include only European animals, but Vachon (1952a) also included material from Morocco, which was most likely not conspecific.

**Remarks:** All material collected outside of the range here proposed should be considered as *Buthus sp.* Only the re-examination of those specimens could reveal their appropriate identity. Vachon (1952a) included animals from the west (Atlantic) coast of Morocco, from Kenitra to El Jadida, within his definition of *B. o. occitanus.* As explained above this material is now considered not to be part of *B. occitanus* and thus remains unnamed. We opted to leave *Androctonus eurilochus* in synonymy with *B. occitanus*, despite the uncertain about the species provenance, to help to stabilise the genus' taxonomy. To further bring stability to *Buthus* taxonomy we propose Franganillo's subspecies, *B. o. tridentatus*, as a junior synonym of *B. occitanus*. Although the type specimen is not known (if it ever existed as such), its type locality is clearly stated as Javier, in Navarre, Spain. Extensive sampling on the left bank of the Ebro River (Sousa 2017) indicates that only *B. occitanus* occurs in this part of Spain and hence we here propose this new synonymy.

#### 42. Buthus orientalis Lourenço and Simon, 2012

http://zoobank.org/E408579C-9287-4EE7-9C32-E754EC925B92 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs6910 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs6623

*Buthus orientalis*: Lourenço and Simon 2012: 10-14, fig. 1-12; Rossi 2013: 191-192; Rossi, Tropea and Yağmur 2013: 5, 7.

**Type material:** 1 F holotype (MNHN N° RS8910), Alexandria (approx. 31.17°, 29.91°), Egypt. Paratypes: 7 M, 13 F (MNHN, N° RS6623), same locality.

Distribution: known only from the type locality.

#### 43. Buthus paris (C. L. Koch, 1839)

*= Androctonus clytoneus*: C. L. Koch 1839a: 70-72, pl. CLXIII, fig. 384 (synonymized by Vachon 1949c: 380-381). Types lost; Africa.

*Androctonus paris*: C. L. Koch 1839a: 25-28, pl. CLI, fig. 352; C. L. Koch 1850: 90. *Androctonus clytonicus* (ISS): Gervais 1844a: 43.

Androctonus clytoneus: C. L. Koch 1850: 90.

*Buthus occitanus paris*: Birula 1903: 107; Birula 1910: 118, 155; Giltay 1929: 196; Werner 1932: 300-305; Vachon 1949c: 380-388, fig. 356, 400, 445-455; Vachon 1951b: 621; Vachon 1952a: 308-316, fig. 356, 400 445-455; Malhomme 1954: 29; Arroyo 1961: 186-189; Le Corroller 1967: 63; Peréz 1974: 23; Levy and Amitai 1980: 16; El-Hennawy 1992: 98, 121; Kovařík 1995: 20; Gantenbein et al., 1998a: 51; Kovařík 1998: 106; Fet and Lowe 2000: 96; Touloun et al. 2001: 2; Gantenbein and Largiadèr 2003: 120, 122; Touloun 2012: 35, 104, 108, fig.5B.

Buthus (Buthus) occitanus paris: Birula 1910. 145, 155; Birula 1917a: 223.

*Buthus paris*: Lourenço 2003: 896-897, fig. 52-56; Kovařík 2006: 2, 6, 8, 15, fig. 10-11; Lourenço 2013: 65-66; Rossi, Tropea and Yağmur 2013: 3, 5, 7; Aboumaâd et al. 2014: 6; Touloun et al. 2014: 77-78; Lourenço and Sadine 2016: 14-15.

**Type material:** Holotype, lost according to Fet and Lowe (2000), Algeria. Vachon (1949c, 1952a) wrote that the types came from Alger without further explanation.

Distribution: the species is currently distributed across Algeria, Morocco and Tunisia. **Remarks:** Because of the description of new species from Algeria, and the lack of both type specimen and locality (beyond the country), a neotype for *B. paris* is necessary to stabilize the taxonomy of Algerian Buthus, which may challenge the status of some newly described species. This is further complicated by recent diagnoses of B. paris (at least in part: Lourenço 2003, Kovařík 2006, Lourenço & Sadine 2016) that differ from those offered by Vachon (1952a). The differences between the different diagnoses include the number of rows in the movable finger, the aspect ratio of the first metasomal segment, the body chaetotaxie, the aculeus to vesicle length and the type of sexual dimorphism of the pedipalp chelae. Vachon (1952a) studied a large number of specimens from the entire Maghreb region, unrivalled by any subsequent study, which leads us to consider Vachon's description as the "gold standard". Vachon himself stated that most of the specimens used in his redescriptions were stored at the MNHN. If this material is ever located, it should have priority in the designation of a neotype. Although Vachon (1952a) did not formally describe any varieties within *B. paris*, he split the specimens that compose the species into three regions: 1) The typical region (from Algiers to northern Tunisia); 2) Specimens from the Oujda region (Morocco); 3) Those from northern Morocco and the Middle Atlas flanks. Interestingly the split of B. paris into these three regions corresponds well with the distribution of the genetic variability in the cox1 gene (Gantenbein and Largiadèr 2003, Sousa et al. 2012, Pedroso et al. 2013), placing B. paris in two different groups: in occitanus which include all B. paris specimens from regions two and three, and tunetanus, which include B. paris specimens from the typical region one, along the split of the two cox1 groups in the middle of Algeria (Fig. 7). If confirmed, this will mean that B. paris does not occur in Morocco. It is unclear if the variety from the third region above might correspond to *B. confluens* Lourenco. Touloun and Boumezzough, 2012, although these authors (page 22) refrained from suggesting this possibility because they could not find any of the material used by Vachon, purportedly to be in the MNHN, to describe this variety.

#### 44. Buthus parroti Vachon, 1949 (new status)

*Buthus atlantis parroti*: Vachon 1949a: 168-169, fig. 346, 350, 352-354, 356, 363; Vachon 1952a: 254-255, fig. 346, 350, 352, 353, 354, 356, 363; Le Corroller 1967: 63; Pérez, 1974 1974: 22; El-Hennawy. 1992: 98, 119; Kovařík 1995: 20; Kovařík 1998: 106; Lourenço 2003: 883-885, fig. 23-26; Fet and Lowe 2000: 91-92; Touloun 2012: 43, fig. 9B; Pedroso et al. 2013: 300.



**Figure II-16.** Photo of a *B. parroti* female syntype (MNHN N° RS1870), from the Ademine Forest, 04-1939, S.W. of Agadir, Morocco (Vachon 1952).

**Type material:** 1 F (MNHN N° RS1870), 1F, 12 juv., syntypes (MNHN), Forest house (approx. 30.31°, -9.33°), Ademine Forest, 40 Km S.W. of Agadir, Morocco; 2 M, 1 F juv., syntypes (MNHN), Taroudant (approx. 30.46°, -8.87°), Morocco.

**Distribution:** Known only from the Sous River Valley. Type localities in Fet and Lowe (2000) were mixed up.

**Remarks:** *B. parroti* was first described as a subspecies of *B. atlantis*. The two species occupy different habitats in Western Morocco; B. parroti is a forest species and B. atlantis is a sand dune dweller (Vachon 1952a). Furthermore, B. atlantis is clearly larger than B. parroti. Additionaly, the first metasomal segment of *B. parroti* is wider than long, the aculeus is shorter than the vesicle and the anal arch has only two lobes (Vachon 1952a), while in *B. atlantis* this segment is longer than wide, the aculeus is as long as or longer than the vesicle and the anal arch has three lobes (Vachon 1952a). Three additional Buthus species occur in the same area of Morocco as B. parroti, namely B. elmoutaouakili Lourenco and Qi, 2006, B. mardochei Simon, 1878 and B. mariefranceae. B. parroti can be distinguished from all three species by the presence of macrosetae in the tergites (Vachon 1952a). Moreover, it can be distinguished from *B. mariefranceae* by its larger size and absence of a dark fifth metasomal segment. Mesosoma colour pattern is not clear for *B. parroti*; the examined specimen at the MNHN (RS1870) (Fig. 16) appears to have two very faint darker stripes, while B. mariefranceae has two very well marked mesosomal dark stripes. B. parroti males show slender pedipalp chelae than females, while there is little if any sexual dimorphism in *B. mardochei*.

#### 45. Buthus prudenti Lourenço and Leguin, 2012

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8913 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8915 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8914

Buthus prudenti: Lourenço and Leguin 2012: 2-6, 8, fig. 1-14; Lourenço 2016a: 76.

**Type material:** 1 M holotype (MNHN N° RS8913), Ouro Labaré (9.38715°, 13.83447°), Bénoué, Cameroon. Paratypes: 7 M, 8 F, same locality; 11 paratypes (MNHN N° RS8914, RS8915), 4 paratypes (CBGP).

**Distribution:** known only from the type locality.

**Remarks:** type locality toponym derived from the coordinates given in the original description, as the type locality given was only Region of Sanguéré-Djoi, Cameroon.

#### 46. Buthus pusillus Lourenço, 2013

*Buthus pusillus*: Lourenço 2013: 64-67, fig. 1-9; Lourenço and Sadine 2016: 14; Rossi, Tropea and Yağmur 2013: 5, 7.

**Type material:** 1 M holotype (ZMH N° A11/13), Tizi Oumalou (36.5102°, 4.3390°), Tizi Ouzou Province, Djurdjura Mountains, Algeria. Paratype: 1 M juv. (ZMH N° A12/13), same location.

**Distribution:** known only from the type locality.

**Remarks:** The locality we present here corresponds to the coordinates given in the paper, although these do not agree with the elevation also reported in the paper: 2150 m a.s.l. This is very close to the maximum altitude of the highest peaks of the Djurdjura Mountains, and much higher than the 935 m a.s.l. of Tizi Oumalou.

#### 47. Buthus rochati Lourenço, 2003

*Buthus rochati*: Lourenço 2003: 893-896, fig. 47-51;Aboumaâd et al. 2014: 6. *Buthus occitanus mardochei panousei*: Vachon 1949c: 373-376, fig. 426-432; Vachon 1952a: 301-304, fig. 426-432; Le Corroller 1967: 63; Pérez 1974: 23; Touloun 2012: 57.

**Type material:** 1 M holotype (MHNG), Tafnidilt Region (as Tafnidit) (approx. 28.56°, -11.03°), Guelmim Region, Morocco. Paratypes: 1 M, 1 F (MHNG); same locality; 6 F (MHNG), west of Tafnidilt Region, Draa River valley; 3 M, 1 F (MHNG), unknown locality. **Distribution:** known distribution confined to the Tafnidilt region of Morocco.

**Remarks:** Lourenço (2003) does not mention the most remarkable diagnostic character given by Vachon (1952a), the interrupted dorso-median keel of the pedipalp patella. However Lourenço's Figure 49 illustrates this character, as it is a copy of Vachon's original drawings, and thus we consider it as part of the species diagnose and the most reliable diagnostic character for *B. rochati.* Vachon (1949) infrasubspecific name is not available as is explained above.

#### 48. Buthus saharicus Saddine, Bissati and Lourenço, 2015

*Buthus saharicus*: Saddine, Bissati and Lourenço 2015: 47-49, fig. 6-8; Lourenço 2016b: fig. 4.

**Type material:** 1 F holotype (MNHN), Ghardaïa Region (approx. 32.300°, 3.833°), in Wadi bed, Algeria. Paratypes: 1 M (UGA), 1 F juv. (MNHN), same locality. **Distribution:** known only from the type locality.

**Remarks:** Saddine *et al.* claimed that *B. saharicus* was the "first true deserticolous species found in Algeria", a bold claim given that *B. tunetanus* (*sensu* Vachon 1949, part) had already been recorded for Beni Abbés by Vachon (1949, 1952), also a desert location in central west Algeria, and albeit neither localities are Erg Desert areas, Beni Abbés is in the border of the Grand Erg Occidental.

#### 49. Buthus tassili Lourenço, 2002

https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8501 https://science.mnhn.fr/institution/mnhn/collection/rs/item/rs8622

*Buthus tassili*: Lourenço 2002: 113-115, fig. 10, 12, 14; Lourenço 2003: 906-909, fig. 80-86; Rossi, Tropea and Yağmur 2013: 3-5, 7.

Buthus occitanus (MIS): Pallary 1929: 134, 140; Pallary 1934: 98-99.

*Buthus occitanus tunetanus neeli*: Gysin 1969: 65-71, fig. 1-5; Peréz 1974: 22; Touloun 2012: 37, 40.

*Buthus occitanus tunetatus*, "Spécimens des régions montagneuses centrales du Sahara": Vachon 1952a: 279.

**Type material:** 1 M holotype (MNHN N° RS8501), Tin Tazarif (approx. 24.466°, 10.466°), Illizi, Algeria. Paratype: 1 F (MNHN N° RS8622), same locality.

**Distribution:** This species is known from a wide area around the Hoggar and Tassili N'Ajjer Mountains, including at least one locality in Libya.

**Remarks:** The Tin Tazarif coordinates given here, standing at 880 m a.s.l., do not match the altitude given for the point by Lourenço (2002), of 1.800 m a.s.l., but correspond well to the map location given by the author in Figure 6 of the same article. Nevertheless there are several locations in the Tassili N'Ajjer Mountains at or above 1.800 m a.s.l., located closer to Jebel Azao, its highest peak. The species ranges from the Hoggar Mountains to the Tassili N'Ajjer Mountains, including Ghat in Libya. Gysin's name, *B. o. t. neeli*, is not available under the ICZN article 10.2 and 45.5, as already stated by Fet and Lowe (2000). Therefore, it cannot enter formal synonymy. Nevertheless, because Gysin's description (1969) brings relevant taxonomic information (figures and new localities) for *B. tassili*, we have decided to clearly state this new informal synonymy. The specimens studied of both species come from the Algerian Hoggar Mountains and share a typical darkened fifth segment of the metasoma (see *B. nigrovesiculosus* above).

#### 50. *Buthus trinacrius* Lourenço and Rossi, 2013

*Buthus trinacrius*: Lourenço and Rossi 2013: 10-12, fig. 1-9. *Buthus europaeus* (MIS): Simon 1879: 97; Simon 1910 (part): 69. *Buthus occitanus* (MIS): Kraepelin (1901): 266.

**Type material:** 1 M holotype, Palermo Province? (approx. 38.05°, 13.32°), Sicily. Paratypes: 1 M, 1 F. All type material in bad conditions (MNHN N° RS3247). **Distribution:** Recorded from Sicily.

**Remarks:** Although Lourenço and Rossi (2013) report that the collector is not mentioned in Simon's notes, Kraepelin (1901), in his list of all the scorpion material present in the MNHN Paris, writes that Letourneur collected the *Buthus* material from Sicily and Corfu (Greece), which causes doubts regarding the correct collection locality of the specimens used to describe this species. Simon (1879) had doubts about the actual existence of *Buthus* in Sicily (when examining the specimens that would eventually be designated as type material for *B. trinacrius*): "*il habite probablement aussi le midi de l'Italie et la Sicile*", and *Buthus* has never been found in mainland Italy. Furthermore, the authors also claimed that Simon (1910) "referred to the almost impossibility to distinguish *Buthus* populations from North of Africa with those from Spain and Sicily", which is only partially correct. Simon (1910) solely referred to the distinction of *Buthus* populations of Algeria and Spain from those of Egypt, although in a subsequent paper, Simon does state that *Buthus* exist in Sicily without any further comments.

#### 51. Buthus tunetatus (Herbst, 1800)

*Scorpio tunetanus* Herbst 1800: 68-69, pl. III, fig. 3 (not pl. II, fig. 2, as listed in the text); Latreille 1804: 122-124.

Androctonus (Leiurus) tunetanus: Ehrenberg in Hemprich and Ehrenberg 1829: 354.

Androctonus (Leiurus) tunetanus genuinus: Ehrenberg in Hemprich and Ehrenberg 1829: 354.

Androctonus (Liurus) tunetanus: Ehrenberg in Hemprich and Ehrenberg 1831 (pages unnumbered)

*Androctonus (Liurus) tunetanus genuinus*: Ehrenberg in Hemprich and Ehrenberg 1831 (pages unnumbered).

*Androctonus tunetanus*: C. L. Koch 1845: 15-19, pl. CCCCI (sic), fig. 968; C. L. Koch 1850: 90.

Buthus occitanus (MIS): Kovařík and Whitman 2005 (part): 106.

*Buthus occitanus tunetanus*: Birula 1903: 107; Birula 1910: 118; Borelli 1914a: 154-155; Borelli 1914b: 461; Borelli 1924: 4-5; Borelli 1928: 351; Giltay 1929: 196-197; Werner 1929: 30-31; Caporiacco 1932: 395-396; Schenkel 1932: 379-380; Werner 1932: 300-305; Pallary 1934: 99; Borelli 1934: 169; Werner 1934b: 84-85, fig. 4; Werner 1936: 173; Caporiacco 1937: 345; Schenkel 1949: 186; Vachon 1949c: 344-353, fig. 381-393; Vachon 1951a: fig. 670; Vachon 1952a: 272-281, fig. 381-393, 670; Vachon 1966: 211; Peréz 1974: 22; Levy and Amitai 1980: 16; El-Hennawy 1992: 98, 121; Kovařík 1995: 20; Kovařík 1997: 179; Gantenbein et al. 1998a: 51; Gantenbein et al. 1998b: 33-39; Kovařík 1998: 106; Fet and Lowe 2000: 97; Lourenço 2002, p. 113, 115, fig. 8-9, 11, 13; Kovařík 2002: 6; Gantenbein and Largiadèr 2003: 120, 122; Ben Othmen et al. 2004: 257; Touloun 2012: 37, 41.

*Buthus (Buthus) occitanus tunetanus*: Birula 1908: 123-124; Birula 1909: 507-508, fig. B; Birula 1910: 156-157; Birula 1917a: 223; Roewer 1943: 206.

*Buthus tunetanus*: Simon 1872: 251-252; Lourenço 2003: 897-899, fig. 57-61; Kovařík 2006: 2, 8, 10, 15, fig. 16-19.; Sadine et al. 2011: 6; Lourenço and Cloudsley-Thompson 2012: 13-16, fig. 8; Lourenço and Simon 2012: 12; Lourenço 2013: 65-66; Rossi, Tropea and Yağmur 2013: 4-5, 7

Scorpion Tunetanus (ISS): Latreille 1817: 106.

Type material: Types lost according to Fet and Lowe (2000), Tunisia.

**Distribution:** The species is currently distributed across Algeria, Libya, Morocco and Tunisia, and doubtfully in the island of Malta.

**Remarks:** Because of the description of new species from Tunisia, and the lack of both type specimen and locality (beyond the country), a neotype for *B. tunetanus* is necessary to stabilize the taxonomy of Tunisian *Buthus*. As explained for *B. paris*, this is further complicated by recent diagnoses of *B. tunetanus* that differ from those offered by Vachon (1952a), and as such if Vachon's *B. tunetanus* material is found in the MNHN it should be given priority in the future designation of a neotype. Vachon (1952a) did not formally described any variety of *B. tunetanus*, but he again split the specimens that compose the species into four regions: 1) the typical region, corresponding to north and central Tunisia; 2) the southern montane region of Algeria, specimens from which have subsequently been described as *B. tassili*; 3) the Algerian Saharan Atlas and the southern region of the High Plateau; and 4) the disjunct desert regions of southern Tunisia, western central Algeria and eastern central Morocco. It is unclear whether region 3 or 4 might either correspond to *B. dunlopi* or *B. saharicus*. As explained in Fet and Lowe (2000), the name *A. (Leiurus) t. genuinus* refers to the nominotypical form of the species and as such the adjective "genuinus" is not an available subspecific name.

#### 52. Buthus yemenensis Lourenço, 2008

Buthus yemenensis: Lourenço 2008: 47-50, fig. 1-7.

**Type material:** 1 F holotype (ZMH N° A33/08), Ma'bar (approx. 14.8°, 44.3°), Dhamar, Yemen.

**Distribution:** Known only from the type locality.

#### 53. Buthus sp.

Buthus albengai (MIS): Habel et al. 2012: 3-4.

*Buthus europaeus* (MIS): Simon 1899: 85; Simon 1910 (part): 68-70, fig. 5, 8. *Buthus malhommei* (MIS): Habel et al. 2012: 3-4.

Buthus occitanus (MIS): Karsch 1881b: 8 (Libya); Pocock 1899: 834 (Africa); Kraepelin 1901a (part): 266; Werner 1902 (part): 598; Chaignon 1904: 83-84 (Tunisia); Tullgren 1909: 2-3 (Egypt); Borelli 1924 (Libya): 4; King 1925: 81 (Sudan); Gough and Hirs 1927: 5, fig. 9 (Egypt); Pallary 1934: 98-99; Werner 1934a: 269, fig. 330 (Morocco); Pallary 1938: 281-282; Sergent 1938: 519-520, pl. 49; Monard 1939: 82-83 (Guinea-Bissau); Moriggi 1941: 84; Sergent 1941a: 355, fig. 1E, 2.7, pl. 35, fig. 7; Sergent 1941b: 447, plate 37; Vachon 1941: 52; Frade 1947: 268 (Guinea-Bissau); Vachon 1952a (part): 262-271, fig. 331-344, 348, 372-380, 591, 641, 657, 663, 679, 687, 696; Vachon 1953: 1021-1024, fig. 12 (Mauritania); Malhomme 1954: 28 (Morocco); Belfield 1956: 44; Kinzelbach 1975: 14, fig. 1; Lamoral and Reynders 1975: 505 (Africa); Levy and Amitai 1980 (part): 15-16; Kinzelbach 1984: 100 (Asia); Kinzelbach 1985: map II (Asia); El-Hennawy 1987: 17 (Egypt); Amr et al. 1988: 374 (Jordan); Michalis and Dolkeras 1989: 265-266 (Greece); El-Hennawy 1992: 98, 101, 119-120 (Arabia); Kovařík 1992a (part): 183; Kovařík 1992b: 90 (Iraq); Amr and El-Oran 1994: 181 (Jordan); Kovařík 1997: 179 (Maghreb); Fet and Lowe 2000 (part): 92-94; Kovařík 2002: 5; Lourenço 2003: 884 (Morocco) Soleglad and Fet 2003: 7 (Morocco); Kaltsas et al. 2008: 215-216 (Libva); Sadine et al. 2012: 33; El-Hennawy 2013: 260; Aboumaâd et al. 2014: 5.

*Buthus occitanus occitanus* (MIS): Pocock 1895: 299 (Egypt); Vachon 1952a (part): 262-271, fig. 331-344, 372-379, 400, 554; Levy and Amitai 1980: 16 (Africa); Kovařík 1995: 20 (Morocco); Kovařík 1997: 179 (Ghana); Gantenbein et al. 1998a: 51, Fet and Lowe 2000 (part): 94-95; Gantenbein and Largiadèr 2003 (part): 120, 122.

Buthus occitanus occitanus, "Afrique occidentale française": Vachon 1952a: 270.

*Buthus occitanus occitanus, "Cote Occidentale du Maroc"*: Vachon 1952a: 268-269, fig. 373-378.

Scorpio occitanus: Dufour 1856 (part): 570.

not *Buthus*: Vachon 1955: 372 (El Fâcher, Djebel Meidob, Darfur, Sudan) (Vachon said that this material was close to but not part of the genus *Buthus*).

We futher propose to transfer a species from the genus *Buthus* to the genus *Androctonus*.

#### Androctonus barbouri (Werner, 1932) (new combination)

*Buthus barbouri*: Werner 1932: 300, fig. 141; Vachon, 1949b: 287-288, fig. 371; Vachon 1952a: 261-262; Perez 1974: 22; El-Hennawy 1992: 98, 119; Kovařík 1995: 20; Kovařík 1998: 106; Fet and Lowe, 2000: 92.

**Type material:** 1 M (type probably lost), Agadir (approx. 30.43°, -9.60°), Morocco.

**Distribution:** known only from the type locality, Agadir, just north of the Sous River the mouth in southern Morocco.

**Remarks:** The species *B. barbouri* was described by Werner from Agadir, Morocco. However, this species cannot be linked to any of the *Buthus* species known from the reported type locality. This problem was already recognized by Vachon (1952a) that considered this species to have been "imperfectly described", but then failed to make any taxonomical or nomenclatural act to fix it. *Buthus barbouri* and *B. marrocanus* are the only fully dark *Buthus* species known from Morocco. *B. marrocanus* was described from the Rabat region, 500 km distant from Agadir and is distinguished from the former species by the shape of the inferior lateral keels of the fifth segment of the metasoma and by the number of pectinal teeth (Vachon, 1952a). Because of the chaetotaxy of the pedipalps Vachon (1952a) considered *B. barbouri* to be closer to *Androctonus mauritanicus* (Pocock, 1902). In agreement with these diagnostic characters, and Vachon's (1952) opinion, we transfer *B. barbouri* to the genus *Androctonus*, with the new combination *Androctonus barbouri* (Werner, 1932). The relationship between A. *barbouri* and *A. mauritanicus bourdoni* Vachon, 1948, a subspecies that also occurs in the Sous River valley should be investigated.

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# Chapter III DEVELOPMENT OF NEW

# **MOLECULAR MARKERS**

#### Paper 2

Sousa, P., Frías-López, C., Harris, D. J., Rozas, J. and Arnedo, M. A. (in preparation). Development of anonymous nuclear markers for *Buthus* scorpions (Scorpiones: Buthidae) using massive parallel sequencing, with an overview of nuclear markers used in Scorpions phylogenetics.

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PAPER 2: Development of Anonymous Nuclear Markers for *Buthus* scorpions (Scorpiones: Buthidae) using massive parallel sequencing, with an overview of nuclear markers used in Scorpions phylogenetics

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### Abstract:

Multilocus datasets are routinely used to reconstruct phylogenetic relationships among groups of organisms, and to uncover phylogeographical patterns underlying species genetic diversity. However, comparatively few markers have been used to infer evolutionary histories in scorpions, of which we offer an overview, and many of the nuclear markers used are too conserved to be useful at or below the species level. Here we used a reduced representation library (RRL) combined with massive parallel sequencing, to develop five new Anonymous Nuclear Markers (ANM) that amplify in the scorpion genus Buthus Leach, 1815. Nucleotide diversity of the ANMs ranged from 2.2% to 5.6% for the five Iberian Buthus mtDNA lineages, and average uncorrected sequence divergence between lineages ranged from 0.23% to 5.28%. These results demonstrate the potential utility of these ANMs to infer the phylogeographical patterns of the Iberian Buthus. Furthermore, we demonstrated that two of the developed ANMs and two other nuclear markers that have been used in Mesobuthus Vachon, 1950, cross-amplify in the Buthidae, at least within the Buthus group of genera, and therefore have the potential to help reconstructing the phylogeny of the Buthidae Family, which contains almost half of all known scorpion species.

### Keywords:

Reduced representation library, N.G.S., nuclear loci, multilocus, Phylogeny, Phylogeography, non-model organisms

## Introduction

The PCR revolution made multilocus DNA sequencing data ever more present, and the number of Loci is increasing fast (e.g. Garrick et al. 2015), especially with the maturation of Next Generation Sequencing (NGS) and Genomics, the implications of which are far reaching (Lemmon and Lemmon, 2013; McCormack et al., 2013; Morey et al., 2013).

Multilocus studies, based on unlinked markers, have many advantages over singlelocus studies (Sánchez-Gracia and Castresana, 2012). These derive from the augmented resolving power they provide, and a combination of both mtDNA and nuDNA has been shown to be particularly desirable (Sánchez-Gracia and Castresana, 2012). Multilocus datasets have a wide range of applications, not limited to phylogenetic reconstruction (Yang and Rannala, 2012), but also species delimitation (Yang and Rannala, 2010), conservation biology (Fennessy et al., 2016), etc. They are also essential for inferring species-trees, which can be different from individual gene-trees (Degnan and Rosenberg, 2009) and have wide impacts on the determination of speciation times (Nichols, 2001).

The development of new nuclear markers in non-model organism can be achieved with different methodologies. These in turn will result in different types of markers that are informative at different levels of the phylogenetic reconstruction (reviewed in Lemmon and Lemmon, 2013; Thomson et al., 2010). The methodologies to obtain new DNA sequences include expressed sequence tag libraries (EST) (e.g. Gantenbein and Keightley 2004), genomic libraries (e.g. Amaral et al. 2009, Bidegaray-Batista et al. 2011) and increasingly, NGS based approaches (Ferreira et al., 2014; Lemmon and Lemmon, 2012). The genomic library preparation can itself be obtain in several ways (reviewed in Lemmon and Lemmon, 2013; McCormack et al., 2013). According to Thomson et al. (2010) these markers can be grouped into three categories: 1) Nuclear Protein Coding Loci (NPCL); 2) Exon-primed Intron-crossing (EPIC); 3) Anonymous Nuclear Markers (ANM). ANM are attractive because they require the least amount of previous knowledge and because they have a strong probability of being highly variable and thus useful at lower levels of phylogenetic reconstruction including species' phylogeny and phylogeography. This characteristic is intrinsic to their development as they are constructed from random portions of the genome, and as most of the genome in Eukaryota is non-coding, amplification of regions of high mutation rate is expected (Thomson et al., 2010).

In Scorpiones, molecular phylogenetic studies have not been numerous. In fact the first Cladistic study in Scorpiones was, according to Soleglad and Fet (2003), that of
Stockwell (1989, unpublished), based on morphological characters. The first molecular phylogenetic studies studying the phylogeny of a genus used allozymes (Gantenbein et al., 2000b, 1998a, 1998b), allozyme and mtDNA (Gantenbein et al., 2000a, 1999; Scherabon et al., 2000) and nuDNA (Ben Ali et al., 2000), many of which already in a multilocus approach. Nevertheless the use of DNA sequences in multilocus datasets has been scarce even in the present, although it is growing, which makes Gantenbein and Keightley (2004) even more noteworthy. These authors developed eight new ANM to reconstruct the evolutionary history of *Mesobuthus gibbosus* (Brullé, 1832) and *M. cyprius* (Gantenbein and Kropf, 2000). Several studies have relied upon the usage of conserved and slow evolving regions of the nuclear rDNA (5.8S, 18S and 28S), but also using the faster evolving Internal transcribed spacers (ITS1 and ITS2) that can be amplified concomitantly (Schlötterer et al., 1994).

The scorpion genus *Buthus* Leach, 1815 (Buthidae C. L. Koch, 1837) currently comprises 52 species that occur in south-western Europe, North Africa and the Middle East. The phylogeography of the Western Mediterranean range of the genus was first evaluated by Gantenbein and Largiadèr (2003) using mtDNA and nuDNA. These authors found three main lineages, namely a European, a Moroccan and a Tunisian lineage. The European populations were further studied by Sousa et al. (2010) using only mtDNA. Their broader geographic sampling uncovered two previous unknown mtDNA lineages, revealing that the evolutionary history of the genus *Buthus* was more complex than previously reported in Iberia. At the same time the taxonomy of the genus in the Iberian Peninsula also changed. For a long time only *B. occitanus* (Amoreux, 1789) was accepted, but *B. ibericus* Lourenço & Vachon, 2004, and *B. montanus* Lourenço & Vachon, 2004 were described and a forth species, *B. elongatus* Rossi, 2012, was also added to the Iberian fauna. Sousa et al. (2012) confirmed that the four Iberian *Buthus* species, together with samples from Northerner Morocco, form one of the four main *Buthus* mtDNA lineages in the Western Mediterranean.

Our objective was to develop new ANMs using a reduced representation library (RRL) combined with NGS (Lemmon and Lemmon, 2012 approach) to improve our knowledge of the phylogeography of the *Buthus* lineages/species found in the Iberian Peninsula (Sousa et al., 2010). As no systematic overview of the nuclear markers used on lower rank phylogenies of Scorpiones has been published we present such overview to promote the usage of comparable datasets in the future.

## Material and methods

## Target species and cross-amplification

We surveyed four *Buthus* individuals from three distinct Iberian mtDNA lineages (*sensu* Sousa et al. 2010). These consisted of three samples from two different mtDNA lineages of *B. ibericus* (Sc1110 and Sc1101 from Alcalá de los Gazules, Spain – lineage 2, and Sc1615 from São Brás de Alportel, Portugal – lineage 1) and one sample from *B. montanus* (Sc1601 from Refugio Poqueira, Capileira, Spain – lineage 4) (Fig. 1).



**Figure III-1. A** – Phylogenetic neighbour-joining tree of the *cox1* mtDNA marker of the 4 *Buthus* individuals used to construct the genomic reduced representation libraries (RRL). **B** – Uncorrected sequence p-distances of the same *cox1* data. All Iberian lineages are part of the *occitanus* group of *Buthus* species (*sensu* Sousa et al Paper 1. Lineage numbering according to Sousa et al (2010).

For testing the variability of the newly design primers, we chose 10 *Buthus* individuals, two from each of the five Iberian mtDNA lineages (*sensu* Sousa et al. 2010). To further test the utility of the primers in a broader taxonomic sample, we cross-amplified them on individuals belonging to Moroccan *Buthus* lineages (*occitanus* group Sc2409 or Sc2533 and *mardochei* group, Sc 1568; *sensu* Sousa et al. Paper 1, other Buthidae genera (*Androctonus mauritanicus*, Sc2408, *Compsobuthus* sp., Sc2591; *Mesobuthus* sp., Sc2520), and two additional families: Scorpionidae Latreille, 1802 (*Scorpio* sp., Sc2405) and Iuridae Thorell, 1876 (*Calchas* sp., Sc2523).

## General lab procedures

Whole genomic DNA was extracted from freshly preserved (96% ethanol) muscle tissue from the whole animal, excluding only the digestive system organs and the exoskeleton, using either the SPEEDTOOLS Tissue DNA Extraction Kit (BIOTOOLS) or phenol/chloroform extraction (two samples).

Polymerase chain reactions (PCR) were performed in a final volume of 25  $\mu$ L using Sigma's REDTaq DNA polymerase with the REDTaq ReadyMix PCR Reaction Mix with MgCl2 (Sigma-Aldrich). General PCR conditions are given in Table 1. Amplified DNA templates were sequenced in both directions using one of the PCR primers and sequenced in an ABI 3700 automated sequencer at the Centres Científics i Tecnològics

de la Universitat de Barcelona (CCiTUB). DNA sequences were edited and assembled using Geneious software v.6.1.8 (Kearse et al., 2012).

In addition to the novel markers, we amplified and sequenced mitochondrial and nuclear markers that have been used in scorpion research. A partial fragment of the *cox1* mitochondrial gene was amplified using the Folmer et al. (1994) primers LCO1490 (GGT CAA CAA ATC ATA AAG ATA TTG G) and HC02198 (TAA ACT TCA GGG TGA CCA AAA AAT CA). We further tested eight nuclear fragments (Table 1). The ribosomal internal transcribed spacer (ITS2) using primers ITS4F (White et al., 1990) and ITS-5.8Sv2 (Agnarsson, 2010). The Histone 3 (H3) using primers H3aF and H3aR (Colgan et al., 1998). The 28S rDNA large subunit domain D3 (28S) using primers 28SO (Hedin and Maddison, 2001) and 28SBv2 (M. Arnedo NEW). The Protein kinase (PK) using primers 03B09F and 03B09R (Gantenbein et al., 2003). And three gene fragments from Gantenbein and Keightley (2004), Methyl transferase (MetT), Defensin 4kD (D4kD) and Lysozyme precursor C (Lys-C). All additional primer sequences can be found in the Supplemental Table 1.

**Table III-1.** Primer sequences for seven anonymous nuclear loci developed from a NGS approach of Iberian *Buthus*. Names indicate the loci, forward and reverse primers, PCR annealing temperature (AT), Extension time (ExT), size of amplicon (bp). Performance taxa tested: IP, Iberian Peninsula *Buthus* lineages; Moroccan *Buthus* mtDNA groups (*sensu* Sousa et al. Paper 1: occ, *occitanus* group; mar, *mardochei* group; A, *Androctonus*; M, *Mesobuthus*; C, *Compsobuthus*; S, Scorpionidae; I, Iuridae. Performance coded as follow: ?, not tested; strikethrough, unsuccessful PCR or Sequencing; or otherwise successful. n.a. unnamed in the publication; H. & M. – Hedin and Maddison, 2001.

Loci	Primer Name	Primer sequence (5'-3')	AT (℃)	ExT (s)	Amplicon size	Performance	Source
c0037	0037F	TGTTTAGCAGATTTCGTCGGA	60°	30s	248	IP, occ, mar, ?	NEW
	0037R	AGCTGACTGTTTAATTCTCGCTG			to 261		
c0061	0061F	ATCAACTCGGATGTAACATCAC	53°	45s	248	IP, occ, mar, <del>A, M, C, S, I</del>	NEW
	0061R	AGCATCAGAAACGTTAGACAAGAG					
c0118	0118F	TCTGCGAGTCACACCTTCAC	60°	30s	366	IP, occ, ?	NEW
	0118R	CCCTAGAACTGCTGTCTGCC					
c0717	0717F	CGGATTCTCTCGCTGAACCG	50°	45s	493	IP, ?	NEW
	0717R	AGGTGTACCTCAAGGCTCTG					
c0791	0791F	CGCTGCCAATGTAGCTCCAG	53°	45s	293	IP,?	NEW
	0791R	GTTCGATTCCCGGCGTGG			to 301		
c0971	0971F	CACGGTTAATGGAAGAAGAGC	53°	45s	467	IP, occ, mar, A, M, <del>S, I</del>	NEW
	0971R	AAGTTCGCATCAGTAAACAGCG			to 500		
c5070	5070F	CGACACTTTGCCAACTTCAAC	64°	30s	780	IP, occ, mar, <del>A, M, C, S, I</del>	NEW
	5070R	GCATTGGTCTGTGGCGAATC			to 845		
28S	28SO,	GAAACTGCTCAAAGGTAAACGG	52°	45s	≈727	IP, occ, mar, A, ?	H. & M.
	28SBv2	TCGGAAGGAACGAGCTAC					NEW
РК	03B09F,	TCTGATGTATGGCAGATGGCAATG	45°	30s	362	IP, occ, mar, A, M, C, <del>S, I</del>	SupT 1
	03B09R	CGAACTCAAGATCCACTCCTGTACTCG					
MetT	n.a.	TGGGTTCCAGCTCGCAGCGGTAACG	60°	30s	456	IP, occ, mar, M, C, <del>S, I</del>	SupT 1
	n.a.	AACTTCGTAGTCGGAATACGAATGTTCTC			to 466		

## Preparation of the genomic reduced representation libraries (RRL)

We obtained a reduced representation genome fragment by digesting the genome DNA with the rare-cutting restriction endonuclease *Notl* (recognition sequence: 5' GC/GGCCGC 3') (New England Biolabs), which generates large genomic fragments (Lambert et al., 2008). We subsequently selected fragments ranging from 2.5 to 3 kb by excising the corresponding bands from the agarose gel (1% concentration). DNA was purified with a QIAquick Gel Extraction Kit (Qiagen). Because of the reduced amount of DNA recovered, we conducted a round of genome re-amplification, using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare), following the manufacturer's isothermal reaction specifications. This method conducts a global amplification via multiple strand displacements (Paez et al., 2004), using the  $\Phi$ 29 DNA polymerase (Blanco et al., 1989) and random hexamer primers. The DNA was purified again with the QIAquick PCR Purification Kit (Qiagen). We constructed four separated libraries (one per individual) that were individually tagged with MID's (multiplexed identifier) barcodes. The DNA sequencing was performed in a 1/2 454 plate of the GS-FLX titanium platform.

### Pre-processed and assembling of NGS data

We processed the 454 reads independently, according to their MID tag. First, adapters and putative contaminants were discarded using the SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) script. We then removed exact duplicate reads (forward and reverse complement) and those with low complexity using the dust algorithm with the PRINSEQ (Schmieder and Edwards, 2011) software; this step reduces the computation time and the number of false nucleotide variants. Moreover, we also removed read fragments with low-quality bases at the ends of the sequences, and all reads shorter than 100 bp with a mean quality score below 20 sequences using NGS QC Toolkit v.2.1 (Patel and Jain, 2012).

The pre-processed reads were used to conduct the *de novo* assembling (independently per each species) using two iterative rounds of CAP3. Then, we mapped the reads to the assembled contigs belonging to the same individual using the algorithm BWA-MEM (bwa-0.6.1) to determine the individual depth and removed contigs and reads related with multiple alignments (generating 4 BAM files, one per species). This excluded for the next SNP discovery step the reads that align to multiple locations and the contigs involved. Then, we performed a second alignment round, mapping all the filtered reads onto the four filtered assemblies (generating 12 BAM files, three per species) in order to identify the variant positions against the individuals used for generating the RRLs. Then we applied several filters to discard putative false nucleotide variants using a combination of SAMtools 'view' (Li et al., 2009) and a number of Perl script developed

*ad hoc*. In particular, we 1) removed the alignments with a CIGAR string with a 10% of hard clipping larger than the length of the aligned sequence; 2) realigned mismatches positions around indels; 3) removed reads that map to multiple locations (using SAMtools view option '-q 1') and removed sequences with the XA:Z flag; and 4) mark duplicated reads and add read groups using picard [http://picard.sourceforge.net.]. Afterwards, we used the filtered files and SAMtools 'mpileup' to obtain the coordinates of the variable positions of every individual sequence against each other and we exclude the nucleotide variant positions with a depth bigger than the two-fold of average coverage (obtained in the first mapping step).

Finally, we conducted the SNP calling step using the above filtered pileup files and *in house* Perl scripts to translate these pileup files to a matrix (using a value of a '0' for the non-variant positions and '1' for the polymorphic positions) to identify contigs mapped for reads belonging to at least two individuals (or the individuals of interest), with a variable region larger than 300 bp, a percentage of variability between 1-10%, and flanked by conserved regions with a length of 30-50 bp. The filtered contigs were visually explored using Geneious software v.6.1.8 (Kearse et al., 2012) to identify some erroneous mapped sequences, for instance, with homopolymers, or contigs with an excess of heterozygotic positions, that might imply a bad assembly (or mapping).

Primer pairs for the ANM (Table 1) were designed with the Primer3 software (Rozen and Skaletsky, 1999) as implemented in the Geneious software v.6.1.8 (Kearse et al., 2012). Several primers were tweaked to guaranty that their 3'-end was a C or G to promote binding, while also retaining annealing temperature, G-C content and other primer design features requirements.

All new sequences obtained in this study are available in GenBank.

## Data analyses

The haplotype phases were resolved using a two-step procedure. First, for sequences that were heterozygous for insertions or deletions, we used Champuru software online v1.0 (Flot, 2007), which implements the method described by Flot et al. (2006). Second, nucleotide polymorphisms were resolved using the Bayesian algorithm implemented in PHASE (Stephens et al., 2001). Phase was run five times per dataset.

The protein coding genes *cox1* and PK were aligned with Muscle (Edgar, 2004) and no indel were found. They were translated to amino acids and show no stop codons. The remaining genes were aligned with the MAFFT (v7.017) method G-INS-i (Katoh et al., 2002; Katoh and Standley, 2013) in Geneious v.6.1.8 (Kearse et al., 2012).

Uncorrected genetic p-distances between mtDNA *cox1* lineages were estimated with MEGA v6.06 (Tamura et al., 2013). Standard deviation was assessed by conducting

1000 bootstraps. Genetic diversity indices were estimated using DnaSP v.5.10.01 (Librado and Rozas, 2009). We calculated the number of segregation sites (S), the number of segregating sites per 100 bp ( $S_{100}$ ), the nucleotide diversity ( $\pi$ ), and the haplotype number (H) and diversity (Hd). Non-neutral evolution was evaluated with Tajima's D test (D) (Tajima, 1989). Recombination was investigated using the minimum number of recombination events ( $R_M$ ) (Hudson and Kaplan, 1985) and the linkage disequilibrium statistic (ZZ), which can also detect intragenic recombination (Rozas et al., 2001). The significance of the results was assessed using coalescent simulations with the algorithm implemented in DnaSP.

We made a bibliographic search for all relevant literature published until December 2016 that presented a molecular phylogeny or phylogeography of the Order Scorpiones, bellow the family rank, in order to a review all nuclear markers used. Studies focusing on venom nuclear markers were not considered.

## Results

We obtained a total of 487,357 raw reads across all four samples, which represent about 0.7% of the genome, assuming a random distribution of restriction sites, or 7.7 Mbp assuming an average genome size of about 1.1 Gbp for scorpions [from 0.90 Gbp in Centruroides vittatus (Hanrahan and Johnston, 2011) to 1.35 Gbp in Mesobuthus martensii (Cao et al., 2013)]. We removed low quality reads and, given the properties of the fragments obtained, searched and discarded the sequences with repetitive motifs, low complexity, and with high levels of entropy, removing 51% of the total reads. After the pre-processing step, we used two iterative rounds of CAP3 (-0 150 -p 90, -0 100 -p 90), and assembled a total of 9,183 contigs (Suppl. Table 2.2) with a N50 of 758. We also reduced the number of duplicate/paralogous sequences performing a previous individual mapping step using the assembled contigs as reference sequences and the reads assembled of the same individual. We therefore, repeated the mapping step, aligning the filtered reads of the four individuals using also the four filtered assemblies as reference sequences (the reads and contigs filtered in the previous step related with multimapping flags). The BAM files obtained were filtered to obtain only the reads with a single/UNIQ alignment performed and without hard clipped bases which reduced the percentage mapping around ~30% (Suppl. Table 2.2). To identify polymorphic positions, we used SAMtools to generate the 'mpileup' file with the alignment information of every individual, through mapping the reads of the other three individuals onto every assembly.

We also removed the alignments with a higher coverage than the two-fold of its average depth. Filtered pileup files were analysed using *in house* Perl script's to identify sequences mapped for the other individuals with a variable region of at least 300 bp, a variability range between 1-10%, and flanked by conserved fragments of 30-50 bp.

We identified 67 contigs that fulfilled the defined rules. These were then individually analysed and 18 were selected (16 different markers and two length variants) for which we design primers and tested for PCR amplification and variability. Only seven markers (ANM) could be amplified and sequenced (Table 1), although only five of them were consistently recovered (Table 1, 2).

**Table III-2.** Summary diversity statistics for 12 nuclear sequence markers for five Iberian *Buthus* lineages plus one Moroccan *Buthus*. N°, number of specimens, IP lin., number of Iberian lineages; Mor, Moroccan mtDNA groups represented (*sensu* Sousa et al. Paper 1, for *occitanus* group: *occ*1 - Sc2409, *occ*2 - Sc2533; The length in bp for each locus (L) after sequences end-trimming, excluding sites with gaps. The summed lengths of indels in bp (Indels). The number of segregating sites (S), the number of segregating sites per 100 bp (S100), haplotype number (H), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ), minimum number of recombination events (RM) of Hudson (1985), linkage disequilibrium statistic (ZZ) of Rozas et al. (2001), Tajima's D test (D) of Tajima (1989). Not significant (ns) and significant (\*) values at P < 0.05 of statistics after 10.000 coalescence simulations. *cox1* is presented twice, with and without Moroccan samples for more appropriate comparison with the different Loci results. 1 – Different specimens from the same lineages were used due to difficulties during sequencing. 2- Includes a previous unidentified Iberian lineage that we used due to limited available results.

Locus	N٥	IP	Mor	L	Indels	S	<b>S</b> 100	π	н	Hd	Rm	ZZ	D	
cox1	11	5	mar.	641	0	136	21.2%	0.088	11	1.000	45 ns	0.006 ns	0.019 ns	
c0037	7	3	mar.	264	21	36	14.8%	0.056	4	0.810	2 ns	-0.002 ns	-0.6 ns	
c0061	11	5	occ1	226	6	27	12.3%	0.037	10	0.992	1 ns	0.002 ns	0.256 ns	
c5070	11	5	mar.	778	31	76	10.2%	0.022	13	0.944	2 *	0.139 ns	-0.875 ns	
c0971	11	5	mar.	451	27	40	9.4%	0.022	0.022 12 0.926 4		4 ns	0.045 ns	-0.689 ns	
c0118	<b>7</b> <sup>1</sup>	5	occ2	366	0	33	9.0%	0.029	10	0.923	6 ns	0.161 ns	-0.093 ns	
MetT	11	5	mar.	385	5	27	7.1%	0.024	13	0.944	4 ns	0.136 ns	0.76 ns	
PK	11	5	mar.	362	0	13	3.6%	0.008	12	0.887	2 ns	-0.014 ns	-0.631 ns	
28S	11 <sup>1</sup>	5	mar.	727	0	7	1.0%	0.004	6	0.801	1 ns	0.015 ns	1.222 ns	
ITS2	4	2	mar.	475	16	26	5.7%	0.032	3	0.833	0 ns	0.042 ns	-0.159 ns	
H3	2	1	mar.	328	0	2	0.6%	0.006	2	1.000	0 ns	0.000	n.a.	
cox1	10	5	none	641	0	118	18.4%	0.080	10	1.000	38 ns	0.031 ns	0.381 ns	
c0717	4	3 <sup>2</sup>	none	493	0	24	4.9%	0.019	7	0.964	2 ns	0.009 ns	-0.019 ns	
c0791	2	<b>2</b> <sup>2</sup>	none	302	10	8	2.7%	0.027	2	1.000	0 ns	0.000	n.a.	

We were able to amplify, albeit with different success rates, six of the former nuclear markers available for scorpion research, (Suppl. Table 2.1). We only amplified two specimens for the **H3**, although due to the extremely low  $S_{100}$  (0.61%) (Table 2) we did not investigate this marker any further. Although, we initially tried to sequence the fragment spawning the **18S** plus **ITS1** region used by Gantenbein and Largiadèr (2003), this proved to be difficult and we used the **ITS2** instead. Although **ITS2** had potential ( $S_{100} = 5.7\%$ ) we did not pursue it due to the lack of *Buthus* sequences available for comparison in Genbank. The **D4kD** and **Lys-C** could not be amplified in any *Buthus* 

samples. The remaining three nuclear markers, Met T, PK and 28S were successfully amplified and sequenced (Table 2).

The success of cross-amplification varied considerably between the loci tested. All amplified the five Iberian lineages tested and the Moroccan representative of the *occitanus* mtDNA group and all that were tested also most amplified the distant Moroccan *mardochei* mtDNA group (Table 1). The loci c0971, 28S, PK and MetT, amplified all the Buthidae genera in which they were tested, but we were unsuccessful in amplifying either the Scorpionidae or luridae samples (Table 1).

Genetic divergences were calculated for the *cox1* and the c0037, c0061, c0118, c0971, c5070, MetT, PK and 28S nuDNA (Suppl. Table 2.3). For the Iberian *Buthus* lineages, estimates ranged from 0.27% (c0971) to 4.07% (c0061) between lineages 1 and 2, 0.34% (28S) to 5.28% (c0037) between lineages 1 and 3, 0.38% (28S) to 4.33% (MetT), between lineages 1 and 4, 0.34% (28S) to 4.84% (c0037) between lineages 1 and 5, 0.23% (c0971) to 2.23% (c5070) between lineages 2 and 3, 0.58% (28S) to 4.19% (c0061) between lineages 2 and 4, 0.28% (28S) to 4.75% (c0061) between lineages 2 and 5, 0.58% (28S) to 4.19% (c0061) between lineages 3 and 4, 0.28% (28S) to 4.76% (c0061) between lineages 3 and 5, 0.28% (28S) to 4.66% (MetT) between lineages 4 and 5 (Suppl. Table 2.3). The *cox1* mtDNA fragment was found to be twice as variable as the ANM c0037 and c0061, ten times more variable than PK and more than twenty times more variable than 28S. Remarkably the ANM c0037 was found to be a little bit more variable (x1.1) than the *cox1* locus when comparing Iberian and Moroccan samples (Suppl. Table 2.3).

No intragenic recombination was detected (Rozas' et al. ZZ), with the data conforming to the expected linkage disequilibrium, although we found that the minimum number of recombination events deviated from what was expected for the Loci c5070 (Hudson and Kaplan's Rm).

**Table III-3.** Nuclear Loci used in 30 molecular phylogenetic or phylogeographic studies of Scorpiones, ordered chronologically. The list does not include works that have relied upon venom markers, including venom gland transcriptomes or cytogenetics, and only include works that have focused below the family rank in Scorpiones. Notes: 1 - internal region sequenced; 2 - only amplifies in *Centruroides vittatus* according to the authors. For primer sequences and references see Supplemental Table 1. a - the authors also sequenced a small portion of the end of 18S and beginning of 28S; b - only the 18S + ITS1 region was sequenced.

Loci or Marker type	Works									
Allozymes	Gantenbein et al., 1998a, 1998b, 2000, 2001; Gantenbein 2004									
ITS1+ 5.8S + ITS2	Ben Ali et al., 2000; Bryson et al., 2014 <sup>a</sup>									
18S + ITS1 + 5.8S	Gantenbein & Largiadèr, 2003 <sup>b</sup> ; Salomone et al., 2007									
18S rRNA	Soleglad & Fet, 2003; Li et al. 2009; González-Santillán & Prendini 2014; Santibáñez-López et al., 2014 Ceccarelli et al., 2016a?									
28S rRNA	Prendini et al., 2003; Bryson et al., 2013a; Bryson et al., 2013b; González-Santillán & Prendini 2014; Santibáñez- López et al., 2014; Talal et al., 2015; Ceccarelli et al., 2016a; Ojanguren-Affilastro et al., 2016; Luna-Ramirez et al., 2017									
Protein kinase	Gantenbein et al., 2003; Gantenbein & Keightley, 2004; Shi et al., 2013									
Chaperonin 10, Defensin, Lysozyme precursor C, Methyl transferase, Unknown protein, Thioredoxin1	Gantenbein & Keightley, 2004									
Serinproteinase inhibitor, Serin-type endopepdidase	Gantenbein & Keightley, 2004; Shi et al., 2013									
non-LTR retrotransposons	Glushkov et al., 2006									
Microsatellites	Ji et al. 2008									
RAPD	Abdel-Rahman et al., 2009									
ITS2	Bryson et al., 2013a; Bryson et al., 2013b; Graham et al., 2013									
ANM (Locus 1075) <sup>2</sup>	Yamashita & Rhoads, 2013									
Genomics	Sharma et al., 2015									
Actin 5C	Ceccarelli et al., 2016b									
wingless	Ceccarelli et al., 2016b									
SNP Bryson et al., 2016										

In Table 3 we present a bibliographic overview of all nuclear markers that have been used in molecular phylogenenetic studies on scorpions in Table 3. We have found 30 published articles up to December 2016, the majority of which (21) have relied on Sanger sequencing of nuclear DNA. Most of the molecular systematics and phylogeographic studies found used a limited number of nuclear Loci using Sanger sequencing (1.86 average Loci per study; 1.50 removing Gantenbein and Keightley 2004 work) (Table 3). For comparison, earlier allozyme studies had an average of 16.8 loci analysed per study.

## Discussion

We were able to successfully design new nuclear markers that were informative at the species and population level in *Buthus* scorpions, using the approach described in Frías-López et al. (2016, supplementary material), based on the combination of restricted representation libraries and massive parallel sequencing (Lemmon and Lemmon, 2012). The novel markers designed in the present study remain anonymous (ANM) because no significant matches were recovered in BLAST searches. Although the assembled complete genome of *M. martensii* has been made available by Cao et al. (2013), it is of limited use due to the lack of accurate annotations.

We found wide disparities in the results when comparing the average uncorrected inter-lineage sequence divergence (Suppl. Table 2.3), as expect if we were successful at amplifying portions of the nuclear genome that are evolving at different rates. Nevertheless, were surprise to find similar but higher divergences using the locus c0037 and not the *cox1* mtDNA loci when comparing Iberian and Moroccan lineages. When comparing only Iberian *Buthus* lineages the *cox1* mtDNA performed as expected, whit at least double the amount of inter-lineage sequence divergence. This might reflect different evolutionary rates in different branches of *Buthus* phylogeny.

Most of the published works found in the bibliographic search have used a limited number of nuclear Loci (Table 3). Most works relied on two nuclear genes, 18S and 28S, probably due to easiness of amplification (Hillis and Dixon 1991). As predicted, these markers were found to be highly conserved and their use for shallow relationship was very limited (Bryson Jr et al., 2013; Talal et al., 2015). We obtained a similar result while testing them in *Buthus* (Suppl. Table 2.3). Comparing NPCL markers, these were found to yield similar results to ours in Ceccarelli et al. (2016) study. However, the PK marker that we tested was found to be much more variable in *Mesobuthus* species (S<sub>100</sub> = 16.5% vs 3.6% in *Buthus*) (we combined three works in this analysis: Gantenbein et al., 2003;

Gantenbein and Keightley, 2004; Shi et al., 2013). As expected, the Internal transcribed spacer (ITS1, ITS2 or combined with portions of rDNA), used in three studies (Bryson Jr et al., 2014, 2013; Salomone et al., 2007) (Table 3), was more variable. These markers were found to be at least as variable ( $S_{100}$  ranging from 10.0% to 20.3%) as the most variable ANM developed in our work (e.g. c0037, 14.8%; c5070, 10.2%). Comparison of interspecific sequence divergence yield similar results. The amount of divergence between two pairs of species calculated with the most variable nuclear markers (ITS) (Salomone et al., 2007) was similar to what we found in *Buthus* with the two most variable ANM (c0037, 4.29%; c0061, 3.62%; average p-distances). The results of interspecific sequence (p-distance) using the PK alignment described above (10 species pairs of *Mesobuthus*, number sequences = 97) was on average 2.04%. This was more than double what we found for *Buthus* (0.90%), but it was very similar to the divergence found between *M. gibbosus* and *M. cyprius* (0.96%), both from the Aegean region. Both marker variability and sequence divergence suggests either an older divergence time for the *Mesobuthus* species studied or an accelerated rate of mutation in the PK marker.

In this study we were able to demonstrate that two new ANM (c0037 and c0971) can be sequenced in three Buthidae genera. This is the most specious scorpion family, comprising almost half of all known scorpion species (Rein, 2016). We also demonstrated that two other markers, PK (Gantenbein et al., 2003), a NPCL marker, and MetT (Gantenbein and Keightley, 2004), an EPIC marker, can both be sequenced beyond the *Mesobuthus* genus. These four markers were applied only in the *Buthus* group of Buthidae genera (*sensu* Fet et al., 2005), but if successfully applied in the broader Buthidae, they can provide a framework for a coherent molecular systematic study of this diverse and venomous scorpion family (Chippaux and Goyffon, 2008), which remains largely unexplored (Fet et al., 2003; Sharma et al., 2015).

The methodological approach we followed proved successful to develop five new ANM that seem promising to investigate evolutionary relationships at least within the genus *Buthus*. Moreover, other massive parallel sequencing techniques that provide greater coverage should facilitate the assembly steps of the genomic RRL pipeline. This approach is also very flexible because the NGS data acquired can be used for other objectives, for example creating microsatellites markers to study recent population-level events in the Iberian *Buthus* species.

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# Chapter IV MEDITERRANEAN BUTHUS -

# **PATTERNS AND PROCESSES**

### Paper 3

Sousa, P., Harris, D. J., Froufe, E. and van der Meijden, A. (2012). Phylogeographic patterns of *Buthus* scorpions (Scorpiones: Buthidae) in the Maghreb and South-Western Europe based on CO1 mtDNA sequences. *Journal of Zoology*, doi: 10.1111/j.1469-7998.2012.00925.x

### Paper 4

Sousa, P., Arnedo, M. A., van der Meijden, A., Kovařík, F., Rossi, A., Yağmur, E. A., Planas, E., Henriques, S. S., Alves, P. C. and Harris, D. J. (in preparation). Can a scorpion cross the sea? Biogeographical answers from a multilocus phylogeny of Mediterranean *Buthus* (Buthidae, Scorpiones).

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### ORIGINAL MANUSCRIPT

## Phylogeographic patterns of Buthus scorpions (Scorpiones: Buthidae) in the Maghreb and South-Western Europe based on CO1 mtDNA sequences

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#### Keywords

Buthus; Scorpiones; Western Mediterranean; CO1; cryptic diversity; phylogeography.

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#### Abstract

The genus Buthus is a medium diverse scorpion genus, with 35 species distributed from Portugal and Morocco ranging eastward to Yemen in the Arabic Peninsula. The bulk of the genus' known species diversity occurs in the Western Mediterranean area. A recent molecular study started to elucidate the patterns of diversity of this genus in the Iberian Peninsula and the Maghreb. Since then, the taxonomy of the genus has changed substantially, with several new species having been described, and with the elevation of former subspecies to species-level. In this study, we assessed the patterns of diversity of Buthus scorpions from across the Maghreb region of North Africa using CO1 DNA sequence data. Based on our dataset of 147 sequences, including 67 new sequences, we recovered four wellsupported deep clades within Buthus scorpions from the Maghreb and Southern Europe. This further strengthens the support for cryptic diversity in the Maghreb region. The broader sampling of the Maghreb permitted a better understanding of the phylogeographic structure in this area. Three clades were restricted to Morocco and appear to have originated at the Atlantic Coast of this country, while the fourth was found throughout the region. We propose a model with two colonizing events to explain the distribution patterns across the Strait of Gibraltar, with an initial colonization from North Africa to Iberia followed by a reinvasion of the Rif Mountains region in Morocco.

## Introduction

The scorpion genus Buthus Leach, 1815 (Scorpiones, Buthidae) received very little taxonomic attention during the last half of the 20th century. In 1952, Vachon greatly modified the genus' taxonomy, transforming a poorly defined and heterogeneous group into a morphologically uniform coherent genus. Few changes were then made until Fet & Lowe (2000) suppressed all infrasubspecific varieties created by Vachon, reducing the number of taxa to five species and 11 subspecies (two of B. atlantis Pocock, 1889 and nine of B. occitanus Amoreux, 1789). However, since then, the genus has gained 30 new species, with the elevation of several subspecies to species level, the redescription of suppressed varieties and with the discovery of new material. The genus is now known from Southern Europe, Iberia and the South of France, Africa, in all countries bordering the Mediterranean, plus Guinea, Mauritania, Niger, Senegal and Sudan (south of the Sahara Desert) and Eritrea, Ethiopia and Somalia in the Horn of Africa, and the Middle East, in Cyprus, Egypt (Sinai), Israel, Jordan and Yemen (Fet & Lowe, 2000; Lourenço, 2002, 2003, 2005a,b, 2008; Lourenço & Slimani, 2004; Lourenço & Vachon, 2004; Lourenço & Geniez, 2005; Kovařík, 2006, 2011; Lourenço & Qi, 2006; Lourenço, Sun & Zhu, 2009; Lourenço, Yağmur & Duhem, 2010; Touloun & Boumezzough, 2011; Yağmur, Koç & Lourenço, 2011). The genus has been cited for other countries such as Djibouti, Gambia, Ghana, Iraq and Lebanon, but these records require additional confirmation (Fet & Lowe, 2000).

The distribution of the 35 species of *Buthus* is not uniform across the range of the genus. Twenty-one species are endemic to the Western Mediterranean area alone, although this is also historically the most surveyed area were the genus is known to occur. Morocco has 13 endemic species and shares two more with Algeria and Tunisia, with the latter country adding two endemic species to this region (Lourenço, 2002; Kovařík, 2006). Additionally, three more are known from the Iberian Peninsula and South of France. Still much remains to be

learned about the distribution of each species; for example, the range of Moroccan species has been shrinking continuously with the splitting of previously described species and the description of new ones. Distribution overlaps occur at least in some areas of the country (P. Sousa, pers. obs.). For most other species occurring outside of the Western Mediterranean area, only type localities are known.

The pattern of higher species numbers in North Africa has only emerged in the last 10 years following the description of most species. Earlier, authors failed to understand the biogeography of the genus; for example Vachon (1952) stated that the genus originated in Europe and then invaded Africa after the Alpine orogeny. However, in 2002, Lourenço already suggested that the bulk of the genus diversity, and its origin, are within North Africa.

Recently, molecular tools have been used to assess the phylogeny of the genus. Gantenbein & Largiadèr (2003), using mitochondrial DNA (mtDNA) and nuclear DNA sequences found three main clades in the Western Mediterranean, namely a European, an Atlas (Morocco) and a Tell-Atlas (Tunisia) clade. The same authors found three distinct lineages in the European clade, the identity of which were confirmed with allozymes (Gantenbein, 2004), possibly predating the reopening of the Strait of Gibraltar circa 5.3 million years before the present (Krijgsman et al., 1999). In Morocco, eight highly divergent, cryptic mitochondrial lineages were found (Gantenbein & Largiadèr, 2003). Later, Sousa et al. (2010), discovered two additional lineages in the Iberian Peninsula using CO1 mtDNA sequences, thus demonstrating the usefulness of this gene as a barcoding marker for this genus, and further demonstrating the complex patterns of diversity uncovered in the Western Mediterranean region.

Unfortunately the morphological identification of *Buthus* species has been rendered very difficult in the past decade. The last published keys for the genus only included 11 (Lourenço, 2003, mostly Morocco) and four species (Kovařík, 2006, only Tunisia). Furthermore, the key from Lourenço (2003) uses ambiguous characters, such as the 'strength' of surface granulation. Other characters, such as the presence or absence of sexual dimorphism first used by Kovařík (2006) may prove informative, if enough specimens are available, but have attracted little attention. Furthermore the identification of juveniles, which change colour, pedipalp, chela and telson shape during ontogeny, cause additional problems (Vachon, 1952; pers. obs.). Given these difficulties, and the apparent presence of cryptic species in the region, a preliminary barcoding approach is needed to delimit the known patterns of diversity, so that morphological characters can then be evaluated in an attempt to refine the taxonomy of Buthus across the region.

In the present study, we assess the patterns of diversity estimated using novel and already available CO1 sequence data from across the Maghreb region of North Africa, an area with 18 known *Buthus* species. We include for the first time specimens from Algeria and four previously unsampled species from Morocco. Our study further reinforces the presence of cryptic diversity in the region. We report the discovery of four well-supported deep clades within *Buthus* scorpions from the Maghreb and Southern Europe. The broader sampling allowed us to greatly refine and modify the phylogeographic patterns from previous studies using CO1 sequence data (Gantenbein & Largiadèr, 2003; Sousa *et al.*, 2010). With the present study, we hope to identify possible areas of ancient refugia of the genus *Buthus* in the Western Mediterranean area, and also to re-evaluate the invasion process of the Iberian Peninsula by this genus in light of recent reassessments of other taxa.

### **Material and methods**

Information and GenBank accession codes of the 64 specimens, captured in Morocco, Algeria and Tunisia, are given in Table 1. Further taxonomic and geographic information are given in Supporting Information Table S1. All specimens were examined morphologically and, when possible, identified (by P. Sousa) to species level following Vachon (1952), Lourenço (2003), Lourenço & Slimani (2004) and Kovařík (2006). All specimens are deposited in the collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Universidade do Porto, Vila do Conde, Portugal. CO1 sequences from previous studies were included in the analyses. Specimens BotTA1, BotTA2 and BotTA3 were mapped according to locality names given by Gantenbein & Largiadèr (2003). Furthermore, the change in taxonomy of Buthus species in Morocco, with the splitting of many species, together with the lack of a useful identification key, required the reinterpretation of most identifications made in Gantenbein & Largiadèr (2003). Only B. atlantis remains a a reliable identification, as this taxon has not experienced changes since the work of Vachon (1952).

For the genetic analyses, whole genomic DNA was extracted from preserved (96% ethanol) muscle tissue (leg) using a standard high-salt protocol (Sambrook, Fritsch & Maniatis, 1989). A fragment of the cytochrome oxidase 1 (CO1) was amplified by polymerase chain reaction (PCR) using the primers LCO1490 and HCO2198 from Folmer *et al.* (1994). The PCR conditions (25  $\mu$ L reactions) were as described in Sousa *et al.* (2010). Amplified DNA templates were sequenced by a commercial company.

Chromatograms were checked by eye using ChromasPro 1.41 (http://www.technelysium.com.au) and the sequences were subsequently aligned using ClustalW as implemented in MEGA 5 (Tamura *et al.*, 2011) using the default settings. The best-fitting models of sequence evolution were determined by JModeltest 0.1 (Posada, 2008) under the AIC criterion. Phylogeny reconstruction was performed using the Bayesian inference method, with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), with 5 000 000 generations, sampling trees every 10th generation (and calculating a consensus tree after omitting the first 12 500 trees). Genetic variability was calculated with DnaSP v.5.10.01 (Librado & Rozas, 2009).

### Results

The alignment used in the phylogeny reconstruction consisted of 147 DNA sequences from *Buthus* specimens, 64 of which are new *Buthus* sequences from specimens collected from 53

P. Sousa et al.

### Table 1 MtDNA Lineage and GenBank accession code of all the samples used in the study

MtDNA		GenBank	MtDNA			MtDNA		GenBank
lineage	Scorpion Id	Code	lineage	Scorpion Id	GenBank Code	lineage	Scorpion Id	Code
A1	Bom AG1	AJ506880	C3	Sc192	JQ775943	D6	Boo IB2	AJ506907
	Bom HA1a	AJ506892	C4	Bom DR1	AJ506887		Boo IB3a	AJ506908
	Boo CB1	AJ506904		Bom DR2a	AJ506888		Boo IB3b	AJ506909
	Sc008	JQ775905		Bom DR2b	AJ506889		Boo IB4	AJ506910
A2	Bom AG2a	AJ506881		Bom DR3	AJ506890		Boo IB6	AJ517296
	Bom AG2b	AJ506882		Bom DR4	AJ506891		EU523755	EU523755
	Bom AG3	AJ506883		Sc066	JQ775929	D7	Boo IB7a	AJ517182
	Bom AG4	AJ506884		Sc182	JQ775938		Boo IB7b	AJ517183
	Sc007	JQ775904		Sc183	JQ775939		Boo IB8	AJ517184
	Sc037	JQ775914		Sc187	JQ775941	D8	Boo IB5a	AJ506911
	Sc039	JQ775915		Sc330	JQ775950		Boo IB5b	AJ506912
	Sc177	JQ775934		Sc394	JQ775956		Sc084	GQ168519
	Sc351	JQ775952	D1	Sc370	JQ775953		Sc089	GQ168520
A3	Bom AA1	AJ506873		Sc373	JQ775954		Sc095	GQ168521
	Bom AA2a	AJ506874		Sc405	JQ775959		Sc100	GQ168525
	Bom AA2b	AJ506875	D2	Bot TA1	AJ506916		Sc104	GQ168526
	Bom AA3	AJ506876		Bot TA2	AJ506917		Sc105	GQ168527
	Bom AA4	AJ506877		Bot TA3	AJ506918		Sc106	GQ168528
	Bom AA5b	AJ506879		Bot TU1	AJ506915		Sc107	GQ168529
	Sc065	JQ775928		Sc402	JQ775958		Sc108	GQ168530
	Sc178	JQ775935		Sc407	JQ775960		Sc109	GQ168531
	Sc191	JQ775942		Sc408	JQ775961		Sc113	GQ168533
A4	Bom AA5a	AJ506878		Sc409	JQ775962		Sc114	GQ168534
	Bom HA2a	AJ506894		Sc410	JQ775963		Sc115	GQ168535
	Bom HA2b	AJ506895		Sc411	JQ775964		Sc116	GQ168536
A5	Bom AS1a	AJ506885		Sc412	JQ775965		Sc112	GQ168532
	Bom AS1b	AJ506886		Sc413	JQ775966		Sc120	GQ168537
	Bom HA1b	AJ506893	D3	Ba AC1	AJ506869		Sc121	GQ168538
	Bom HA4a	AJ506898		Ba AC2a	AJ506870		Sc157	GQ168539
	Bom HA4b	AJ506899		Ba AC2b	AJ514323		Sc158	GQ168540
	Sc013	JQ775909		Ba AC3	AJ506871		Sc161	GQ168541
	Sc043	JQ775916		Ba AC4	AJ506872		Sc190	GQ168542
	Sc052	JQ775921		Bom HA5a	AJ506900	D9	Sc001	FJ198055
	Sc055	JQ775923		Bom HA5b	AJ507584		Sc059	JQ775924
	Sc061	JQ775925		Sc006	JQ775903	D10	Bop MA1	AJ506913
	Sc064	JQ775927		Sc023	JQ775911		Bop MA2	AJ506914
	Sc214	JQ775945		Sc053	JQ775922		Sc029	JQ775912
	Sc266	JQ775946		Sc376	JQ775955		Sc030	JQ775913
	Sc277	JQ775947	D4	Sc098	GQ168523		Sc044	JQ775917
	Sc278	JQ775948		Sc099	GQ168524		Sc045	JQ775918
	Sc331	JQ775951	D5	Sc002	JF820096		Sc063	JQ775926
B1	Sc205	JQ775944		Sc004	FJ198056		Sc071	JQ775930
B2	Bom HA3a	AJ506896		Sc010	JQ775906		Sc078	JQ775931
	Bom HA3b	AJ506897		Sc011	JQ775907		Sc174	JQ775932
	Bom HA6a	AJ506901		Sc012	JQ775908		Sc180	JQ775936
	Bom HA6b	AJ506902		Sc014	JQ775910		Sc176	JQ775933
	Sc047	JQ775919		Sc050	JQ775920		Sc329	JQ775949
C1	Sc401	JQ775957		Sc096	GQ168522	Outgroup		AF370829
C2	Sc181	JQ775937	D6	Boo IB1a	AJ506905		Sc292	JF820097
	Sc185	JQ775940		Boo IB1b	AJ506906			

For further information on the geographic locations of the samples used refer to the supplementary material. Each sub-clade group number are shown in bold.

MtDNA = mitochondrial DNA.

P. Sousa *et al.* 



Figure 1 Phylogram depicting phylogenetic relationships estimated using Bayesian inference. Bayesian posterior probabilities are presented below nodes, except for those within the subclade level, that have been omitted.

locations across Morocco, Tunisia and Northern Algeria (Table 1 and Fig. 2a; for further detail, see Supporting Information Table S1). Specimens Sc010, Sc011, Sc012, Sc014 and Sc030, have no precise sampling information and are not represented in Fig. 2a and were collected in the Southern region of Tangier (North of Morocco). Additionally two species of the genus *Androctonus* Ehrenberg, 1828, *A. australis* (Linnaeus, 1758) and *A. mauritanicus* (Pocock, 1902) were included as outgroups (see Table 1). The taxonomy of *Buthus* remains difficult, with new species being described every year, but with a clear lack of a useful identification key. Nevertheless, we were able to obtain the first published sequences for *Buthus draa* Lourenço & Slimani, 2004, *B. malhommei* Vachon, 1949, *B. mariefranceae* Lourenço, 2003 and *B. maroccanus* Birula, 1903.

We identified 120 haplotypes in the alignment. Sequences were 624 bp long and contained 138 polymorphic and 99 par-

simony informative sites. Levels of nucleotide variability found in the analysed *Buthus* sequences were high (Hd = 0.9974,  $\pi = 0.08127$ ). A single three-base pair indel was observed in one specimen, Sc045, which lacked one amino acid (Serine) compared with all other analysed samples. All sequences were translated to amino acid, and no unexpected stop codons were observed.

Four highly supported clades were resolved, although the relationships between them lacked support (Fig. 1; for further detail see Supporting Information Fig. S1). Clades A, B and C are restricted to Morocco (Figs 1 and 2b). Clade A, comprising 41 specimens (28%), is distributed in the Anti-Atlas, High Atlas, the Oued Sous valley and the plain between the Atlantic Ocean and the Atlas mountains, as far North as Casablanca (Table 1 and Fig. 1). Clade B, with only six specimens (4%), is restricted to the Upper Oued Dra valley, in the Ouarzazate region. Clade C, with 15 specimens (10%), is restricted to the



Figure 2 (a) Map showing overall distribution of the four Buthus clades retrieved; (b) Map showing the distribution of Buthus subclades, as depicted in Fig. 1, present in Morocco.

South of the High and Anti-Atlas, superimposing roughly to the Oued Dra valley excluding the higher altitudes. Clade D is the largest, with 85 specimens representing 58%, and has the widest distribution (Figs 1 and 3). It ranges from Europe (Iberian Peninsula and the South of France) and throughout the entire Maghreb, excluding the inland desert regions. In Morocco, it reaches as far South as Agadir, but does not reach inland beyond the High Atlas.

A further analysis of each clade shows additional interesting geographic patterns. Clade A can be divided into six wellsupported subclades (Figs 1 and 2b). The basal subclade A1 comprises coastal specimens and one collected in Oukaimeden. Subclade A2 has a large range, occupying the plain that reaches from the High Atlas to the Atlantic Ocean, between Agadir and Casablanca. This subclade includes a specimen identified as *B. malhommei*. Subclade A3 is restricted to the Oued Sous valley and the Tafraout area of the Anti-Atlas. Subclade A4 occurs in both the Anti-Atlas and the High Atlas whereas subclade A5 only occurs in the High Atlas. Clade B is found only in the Upper Oued Dra valley, in the Ouarzazate region (subclade B2), and in a different watershed 100 km to the East of Ouarzazate (B1) (Figs 1 and 2b).

In clade C, most subclades are represented by a single specimen. Subclades C1 and C3 are located around Guelmim and are composed of *B. mariefranceae* (Figs 1 and 2b). Subclade C2 occurs further North, between Guelmim and Tafraout. Subclade C4 distribution roughly overlaps the Oued Dra valley but not at higher elevation (>1.500 m) where Clade B was found and includes specimens identified as *B. draa* and others with a morphology that is neither entirely *B. mariefranceae* nor *B. draa*, although it resembles the latter species more closely.

Clade D is divided into 10 well-supported subclades, although the branching order is poorly supported (Fig. 1). Subclade D1 is restricted to Algeria whereas Subclade D2 occurs both in Algeria and Tunisia (Fig. 3). Their geographic distributions fits well with the known distributions of *B. paris* and *B. tunetanus*, respectively. Subclade D3 is distributed

Phylogeography of West Mediterranean Buthus



Figure 3 Map showing the distribution of Buthus clade D subclades as depicted in Fig. 1.

between the Atlantic coast North of Agadir, the Northeast area of Morocco and the middle of Northern Algeria, and a single point in the middle of the High Atlas, which includes B. atlantis as identified by Gantenbein & Largiadèr (2003), and that fits well with the known distribution of this species (Vachon, 1952). Subclade D5 groups specimens that are distributed on both sides of the Strait of Gibraltar, in Morocco to the South and West of the Rif Mountains, and in Spain, South of Sierra de Segura. Subclade D9 occurs in the Fès-Meknès region. Subclade D10 occurs between Marrakesh and North of Béni Mellal, and also in the Rabbat region and includes a specimen identified as *B. marrocanus*, with a uniform dark brown colour, although it is roughly 25 km NW from Boulhaut, the closest known locality for this species (Vachon, 1952). Subclades D4, D6, D7 and D8, from the Iberian Peninsula, have been discussed in detail in Sousa et al. (2010).

### Discussion

Our study considerably increases our understanding regarding the distribution of *Buthus* scorpions in the Western Mediterranean region. In 1952, Vachon tried for the first time to discern patterns regarding the diversity of the genus. He hypothesized in this work that the genus had evolved from an initial centre in the Iberian Peninsula, and later colonized Africa. Lourenço (2002) on the other hand suggested that the centre of diversity of the genus *Buthus* is in Africa, not Southern Europe. The work of Gantenbein & Largiadèr (2003) gave an unprecedented insight into the genus, using molecular data to infer relationships between different taxa. In particular, they highlighted the high genetic diversity found in the Maghreb. Gantenbein & Largiadèr (2003) and Gantenbein (2004) found three main areas of divergence, corresponding to three very divergent clades: the European, the Atlas (Morocco) and the Tell-Atlas (Tunisia), although these were better resolved with the nuclear than with the mitochondrial data. Our findings partially support this pattern, although the European and Tell-Atlas clades were shown to group together with part of the Moroccan samples, where we recovered a high diversity with three exclusive clades (Fig. 2a). It is therefore reasonable to propose that this region of North Africa has been the origin of the genus diversity. However, there is a lack of support to resolve the relationships between the four clades. Samples from the eastern and southern portions of the genus' distribution are needed to test and refine this biogeographic hypothesis.

The absence of a reliable useful identification key prevented us from assigning the majority of specimens to the species level. This makes the splitting of subclades in the resolved tree harder, but we expect that most subclades, especially in Morocco, include multiple species, described or not. This is well supported when comparing the large intrageneric genetic distances in this study with available data for other genera. The average genetic distance between clades ranges from 8.5% to 10.5% (Table 2), with an overall mean distance of 8.8%[standard deviation (SD) = 1.0%] (Kimura 2-P distances calculated in MEGA 5). Even larger genetic distances were found when comparing different subclades, with a maximum of 13.1% between subclades C2 and D3, and a minimum of 3.4% between subclades C3 and C4 (Table 3). These values coincide with known genetic distances for other arthropod genera based on CO1 sequences, such as Centruroides Marx, 1890, with an average genetic distance between species of 11.2% (sD = 2.6%) and Mesobuthus Pocock, 1900, which showed an

average genetic distance between species of 15% (sD = 2.4%) (genetic distances calculated in Sousa *et al.*, 2011 based on sequences available in GenBank). Similar genetic distances between species were also found in two genera of Araneae: *Modisimus* Simon, 1893, mostly island-based spiders, with distances between 7.3% and 8.5% (Huber, Fischer & Astrin, 2010) and *Aphonopelma* Pocock, 1901, with a minimum of 6.8% and a maximum of 12.7% separating seven described or new spider species (Hamilton, Formanowicz & Bond, 2011).

Despite the lack of solid species identification, and continuing from the work of Gantenbein & Largiadèr (2003), we demonstrated that specimens from Algeria and Tunisia are related with specimens from Morocco and the Iberian Peninsula, as they are all grouped in Clade D (Figs 1 and 3). The founding of subclade D5, that includes samples from North of Morocco and the South of Spain has geographic coherence. Gantenbein & Largiadèr (2003) were unable to fully address the issue of direction of colonization events to or from Europe because of a lack of resolution. Based on our estimate of relationships, the most parsimonious scenario would be a

Table 2 Pairwise net sequence divergence (Kimura 2-parameter) among the four clades of Buthus sequences, and the within-clade sequence divergence values

				Within clade
	Clade A	Clade B	Clade C	estimates
Clade A	-			0.065
Clade B	0.088	_		0.017
Clade C	0.105	0.089	-	0.038
Clade D	0.093	0.085	0.105	0.073

single invasion of Europe, followed by a later reinvasion from Southern Iberia to the Rif Mountains region. Interestingly, exactly the same pattern appears to occur in the wall lizard Podarcis Wagler, 1830 (Kaliontzopoulou et al., 2011). However, relationships are not strongly supported, and therefore, this biogeographic hypothesis must be treated with caution. Other biogeographic questions also arise from the analysis of Clade D. It is apparent that Algerian and Tunisian samples group together, and that subclade D1 has a deep internal split that may coincide with the species *B. paris* and B. tunetanus, although, since the work of Kovařík (2006), two other species, B. chambiensis Kovařík, 2006 and B. dunlopi Kovařík, 2006, are known from Tunisia. The other subclades that compose D are present North of the High and Middle Atlas and a single specimen further East in the Tell-Atlas. although the disjunct distribution of subclade D2 remains partially unknown. The grouping of B. atlantis with B. paris and *B. tunetanus* is not compatible with the hypothesis of two 'complex' in the Buthus genus, suggested by Vachon (1952) based on morphological affinities. This author separated B. atlantis from all the other species of the genus that were grouped in a 'Buthus occitanus' complex. Later, Lourenço & Geniez (2005) placed B. bonito Lourenço & Geniez, 2005 in the 'Buthus atlantis' complex. This nomenclature is presently still in use, as Lourenço et al. (2010) and Touloun & Boumezzough (2011) placed the new species they describe in the 'Buthus occitanus' complex. Our results suggest that such a group would not be monophyletic, unless it also included B. atlantis.

The other three clades were only found in Morocco, and show little overlap with clade D (Fig. 2b), although areas such as the Middle Atlas are less well represented in the sampling.

Table 3 Net pairwise sequence divergence (Kimura 2-parameter) among the subclades of Buthus

	A1	A2	A3	A4	A5	В	C1	C2	C3	C4	D1	D2	D3	D4	D5	D6	D7	D8	D9
A1	-																		
A2	0.090	-																	
A3	0.086	0.077	_																
A4	0.102	0.093	0.074	-															
A5	0.085	0.086	0.069	0.060	_														
В	0.091	0.094	0.090	0.093	0.082	_													
C1	0.102	0.098	0.080	0.103	0.092	0.081	_												
C2	0.107	0.109	0.100	0.117	0.108	0.085	0.049	_											
C3	0.090	0.100	0.088	0.122	0.103	0.089	0.070	0.046	_										
C4	0.104	0.100	0.103	0.124	0.108	0.091	0.074	0.062	0.034	-									
D1	0.087	0.091	0.078	0.095	0.092	0.083	0.086	0.092	0.090	0.099	-								
D2	0.107	0.095	0.086	0.114	0.104	0.095	0.108	0.108	0.101	0.105	0.079	-							
D3	0.105	0.089	0.092	0.107	0.102	0.092	0.118	0.131	0.123	0.118	0.090	0.099	_						
D4	0.088	0.095	0.086	0.093	0.077	0.071	0.099	0.118	0.109	0.109	0.105	0.100	0.090	_					
D5	0.098	0.089	0.087	0.102	0.099	0.076	0.096	0.098	0.101	0.100	0.094	0.082	0.087	0.079	-				
D6	0.083	0.080	0.076	0.097	0.082	0.091	0.100	0.106	0.074	0.093	0.084	0.090	0.090	0.079	0.087	-			
D7	0.105	0.083	0.089	0.090	0.085	0.078	0.103	0.106	0.108	0.110	0.093	0.091	0.083	0.071	0.068	0.072	_		
D8	0.093	0.095	0.082	0.093	0.089	0.076	0.084	0.104	0.109	0.107	0.072	0.081	0.085	0.077	0.088	0.072	0.064	_	
D9	0.079	0.090	0.093	0.110	0.098	0.082	0.103	0.112	0.105	0.110	0.089	0.103	0.083	0.076	0.084	0.072	0.084	0.077	-
D10	0.099	0.088	0.092	0.115	0.102	0.095	0.097	0.105	0.094	0.099	0.088	0.091	0.087	0.088	0.083	0.078	0.094	0.093	0.088

Subclades B1 and B2 were analysed together.

P. Sousa *et al*.



Figure 4 Detailed map of the High Atlas region of Morocco representing all Buthus subclades present in the area, as depicted in Fig. 1.

The distribution of diversity of these clades and of their subclades suggest the influence of the High Atlas and the Anti-Atlas, and also of several river valleys, namely the Dra and Sous, and to a less extent, the Oum Er-Rbia and Sebou in the North. River systems also seem to have played a role in shaping the distribution of *Hottentotta* Birula, 1908 scorpions (Sousa *et al.*, 2011).

Both clades A and B have their basal subclades at the Atlantic coast of Morocco (Fig. 2b), suggesting an earlier occupation of lower patches of terrain by Buthus scorpions, perhaps an area with more stable climatic conditions given its proximity to the ocean and mild orography (Pokras & Ruddiman, 1989; Feakins & deMenocal, 2010). Also, this is coherent with a separation promoted by the Anti-Atlas Mountain and related river systems, namely the Dra, and while it is accepted that the mountains that compose the Atlas system have been uplifted around the same time period, during Alpine orogeny (uplift began about 40 Mya -Michard et al., 2008), the Anti-atlas mountains are composed of much older rocks, that have suffered changes at least since the Variscan (also known as Hercynian) orogeny (Late Palaeozoic), although the complex geological settings of this mountain range is still far from fully understood (Malusà et al., 2007; Michard et al., 2008). Such geological uplifts and subsequent associated alterations of the climatic patterns may have promoted the further diversification in both clades.

The high mountain diversity of this genus appears to be linked primarily with clade A, as all other clades can also be found in the High Atlas, but mostly at lower altitudes or in valleys (Fig. 4). Clearly, the High Atlas is a region of both high endemicity and complexity, particularly the western

region. Only B. lienhardi Lourenço, 2003 was, until recently, known from the higher regions of the High Atlas. Nevertheless our analysis has revealed that in this area of the High Atlas Mountain that extends between Marrakech and Ouarzazate, three of the four major clades occur (Fig. 4). Moreover, clade C occurs to the south of Ouarzazate. Again, there is geographic coherency in the distribution of the clades, with subclade B2 restricted to the lower region of Ouarzazate and subclade D3 occurring in the lower portions of the northern slope of the High Atlas, the only exception being BomHA5 that was found to the south of the High Atlas. Well-supported subclades A1, A2, A4 and A5 also all occur there. Subclades A4 and A5 seem to be restricted to the higher portions of the High and Anti-Atlas, but subclades A1 and A2 occur from the sea level up to 2600 m a.s.l, thus strongly implying that B. lienhardi may be a paraphyletic species or a complex of cryptic diversity. This would fit well with the recent description of a new species in the region of Boumalene, B. boumalenii Touloun & Boumezzough, 2011, geographically connected with clade B2 through the Oued Dades.

The situation of *B. mariefranceae* is also complex. This species grouped in Clade C, in geographically close subclades C1 and C3, with two specimens of *Buthus* scorpions morphologically very distinct from subclade C2 (Fig. 1). This suggest the paraphyly of *B. mariefranceae*, although the limited sampling impedes at present the reassessment of this species' taxonomy.

Although much remains to be understood about the radiation of *Buthus* scorpions in North Africa and Southern Europe, and especially in Morocco, we have demonstrated that at least, a part of this diversity probably originated on the Atlantic coast of Morocco, possibly from species adapted to less arid conditions. Subsequent geological changes in the landscape, namely related with tectonic movements that raised the Atlas Mountains in Morocco, and climatic changes, related or not with the former, may have led to the differentiation of multiple genetic lineages, with new species regularly described from this area. This would support the hypothesis that the High Atlas functioned primarily as a north-south barrier to contact between older clades, and only secondarily as a source of diversity and speciation. This putative role of

barrier to contact between older clades, and only secondarily as a source of diversity and speciation. This putative role of the High Atlas as a geographical barrier to species dispersal has, at least partially, also been inferred for other animal species, such as *Mauremys leprosa* Schweigger, 1812, a freshwater turtle (Fritz *et al.*, 2006) and *Podarcis* Wagler, 1830 wall lizards (Pinho, Ferrand & Harris, 2006), and may also have functioned as glacial refugia for the latter (Pinho, Harris & Ferrand, 2007).

Further sampling of the eastern and southern distribution of the genus, combined with the use of additional nuclear markers can help shed light to a genus that seems to be intimately connected with changes of North African climate and orography.

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P. Sousa et al.

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### **Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** This is the complete phylogram represented in Figure 1. All individual specimens, together with Bayesian posterior probabilities for all nodes, are given. Sub-clade colours correspond to the colours used in Figures 2, 3 and 4.

**Table S1.** This is an enlarged version of Table 1, which besides information on the MtDNA Lineage and Genbank accession code of all the samples used in the study, also provides geographic location and taxonomic identity for all the samples used in the study. Coordinates are in the WGS84 datum, in decimal degrees. Identifications made by Gantenbein & Largiadèr (2003), are indicated by an a.

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PAPER 4: Can a scorpion cross the sea? Biogeographical answers from a multilocus phylogeny of Mediterranean *Buthus* (Buthidae, Scorpiones).

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## Abstract

The scorpion genus Buthus Leach, 1815 has 53 species distributed in semi-arid areas of North Africa, the Middle East, and the Iberian Peninsula. Several isolated species are known, both in Mediterranean and inland "Mountainous islands" of the Sahara, and several other exist in the Sahel region, which gives it a peri-Saharan distribution and accounts for an excellent model to understand the evolution of the Saharan Desert. Our multilocus phylogeny returned the same five species-groups that had been proposed for the genus, although we had a better understanding of their relationships. We tested several different calibration using the Beast software and found that this early diversification unfolded in the Tortonian to Messinian Ages (6.3 to 9.5 Ma). Furthermore, we propose that these groups can be divided in two clades, to the North and South of the High-Atlas Mountains, but our divergence time's link this split with climatic drivers, as it fits well with recent findings that propose the onset of the Sahara Desert with aridification of North Africa at 7 Ma. The role the second stage of the High-Atlas Mountains uplift might have played is difficult to understand because its timings are not consensual an range from the Miocene to the Pleistocene. We propose a biogeographical scenario in which the Messinian Salinity Crisis (MSC) acted as the vicariant event that split Iberian from North Morocco Buthus scorpions, and a much younger (Upper Pleistocene) dispersal over water to the island of Cyprus by B. kunti Yağmur at al., 2011 (our it's ancestrals). The latter has been found in several animal and plant taxa, but is a remarkable achievement for animals always depicted as poor dispersers. We also call attention to the fact that the "Mesobuthus clock", used in several scorpion phylogenetic works does not fit the biogeographic history of Buthus, as it yielded a much earlier history that would require among others, two over water dispersals. Finally, we also raise taxonomical doubts regarding the validity of some species-pairs, and propose that sampling type-localities can help to disentangle these problems.

## Keywords:

Messinian Salinity Crisis, Beast, Calibration, Miocene, Atlas Mountains System, Morocco, Sahara, Cyprus, Ethiopia

## Introduction

The Mediterranean Sea has long been viewed as a Biogeographic barrier, although recently is role as a mediator rather than as a barrier is slowly emerging (Husemann *et al.*, 2014).

The Mediterranean Sea achieved its present configuration around 10 Ma, when the Betic-Rif arch reached broadly the position it has today in both sides of what is now the Strait of Gibraltar. Before, The Arabian plate had already divided the Neo-Tethys Ocean into a Proto Mediterranean and cut its connection to the Indian Ocean (circa 43 Ma) (Müller and Seton, 2015), although a gateway between both water masses existed until 19 Ma (Rögl, 1998; Harzhauser et al., 2007). At the end of the Miocene the Mediterranean was separated from the Atlantic Ocean and dried out in a phenomenal scale. This event, known as the Messinian Salinity Crisis (MSC) occurred from 5.971 to 5.33 Ma (Roveri et al., 2014), and during this time several land bridge were formed between Europe and North Africa (Hsü et al., 1977), although their exact timing is still debated (Bache *et al.*, 2012). The MSC, although short in geological duration, , left a profound vicariant mark in the phylogeography of many taxa around the Mediterranean (*e.g.* Kaliontzopoulou *et al.*, 2011; Velo-Antón *et al.*, 2012; Maia-Carvalho *et al.*, 2014; Gvoždík *et al.*, 2015; Froufe *et al.*, 2016) (reviewed in Husemann et al., 2014).

Low vagility species with a long history of persistence trough time, such as most scorpions (Polis et al., 1985), are ideal for exploring the evolutionary history of the Mediterranean region. The scorpion genus Buthus Leach, 1815 is the fourth most specious member of the Buthidae C.L.Koch, 1837, the largest scorpion family. The genus can be found in semi-arid areas of North Africa, the Middle East, and the Iberian Peninsula extending up to the south of France. Several isolated species are known, both from Mediterranean islands like Cyprus (Yağmur et al., 2011) and Sicily (Lourenço and Rossi, 2013), but also from inland islands, deeply isolated in Mountain regions in the middle of the Sahara. Notably, some species have also been found south of the Sahel region (e.g. Lourenço, 2005; Lourenço & Leguin, 2012), all of which demonstrate these scorpions ability at persisting and/or crossing the Saharan Desert. Gantenbein and Largiadèr (2003) using mitochondrial (mtDNA) and nuclear DNA (nuDNA) sequences, were the first to evaluate the genetic diversity of the genus. They found three main clades in the Western Mediterranean, namely a European, an Atlas (Morocco) and a Tell-Atlas (Tunisia) clade. The same authors also found three distinct lineages in the European clade, which were also supported with allozyme data (Gantenbein, 2004). Sousa et al. (2012) but relying on a wider sampling across the Maghreb uncovered a much higher level of genetic diversity in Morocco than previously reported. Sousa et al. (2012) found

four main *cox1* mtDNA lineages, three of which were exclusive to Morocco, with similar patterns reported also by Habel et al. (2012). The forth lineage was further split by Pedroso et al. (2013) using a longer fragment of the mitochondria, into a North Morocco plus South-western Europe and an Algerian plus Tunisian groups.

The "molecular evolutionary clock" (MeC) (Zuckerkandl and Pauling, 1965) is deeply intertwined with modern molecular phylogenetic methods (Felsenstein, 2004; Yang, 2014). The MeC can be used to explicitly calibrate phylogenies, giving them biological meaningful time (Ho and Duchêne, 2014). Phylogenies can be calibrate with a priory known rates of nucleotide substitution (e.g. Brower, 1994; Papadopoulou et al., 2010). However, it is well understood that the MeC will vary along the evolutionary time and between different lineages within a phylogeny, which makes these secondary calibrations problematic (Hipsley and Müller, 2014). Other calibrations use node age constraints derived from fossil or biogeographical events properly dated and meaningful for the phylogeny in question (Ho and Duchêne, 2014; Donoghue and Yang, 2016). Age constrains can be introduced as point calibrations, hard or soft bounds and as parametric distributions (review in Ho and Phillips, 2009), that convey different levels of uncertainty in the calibration being used. Biogeographically decisive geological and climatic events can both be used in calibrations (Ho et al., 2015), although great consideration must be exerted in their use because several pitfalls in the reasoning supporting their usage have been identified, although several also apply to fossils (Hipsley and Müller, 2014; Ho and Duchêne, 2014; Baets et al., 2016).

In the present study we used the biogeographical calibration potential of the MSC together with a multilocus dataset sampled over the entire North African distribution of *Buthus* scorpions and a sample from south of the Sahara Desert to: 1) estimate a time frame for the early diversification of *Buthus*; 2) evaluate the biogeographic scenario for *Buthus* speciation and 3) use pairwise genetic distances to evaluate the genus taxonomy.

## Materials and methods

## 2.1. Data collection

We collected 55 *Buthus* specimens from 44 new localities (Table 1) that were used to construct our multilocus dataset. These included for the first time samples from the Eastern Mediterranean, namely Cyprus (one specimen), Egypt (two specimens), Israel (one specimen), Jordan (two specimens), and south of the Sahara Desert, Ethiopia (one specimen) (Table 1). We further added one new locality from Algeria, France and Portugal, one specimen from each, eight new specimens from seven localities from Tunisia, 12 new specimens, one per locality, from Spain, and 22 new specimens from 16 Moroccan localities. These include three specimens from each of the five lineages identified in Sousa et al. (2010) (except for lineage 2 with only two specimens) (Table 1). We included samples of three additional Buthidae genera as outgroups: *Androctonus* Ehrenberg, 1828, *Compsobuthus* Vachon, 1949 and *Mesobuthus* Vachon, 1950.



**Figure IV.II-1.** Map of *Buthus* sampled localities in the Western Mediterranean for this study. For a full overview of sampled localities see Fig. 2 and Fig. 9 for the Eastern Mediterranean localities. Sampling numbers correspond to Table 1.

For the phylogeographical analysis, we used a total of 370 *Buthus* terminals, for which the mitochondrial genes cytochrome c oxidase subunit 1 (*cox1*, 370 sequences) and the 16S rRNA (16S, 163 sequences) were available. These included the 52 *Buthus* 

specimens (42 *cox1* and 40 16S sequences) from the multilocus dataset and ten new specimens (nine sequences from both mtDNA markers) exclusive of this analysis (Table 1). The remaining 433 sequences (319 *cox1* and 114 16S sequences) were retrieved from eight previous studies (Gantenbein and Largiadèr, 2003; Froufe et al., 2008; Sousa et al., 2010, 2011, 2012; Husemann et al., 2012; Habel et al., 2012; Pedroso et al., 2013).

All newly available specimens were examined morphologically. Although identification keys for the genus are difficult to implement (Sousa et al., Paper 1, we made an effort to identified specimens to species level following the available literature (Vachon, 1952; Lourenço, 2003; Lourenço and Vachon, 2004; Kovařík, 2006, 2011; Yağmur et al., 2011; Lourenço and Leguin, 2012; Lourenço et al., 2012; Rossi, 2012; Rossi et al., 2013; Teruel and Melic, 2015). Specimens from the Maghreb and Egypt were identified by P. Sousa; from Cyprus and Jordan by E. A. Yağmur, from Ethiopia by F. Kovařík and from Israel by A. Rossi. Three *Buthus* species identifications were based on immatures and are considered tentative because they were solely based on their proximity to the type locality of nominal species: *B. chambiensis* Kovařík, 2006, *B. aureus* Lourenço & Sadine, 2016 and *B. pusillus* Lourenço, 2013. The first was collected at the type locality (Jebel Chambi Mountains), the latter two species were collected at 13 and 20 km from their type locality, respectively. The specimens' available from Egypt, Israel and Jordan could not be assigned to any nominal species.

All specimens are deposited in the collection of CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Universidade do Porto, Vila do Conde, Portugal, except when stated otherwise in Table 1. **Table IV.II-1.** List of specimens sequenced in this study. Specimens used only in the mtDNA dataset appear before the outgroups. mtDNA: main clade as named in Sousa et al., (submited), Country: Alg. – Algeria, Mor. – Morocco, Tun. – Tunisia; Loc. Corresponds to the locality number in Figs. 1, 2 and 9; Geographic coordinates are given in WGS84; GenBank accession codes for the markers used: / – none sequenced, n.a. – specimen for the mtDNA dataset only; Collec. gives the collection were each specimen voucher or leg sample is located.

ID	Таха	mtDNA	Countr.	Loc.	Lat.	Long.	cox1	16S	PK	0971	0061	5070	28S	Collec.
Sc0375	B. sp.	tunetanus	Alg.	1	35.303	7.653	KF824988	KF825029	1	yes	1	/	/	Cibio
Sc0376	B. sp.	tunetanus	Alg.	2	35.170	2.217	JQ775955	KF825030	1	/	/	/	/	Cibio
Sc0491	B. boumalenii	boumalenii	Mor.	3	31.246	-6.104	KF824993	KF825034	yes	/	/	/	/	Cibio
Sc0548	B. draa	rochati	Mor.	4	30.746	-6.449	KF824997	KF825038	yes	/	/	1	/	Cibio
Sc0898	B. sp.	tunetanus	Tun.	5	35.555	8.681	KF825014	KF825052	yes	yes	/	1	/	Cibio
Sc0900	B. sp.	tunetanus	Tun.	6	34.712	8.517	KF825015	KF825053	yes	yes	yes	/	/	Cibio
Sc0930	B. sp.	tunetanus	Tun.	7	33.533	9.991	yes*	KF825057	yes	/	1	/	/	Cibio
Sc0941	B. sp.	tunetanus	Tun.	8	32.785	10.373	KF825020	KF825058	yes	yes	/	1	/	Cibio
Sc0955	B. sp.	tunetanus	Egypt	9	31.279	27.055	yes	yes	yes	yes	yes	1	/	Cibio
Sc0956	B. sp.	tunetanus	Egypt	9	31.279	27.055	yes	yes	yes	yes	yes	/	/	Cibio
Sc1096	B. halius lin. 1	occitanus	Spain	10	37.888	-6.562	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc1100	B. halius lin. 2	occitanus	Spain	11	36.388	-5.651	yes	yes	yes	yes	yes	yes	1	Cibio
Sc1125	<i>B. sp.</i> lin 4	occitanus	Spain	12	37.283	-3.252	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc1505	B. draa	rochati	Mor.	13	29.727	-7.975	KF824981	KF825073	yes	yes	/	/	yes	Cibio
Sc1534	B. bonito	rochati	Mor.	14	28.017	-12.203	yes	yes	yes	yes	/	1	/	Cibio
Sc1535	B. bonito	rochati	Mor.	14	28.017	-12.203	KF824985	KF825077	/	yes	/	/	/	Cibio
Sc1537	B. bonito	rochati	Mor.	14	28.017	-12.203	yes	yes	yes	yes	/	1	/	Cibio
Sc1538	B. elmoutaouakili	mardochei	Mor.	15	30.059	-9.084	yes	yes	yes	yes	/	/	/	Cibio
Sc1565	B. elmoutaouakili	mardochei	Mor.	15	30.059	-9.084	yes	yes	yes	/	/	1	/	Cibio
Sc1548	B. sp.	mardochei	Mor.	16	30.159	-8.481	yes	yes	yes	yes	/	yes	yes	Cibio
Sc1568	B. sp.	mardochei	Mor.	17	29.765	-9.136	yes	yes	yes	yes	1	yes	yes	Cibio
Sc1590	<i>B. sp.</i> lin 4	occitanus	Spain	18	37.127	-3.214	yes	yes	yes	yes	yes	/	/	Cibio
Sc1722	B. halius lin. 2	occitanus	Spain	19	36.625	-5.668	yes	yes	yes	yes	yes	yes	/	Cibio
Sc1752	B. occitanus lin. 3	occitanus	Spain	20	36.657	-5.098	yes	yes	yes	yes	yes	yes	/	Cibio
ID	Таха	mtDNA	Country	Loc.	Lat.	Long.	cox1	16S	PK	0971	0061	5070	28S	Collec.
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Sc1795	<i>B. sp.</i> lin 4	occitanus	Spain	21	37.128	-2.592	yes	yes	yes	yes	yes	yes	1	Cibio
Sc1843	<i>B. occitanus</i> lin. 5	occitanus	Spain	22	39.918	-0.016	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2026	<i>B. occitanus</i> lin. 5	occitanus	Spain	23	40.947	-1.303	yes	yes	yes	yes	yes	yes	1	Cibio
Sc2100	B. occitanus lin. 3	occitanus	Spain	24	36.528	-4.900	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2111	B. halius lin. 1	occitanus	Spain	25	38.844	-4.027	yes	yes	yes	yes	yes	/	1	Cibio
Sc2365	<i>B. occitanus</i> lin. 5	occitanus	France	26	43.490	3.555	yes	yes	yes	yes	yes	yes	/	Cibio
Sc2371	B. halius lin. 1	occitanus	Port.	27	41.360	-7.810	yes	yes	yes	yes	yes	yes	1	Cibio
Sc2379	B. awashensis	tunetanus	Ethiopia	28	9.577	41.839	yes	yes	yes	yes	yes	yes	yes	F. Kovařík
Sc2388	B. rochati	rochati	Mor.	29	28.221	-11.750	yes	yes	yes	yes	/	/	1	Cibio
Sc2404	B. sp.	mardochei	Mor.	30	32.898	-8.499	yes	yes	yes	/	1	/	1	Cibio
Sc2406	B. confluens	occitanus	Mor.	31	34.301	-5.290	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2409	B. sp.	occitanus	Mor.	32	33.760	-5.954	yes	yes	yes	/	yes	/	yes	Cibio
Sc2410	B. sp.	occitanus	Mor.	32	33.760	-5.954	yes	yes	yes	yes	1	yes	yes	Cibio
Sc2414	B. confluens	occitanus	Mor.	33	34.916	-5.539	yes	yes	yes	yes	/	/	yes	Cibio
Sc2419	B. confluens	occitanus	Mor.	34	35.396	-5.372	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2420	B. confluens	occitanus	Mor.	34	35.396	-5.372	yes	1	yes	yes	yes	yes	yes	Cibio
Sc2423	B. confluens	occitanus	Mor.	35	35.305	-6.027	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2424	B. confluens	occitanus	Mor.	35	35.305	-6.027	yes	yes	yes	yes	yes	/	yes	Cibio
Sc2427	B. occitanus lin. 3	occitanus	Spain	36	36.437	-5.157	yes	yes	yes	yes	yes	yes	yes	Cibio
Sc2496	B. sp.	tunetanus	Tun.	37	35.314	9.338	yes	yes	yes	/	yes	/	/	Cibio
Sc2497	B. chambiensis	tunetanus	Tun.	38	35.206	8.678	yes	yes	yes	/	1	/	/	Cibio
Sc2498	B. sp.	tunetanus	Tun.	39	35.908	8.571	yes	yes	yes	/	1	/	/	Cibio
Sc2533	B. sp.	occitanus	Mor.	40	34.028	-6.708	yes	yes	yes	yes	1	/	/	Cibio
Sc2569	B. kunti	tunetanus	Cyprus	41	35.691	34.562	yes	yes	yes	/	yes	/	/	E. Yagmur
Sc2570	B. sp.	tunetanus	Jordan	42	29.536	35.414	yes	yes	yes	yes	1	/	1	E. Yagmur
Sc2571	B. sp.	tunetanus	Jordan	42	29.536	35.414	yes	1	yes	yes	yes	/	/	E. Yagmur
Sc2590	B. sp.	tunetanus	Israel	43	32.609	35.077	yes	yes	yes	yes	yes	1	1	A. Rossi
Sc2757	B. malhommei	occitanus	Mor.	44	32.661	-7.793	yes	yes	yes	/	1	1	1	Cibio

ID	Таха	mtDNA	Country	Loc.	Lat.	Long.	cox1	16S	PK	0971	0061	5070	28S	Collec.
Sc0050	B. confluens	occitanus	Mor.	45	34.053	-4.170	JQ775920	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc0372	B. pusillus	tunetanus	Alg.	46	36.448	4.125	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc0495	B. boumalenii	boumalenii	Mor.	3	31.246	-6.104	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2407	B. sp.	occitanus	Mor.	47	33.821	-5.970	yes	1	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2495	B. sp.	tunetanus	Tun.	37	35.314	9.338	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2499	B. sp.	tunetanus	Tun.	48	35.751	8.362	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2500	B. sp.	tunetanus	Tun.	48	35.751	8.362	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2525	B. sp.	tunetanus	Tun.	49	36.481	8.325	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2585	B. sp.	occitanus	Mor.	50	33.444	-5.047	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2587	B. sp.	mardochei	Mor.	51	XXX	XXX	yes	yes	n.a.	n.a.	n.a.	n.a.	n.a.	Cibio
Sc2408	A. mauritanicus	outgroup	Mor.	52	33.869	-5.921	yes	yes	yes	yes	yes	/	yes	Cibio
Sc2520	Mesobuthus. sp.	outgroup	Greece	53	26.010	35.048	yes	yes	yes	/	yes	/	yes	Cibio
Sc2591	Compsobuthus. sp.	outgroup	Oman	54	17.486	56.028	yes	yes	yes	yes	1	/	/	Cibio

#### 2.2. DNA extraction, PCR and Sequencing

Whole genomic DNA was extracted from freshly preserved (96% ethanol) muscle tissue (leg) using the SPEEDTOOLS Tissue DNA Extraction Kit (BIOTOOLS). Seven gene regions where amplified, two mitochondrial fragments *cox*1 and 16S, and five nuclear gene fragments, a Protein Kinase like (PK) gene fragment, the 28S rDNA large subunit domain D3 (28S) and three Anonymous Nuclear Markers (ANM) developed for the present study, c0061, c0971 and c5070 (Paper 2). Mitochondrial primers and Polymerase chain reactions (PCR) conditions are presented in Table 2. Nuclear primers (Nunn et al., 1996; Gantenbein et al., 2003; Sousa et al., in prep 1) and PCR conditions follow the methodology described in Sousa et al. (in prep 1). PCR were performed in a final volume of 25  $\mu$ L using Sigma's REDTaq DNA polymerase with the REDTaq ReadyMix PCR Reaction Mix with MgCl2 (Sigma-Aldrich). Amplified DNA templates were sequenced in both directions in an ABI 3700 automated sequencer at the Scientific and technological Centers UB (CCiTUB, http://www.ccit.ub.edu)

Table IV.II-2. Primer sequences and PCR annealing temperatures used with the mtDNA markers.
Primers name, sequenced and Reference are also given. All PCR were run for 35 cycles, with
annealing and extension times for all pairs set at 45s.

Marker	Annealing	Primer	Sequence (5' to 3')	Reference
Marker	Temp.	name		Reference
cov1	130	LCO1490	GGTCAACAAATCATAAAGATATTGG	(Entmoriation 1004)
COXT	43	HC02198	TAAACTTCAGGGTGACCAAAAAATCA	
160	170	16SB2 (LRJ-12864)	CTCCGGTTTGAACTCAGATCA	(Palumbi, 1996)
103	47	16SA (LR-J- 13417)	ATGTTTTTGTTAAACAGGCG	(Simon et al., 1994)
16S		18-mer	CGATTTGAACTCAGATCA	(Simon et al., 1994)
(smaller)	50°	20-mer	GTGCAAAGGTAGCATAATCA	(Gantenbein et al., 1999)

DNA sequences were edited and assembled using Geneious software v.6.1.8 (Kearse et al., 2012). All multiple sequence alignments were exported to the required software formats with the help of the online program ALTER (Glez-Peña et al., 2010) (http://sing.ei.uvigo.es/ALTER/). All sequences newly obtained in this study were submitted to GenBank (Table1).

## 2.3. Alignment, phasing, and best-fitting nucleotide model selection

The two protein coding genes (*cox1* and PK) were aligned with Muscle (Edgar, 2004). No indels were detected and when translated to amino acids no stop codons were identified. The remaining genes were aligned using the MAFFT (v7.017) plugin in Geneious using the G-INS-i algorithm (Katoh et al., 2005; Katoh and Standley, 2013),

except for the 16S gene that was aligned using the MAFFT (v7.305) version available on the CIPRES Science Gateway (CIPRES) (Miller et al., 2010), with the Q-INS-I algorithm (Katoh and Toh, 2008).

For the nuclear genes, allelic phases were resolved using two complementary methods, depending on the existence of indels. For heterozygous sequences with insertions or deletions (all ANM fragments), we used the online Champuru software v1.0 (Flot, 2007), which implements the method described by Flot et al. (2006). For nucleotide heterozygotes, we used the Bayesian algorithm implemented in PHASE (Stephens et al., 2001), using the known phases of alleles determined with Champuru, when available. Phase was run five times per dataset, and the most probable pair of alleles for each heterozygous individual was retained for the downstream analyses (Bryson Jr et al., 2014). Remaining positions were coded missing data (N).

Poorly aligned positions from the genes with alignment gaps (16S and the threeANM) were subsequently eliminated with the help of Gblocks V.0.91b (Castresana, 2000;TalaveraandCastresana,2007)(http://molevol.cmima.csic.es/castresana/Gblocks\_server.html).

The best-fitting nucleotide substitution model for each marker were determined using jModelTest2 (v2.1.6) (Guindon and Gascuel, 2003; Darriba et al., 2012) under the Bayesian information criterion (BIC) (Schwarz, 1978) run on CIPRES (Miller et al., 2010).

## 2.4. Mitochondrial phylogeography and preliminary species delimitation

A mitochondrial based phylogeographic analysis and preliminary species delimitation was conducted on two datasets, one with *cox1* only (mtDNA Dt1, 370 terminals, 658 bp) and the other with the concatenated *cox1* and 16S (mtDNA Dt2, 163 terminals, 841 bp), plus the same three outgroups of the multilocus dataset. The datasets were reduced to 315 and 134 haplotypes respectively (including the three outgroups), to decrease computational time.

Tree inference was conducted using Bayesian inference as implemented in the software MrBayes (v3.2.6) (Ronquist et al., 2012) run on CIPRES (Miller et al., 2010). MrBayes was conducted with two runs, each with 8 chains and with a lowered temperature of 0.05 for better chain swapping concluded after preliminary analyses failed to converge satisfactorily, for 50 million generations, sampling trees every 5.000 generations (and calculating a consensus tree after omitting 25% of the trees). Convergence was evaluated with the PSRF+ parameter in Mr Bayes, the ESS values calculated in Tracer v1.6 (Rambaut et al., 2014) and monitored with the program AWTY (Nylander et al., 2008).

Due to the inherent difficulties in *Buthus* morphological identification, a preliminary species delimitation based on mitochondrial data (mtDNA Dt1) was conducted using two different approaches, the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2011) (wwwabi.snv.jussieu.fr/public/abgd/ abgdweb.html), and the General Mixed Yule Coalescent (GMYC) (Pons et al., 2006; Monaghan et al., 2009; Fujisawa and Barraclough, 2013) (http://species.h-its.org/gmyc/). The ultrametric tree required for the GMYC analyses was obtained with Beast (details on the Beast run are detailed below).

To better assess the ABGD results, we reduced mtDNA Dt1 dataset to only 48 haplotypes (mtDNA Dt3) that represent, from our accumulated experience, the dataset that can be most parsimoniously attributed to *Buthus* species (Suppl. Table 4.1). We used two criteria: morphological identification, for those species that have readily identifiable characters; geographical proximity, for species that were collected close to the species type locality. In areas where different *Buthus* species may occur in sympatry, we required less than 5km of proximity to the type locality, for species from areas where no conspecific have been reported (outside of Morocco), a proximity of up to 20 km was tolerated. These criteria were also used in combination, resulting in a third, hybrid criteria (Suppl. Table 4.1). We also evaluated evolutionary divergence by estimating uncorrected p-distances between and within the *Buthus* species present in the mtDNA Dt3 datset using MEGA v6.06 (Tamura et al., 2013), with variability assessed conducting 1,000 bootstraps.

#### 2.5. Multilocus phylogeny and divergence time reconstruction

The multilocus concatenated dataset comprised 55 specimens, including the three above mentioned outgroups (Table 1) that represent must of the Mediterranean range of the *Buthus* genus. From these, 46 had never been sequenced before, and none had nuclear sequences available.

We estimated standard genetic diversity indices for the concatenated data matrix with DnaSP v.5.10.01 (Librado and Rozas, 2009). We calculated the number of segregation sites (S), the number of segregating sites per 100 bp ( $S_{100}$ ), the nucleotide diversity ( $\pi$ ), and the number (H) and diversity of haplotypes (Hd). Non-neutral evolution was evaluated with Tajima's D test (D) (Tajima, 1989), recombination with the determination of the minimum number of recombination events ( $R_M$ ) (Hudson and Kaplan, 1985) and with the linkage disequilibrium statistic (ZZ), which can also detect intragenic recombination (Rozas et al., 2001). The significance of the test results was assessed using 1.000 coalescent simulations with the algorithm implemented in DnaSP.

Phylogeny reconstruction was performed using Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Maximum Likelihood reconstruction was performed with RAxML-HPC2 Workflow (v8.2.9) (Stamatakis, 2014) run on CIPRES (Miller et al., 2010), for which we conducted 20 runs to find the best ML tree topology using the best-fitting partition model found using jModelTest2 (v2.1.6). A final optimization of branch lengths and model parameters was then performed. We conducted a thorough nonparametric bootstrap with 1,000 replicates.

Bayesian inference was performed with MrBayes (v3.2.6) (Ronquist et al., 2012) run on CIPRES (Miller et al., 2010). MrBayes analysis was conducted with two runs, each with 8 chains and with a lowered temperature of 0.05 for better chain swapping (concluded after preliminary analyses), for 100 million generations, sampling trees every 10,000 generation, and calculating a consensus tree after omitting the first 25% of the trees as burnin. Convergence of chains and correct mixing was assessed has explained above.

The same concatenated data set was subsequently used for estimating time divergences in a Bayesian framework as implemented in Beast v1.8.4 (Drummond et al., 2012) on CIPRES (Miller et al., 2010), although we only constrained *Mesobuthus* and *Compsobuthus* as outgroups. We performed several preliminary runs to best tune the parameters for the program. A clock-like behaviour of our data set was rejected by the standard deviation (SD) and coefficient of variation values of preliminary runs using the lognormal uncorrelated relaxed clock (ULC) (Peng et al., 2006) and hence all analyses were conducted under the ULC model. The Birth-Death was selected as tree prior and the Continuous-time Markov chains (CMTC) (Ferreira and Suchard, 2008) as prior for the clock rate of each partition, whenever prior information was unavailable.

Absolute divergence times were estimated using six alternative schemes.

**Table IV.II-3.** Rates of mtDNA nucleotide substitutions used in this study. Values as substitution/site/million year. Within brackets are the standard deviation values used. \*- Sd value not available, selected by us; <sup>a</sup> – a Sd of 0.3 proved too high, we used 0.005.

Rate	16S	cox1
<i>Mesobuthus</i> (Gantenbein and Largiadèr, 2002; Gantenbein et al., 2005)	0.00515 (0.006)	0.007 (0.003)
<b>UNI 1</b> (Papadopoulou <i>et. al</i> , 2010)	0.0053 (0.003)*	0.0177 (0.003)*
<b>UNI 2</b> (Brower, 1994)	0.011	5 (0.3)ª

Three of them consisted in assigning different mitochondrial DNA substitutions rates available in the literature, as follows: 1) *cox1* and 16S mtDNA rates estimated for *Mesobuthus* scorpions (Gantenbein and Largiadèr, 2002; Gantenbein et al., 2005),

hereafter referred as the *Mesobuthus* scheme; 2) *cox1* and 16S mtDNA rates for arthropods (UNI 1 scheme) (Papadopoulou et al., 2010) and 3) combined mtDNA rate for arthropods (UNI 2 scheme) (Brower, 1994; Parmakelis et al., 2013). The substitutions rates implemented are summarized in Table 3.

**Table IV.II-4.** Priors used for the three different Beast analyses that used the Messinian Salinity Crisis as a vicariant event. \* –mean in real space; n.a. – not applicable.

Clock scheme	Mean	St. Dev.	offset
IP Exp	0.22	n.a.	5.321
IP LogN	5.3*	0.6	5.225
Сур Ехр	0.06	n.a.	5.33

A second set of schemes were based on the vicariant events associated to the desiccation of the Mediterranean during the Messinian Salinity Crisis (MSC). The MSC onset has been estimated at 5.96 Ma (±0.002 Ma) (Krijgsman et al., 1999; Krijgsman, 2002) and the ending of the MSC ended at 5.33 Ma, corresponding to the transition from the Messinian to the Zanclean stage (Lourens et al., 1996; Van Couvering et al., 2000). Following Andújar et al. (2012), we assigned an exponential prior to the stem of the most recent common ancestor (tmrca) of the Iberian clade such as the duration of the MSC was included in the 95% interval of the prior (IP Exp scheme, Table 4). Alternatively, we defined a lognormal prior (IP LogN scheme, Table 4) to give a lesser emphasis to the ending of the MSC, since recent studies suggest that an Atlantic-Mediterranean connection may have existed after the onset of the MSC (Manzi et al., 2013; Roveri et al., 2014). Finally, following Plötner et al., (2010), we assumed that Cyprus was only connect to the Anatolia Peninsula during the Lago Mare phase of the MSC (Robertson, 1998), dated from 5.55 to 5.33 Ma (Hilgen et al., 2007; Roveri et al., 2014), to assign an exponential prior to the stem of *B. kunti* (Cyp Exp scheme, Table 4). Beast analyses were ran twice for 100 million generations, and checked for convergence of variable estimates with the calculation of the effective sample size (ESS) in Tracer v1.6 (Rambaut et al., 2014) and for convergence of tree space with AWTY (Nylander et al., 2008).

# Results

We present for the first time molecular data for seven *Buthus* species: *B. aureus*, *B. awashensis* Kovařík, 2011, *B. chambiensis*, *B. confluens* Lourenço, Touloun & Boumezzough, 2012, *B. kunti* Yağmur, Koc & Lourenço, 2011, *B. pusillus* and *B. rochati* Lourenço, 2003 (Table 1).

## 3.1 Buthus mtDNA

## 3.1.1 Buthus mtDNA phylogeny and biogeography

The inferred trees for the mtDNA Dt1 and mtDNA Dt2 matrices, using the GTR + Gamma nucleotide substitution model for both mtDNA markers as selected under the Akaike information criterion (AIC) using jModelTest2, yielded the same five main lineages already identified in previous studies (Sousa et al., 2012; Pedroso et al., 2013), and relationships within each lineage remained unsupported. However, the new data allowed us to refine the composition of each lineage, which now include more than half of all known *Buthus* species (Table 5).

**Table IV.II-5.** Upoated composition of the five species groups identified within *Buthus*, as proposed by Sousa et al. (2012) and Pedroso et al. (2013), based on the cox1 mtDNA marker or species' known distribution. We assign 31 out of the 52 known *Buthus* species (60%). ° – assignments based on the new *cox1* sequences; <sup>a</sup> – assignments based on the known distribution of the species.

group	species	group	species
boumalenii	B. boumalenii	mardochei	B. elmoutaouakili
			B. lienhardi
occitanus	B. albengai ª		B. mardochei
	B. atlantis		B. parroti
	B. confluens	tunetanus	B. adrianae <sup>a</sup>
	B. elongatus		B. amri ª
	B. ibericus		B. aures
	B. malhommei		B. barcaeus <sup>a</sup>
	B. maroccanus		B. chambiensis °
	B. montanus		B. dunlopi ª
	B. occitanus		B. egyptiensis
			B. israelis <sup>a</sup>
rochati	B. bonito		B. kunti °
	B. draa		B. lourencoi ª
	B. mariefranceae		B. orientalis <sup>a</sup>
	B. rochati °		B. pusillus
			B. tunetatus

Following the lineage nomenclature used by Sousa et al. (Paper 1, the updated distribution areas of the five mtDNA groups are as follows:

*boumalenii* (Fig. 1 and 2); now includes the Upper Oued Dra valley (including the Oued Dades) and extends south into the border with Algeria, always in the eastern bank of the Dra river, and extending east to the Oued Todgha with the additional sampling of Habel et al. (2012) and Husemann et al. (2012). There are two outliers found in the West bank of the Dra, the specimens Ha 9-62-2, 3 (Habel et al., 2012).

occitanus (Fig. 1 and 2); south-western Europe, all of Morocco north of the central and eastern portions of the High Atlas and also the Atlantic shore south into the Sous River valley, and east into Algeria, roughly until Alger's longitude. Two outliers were collected south of the High Atlas: Bom Ha5a; b (Gantenbein and Largiadèr, 2003).

*mardochei* (Fig. 1 and 2); Sous river valley of Morocco, including the northern slopes of the Anti-Atlas, and North until the Oum-er-Rbia river, including the western portion of the High Atlas. Sc0061 is an outlier collect in the Middle Atlas (Sousa et al., 2012).



**Figure IV.II-2.** Map of the known distribution of the five main mtDNA lineages within the genus *Buthus*. Arrows represent potential dispersal routes for the *tunetanus* group, according to the Beast IP LogN analysis (see also Fig. 7). A –The western Mediterranean region depicted in Fig. 1; B – The Eastern Mediterranean region depicted in Fig. 9. Type localities were presented to offer a full understanding of the known distribution of the genus. For further details on type localities see Sousa et al. (submitted). 28 represents the single Ethiopian sampled locality.

*rochati* (Fig. 1 and 2); occupies most of the Oued Dra watershed and the Anti-Atlas except its northern slopes, below 1550 m a.s.l. in the Atlantic shore of Morocco it extends north until the Oued Massa, a watershed in the north-western slope of the Anti-Atlas and south to the Khenifiss Iagoon<del>, 70 Km north of Tarfaya</del>.

*tunetanus* (Fig. 1, 2 and 9); North Africa east of Alger and north of the Sahara Desert, into the Middle East and Cyprus in Asia. Moreover the Ethiopian sample also belongs to this group.

## 3.1.2 Buthus mtDNA preliminary species delimitation

Both methods implemented for conducting a preliminary hypothesis on *Buthus* species delimitation based on single locus data failed to achieve a biologically meaningful result. The ABGD could not find a barcoding gap in the pairwise distances calculated and thus could not provide reliable results (Fig. 3A). GMYC resolved a large number of groups (490 ML entities) with limited useful meaning.



**Figure IV.II-3.** Histograms of *Buthus* sequences uncorrected p-distances. In red is the accumulation curve. A - mtDNA Dt1; B - mtDNA Dt3. Note the lack of a clear bimodal distribution.

The mtDNA Dt3 dataset had 18 *Buthus* species plus three *Buthus* morphospecies that given their geographical origin we considered important to include (Table 6), which represents almost a third of all known *Buthus* species. The frequency distribution of the pairwise distances exhibits a normal-like distribution without a clear gap, although a drop at 8% exists that we use as a breaking point for summarizing the results. Of the resulting 210 pairwise species comparisons 92% had a value of 8.5% (Table 6, Fig. 3B), and 6.7% had a value smaller than 8%. Moreover, three species pairwise comparisons had a value of less than 3.0% (Table 6), a value that has been used as a clear transition between infra and inter-specific divergence (Hebert et al., 2003). Although we could only calculate within species divergence for 12 species, five (40%) had more than 3%, which is above

what is usually assumed as a barcoding gap, a result even more remarkable given the

reduced number of animals per species.

**Table IV.II-6.** Mean sequence divergence (uncorrected p-distances, values in percentage) of *Buthus* species represented in mtDNA Dt3. Below the diagonal are between species distances. Above the diagonal is the standard error calculated with a 1,000 bootstraps. When available, within species distances are represented in the diagonal. N – number of sequences per species; mtDNA – the main mtDNA group each species belongs to; a – only a subset was used, choosing animals from the upper part of the species distribution. Calculated in Mega 6.

ID	Tava	N	mtDNA	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
	Idxa	IN	IIIUDNA	1	4 4 0	3	4	J	1.00	1 0.1	0	9	10	1 10	12	13	14	10	10	1/	10	19	20	21
1 /	s. atlantis	4	OCCI.	0.21	1.16	1.38	1.52	1.39	1.39	1.24	1.35	1.33	1.32	1.16	1.23	1.24	1.42	1.29	1.39	1.40	1.42	1.33	1.33	1.34
2 1	3. aureus	2	tune.	9.11	3.54	1.06	1.20	1.18	0.80	1.02	1.09	1.06	0.97	0.97	0.96	1.03	1.18	1.03	1.17	1.06	1.08	0.77	0.95	0.99
3 1	3. awashensis	1	tune.	11.92	8.88	n.a.	1.23	1.37	1.22	1.07	1.24	1.22	1.17	1.12	1.01	1.08	1.08	1.07	1.37	1.40	1.18	1.15	1.11	1.13
4	3. bonito	3	roch.	13.29	9.96	11.96	0.44	1.38	1.20	1.14	1.00	1.16	1.25	0.99	1.06	0.96	1.21	1.16	1.31	1.34	0.20	1.12	1.28	1.19
5	3. boumalenii	1	boum.	10.89	8.59	9.85	11.21	n.a.	1.41	1.21	1.31	1.40	1.25	1.26	1.29	1.17	1.40	1.31	1.46	1.49	1.40	1.31	1.20	1.25
6 /	3. chambiensis	1	tune.	11.52	4.93	11.40	9.73	10.06	n.a.	1.10	1.24	1.36	1.04	1.10	1.01	1.17	1.21	1.05	1.41	1.26	1.12	0.89	1.04	1.07
7	3. confluens	4	occi.	10.39	8.97	10.57	10.92	9.21	10.42	2.33	1.16	1.17	1.15	1.05	0.97	1.03	1.15	0.99	1.33	1.34	1.11	1.12	1.14	1.17
8	3 draa <sup>a</sup>	7	roch	12.06	9.53	11.89	8.07	10.80	11 27	10.86	3 60	1 13	1 20	1 04	1.03	0.78	1 15	1 17	1.35	1 24	0.93	1 10	1 16	1.08
0	, almoutoouokili	2	mord	11 /6	9.64	10.52	10.60	10.00	11 /0	10.00	10.26	1.70	1.20	0.97	1.07	1.05	1.22	1.22	0.51	1 20	1 20	1.24	1 21	1.00
91	5. emioulaouakim	2	maru.	11.40	0.04	10.52	10.00	10.70	11.40	10.92	10.30	1.79	1.23	0.07	1.07	1.05	1.23	1.22	0.51	1.30	1.20	1.24	1.21	1.21
10 1	3. kunti	1	tune.	11.47	7.33	11.85	12.13	9.42	8.36	10.87	11.83	10.87	n.a.	1.08	1.08	1.12	1.30	1.16	1.32	1.30	1.25	0.92	0.63	1.13
11 /	3. lienhardi	5	mard.	11.14	10.07	11.37	9.90	11.00	11.50	11.67	10.94	8.03	11.19	6.44	0.97	0.96	1.09	1.00	0.92	1.14	0.97	1.01	1.10	1.04
12	3. malhommei	1	occi.	9.90	8.22	9.93	10.38	11.16	8.39	9.43	10.61	9.49	9.93	10.15	n.a.	1.17	0.97	1.02	1.36	1.38	1.22	1.13	1.17	1.04
13	3. mariefranceae	3	roch.	12.00	9.64	10.66	8.25	9.35	11.25	10.41	7.11	9.42	11.05	10.38	10.69	5.88	1.05	1.10	1.12	1.21	0.93	1.05	1.09	1.10
14	3. maroccanus	2	occi.	10.66	9.15	9.68	10.39	10.50	10.04	9.58	10.29	9.57	11.78	11.00	6.77	9.89	1.87	1.11	1.30	1.37	1.10	1.12	1.18	1.06
15	3. occitanus	3	occi.	11.68	8.81	10.98	11.51	11.06	10.06	9.76	11.93	11.35	10.87	10.99	9.91	11.41	10.39	3.26	1.31	1.31	1.17	1.10	1.17	1.09
16	3. parroti	1	mard.	11.09	8.46	10.47	10.26	11.35	10.68	10.58	11.02	1.39	10.04	7.04	9.84	9.19	10.06	9.97	n.a.	1.48	1.30	1.35	1.35	1.32
17	3. pusillus	1	tune.	11.37	7.18	11.13	11.84	10.00	8.75	10.98	10.67	11.02	9.34	10.63	10.58	9.93	11.26	10.74	9.16	n.a.	1.38	1.21	1.35	1.26
18	3. rochati	1	roch.	13.06	9.76	11.70	0.40	11.56	9.73	10.76	7.77	10.87	12.77	10.07	10.06	8.18	10.36	11.79	10.26	11.73	n.a.	1.16	1.30	1.19
19 I	Egypt	2	tune.	10.63	5.71	10.20	10.70	9.64	7.15	10.74	10.58	10.86	6.54	10.66	9.94	10.40	10.46	9.51	9.40	8.05	11.11	0.30	0.95	1.09
20 I	srael	1	tune.	10.89	7.11	11.40	12.42	8.99	8.66	10.42	11.14	10.57	2.89	11.32	10.50	10.96	10.70	10.62	9.40	9.74	12.77	6.39	n.a.	1.12
21 、	ordan	2	tune.	10.94	8.24	9.95	11.28	9.10	9.95	10.46	10.23	10.26	10.41	10.37	8.91	10.82	9.08	10.59	9.51	9.24	11.32	9.06	9.80	0.15

#### 3.2 Buthus Multilocus dataset

The concatenated multilocus dataset included 110 terminals (phased alleles of 55 individuals) and 3,522 aligned characters of seven gene fragments (cox1, 16S, PK, c0061, c0971, c5070 and 28S), 553 of which were parsimony-informative (Table 7). The most informative markers, according to the net percentage of segregating sites, were both mtDNA gene fragments and the ANM c0061 and c0971 (Table 7).

**Table IV.II-7.** Summary diversity statistics for the multilocus dataset, and for the mtDNA dataset 1 (first line). N, number of individuals (mtDNA) / chromosomes (nucDNA). N, number of specimens. Length, number of nucleotides for each locus before and after removing poorly aligned positions with Gblocks. Length information includes outgroup's sequences, which were not used in the remaining columns. Netsites, number of nucleotides excluding sites with alignment gaps. S, number of number of segregating sites. Eta, total number of mutations. H, haplotype number. Hd, haplotype diversity. Pi, nucleotide diversity. Statistics were calculated in DnaSP v.5.10.01.

	Ν	lenght		Ne	tSites		S	S/Net		Eta	Н	Hd	Pi
		Inic	Gb	#	%	#	%	%	#	%			
cox1	312	658	n.a.	274	41.6%	116	17.6%	42.3%	177	26.9%	235	0.997	0.066
cox1	52	658	641	455	71.0%	136	21.2%	29.9%	201	31.4%	49	0.998	0.096
16S	50	491	378	281	74.3%	99	26.2%	35.2%	150	39.7%	45	0.996	0.103
PK	98	354	354	354	100.0%	17	4.8%	4.8%	20	5.6%	21	0.882	0.006
c0971	80	558	428	414	96.7%	101	23.6%	24.4%	115	26.9%	45	0.976	0.042
c0061	56	250	221	217	98.2%	67	30.3%	30.9%	72	32.6%	24	0.956	0.054
c5070	40	784	773	736	95.2%	103	13.3%	14.0%	110	14.2%	23	0.962	0.023
28S	34	738	727	727	100.0%	12	1.7%	1.7%	14	1.9%	9	0.872	0.004

## 3.2.1 Multilocus phylogeny

We resolved the same five main groups with our concatenated multilocus dataset that had been resolved with the mtDNA alone. Although we could not resolve the placement of the *boumalenii* group with support in the Beast analyses (Fig. 6), both the ML and BI (MrBayes) trees retrieve this as clade as sister to the other Buthus main groups with strong support (Suppl. Figs. 4.1, 4.2). Furthermore we were able to determine that the remaining four groups can be arranged into two clades: mardochei with rochati, and occitanus with tunetanus (Fig. 6, Suppl. Figs. 4.1, 4.2). Nevertheless strong support was only achieved with the Beast analyses for the latter, despite less information being available for the first pair as we had a smaller number of specimens. Also, all Eastern Mediterranean specimens analysed grouped, as expected, within the *tunetanus* main group (Fig. 6, Suppl. Figs. 4.1, 4.2). This group included *B. awashensis* (the Ethiopian species), which was found, surprisingly, to form a clade with the Jordan samples (without strong support except for the MrBayes tree) and not with the closer, geographically, Egyptian samples. Moreover, B. kunti (from Cyprus) was included in the same clade as the Israeli and Egyptian samples, which formed a sister Clade to the Tunisian samples, strongly supported in all analyses (Fig. 6, Suppl. Figs. 4.1, 4.2).

The Iberian Peninsula clade (Fig. 6), although not always retrieved with strong support in the BI or ML analyses, was present in the best resolved ML tree and fits well with our data, and for these reasons we used its separation from North Africa as a calibration point. The Iberian clade is the object of a separated study and thus will not be analysed in detail in the present study.

## 3.2.2 Substitution rate estimates – different Beast calibrations comparisons

All six different Beast analyses gave the same results regarding how clock like our seven markers were, relaying both on the SD of the ULC distribution (ucld.stdev) and on the coefficient of variation (CofV) (Suppl. Table 4.2). As explained in the Methods section none of the markers exhibited a strict molecular clock behaviour (although 16S was close in all but the IP Exp, Suppl. Table 4.2). Nevertheless both mtDNA markers behaved securely under a relaxed molecular clock model (estimates below 1), and PK and c5070 were very close to such behaviour with the ucld.stdev parameter (estimates just above 1), but deviated more in the CofV parameter (Suppl. Table 4.2). As such we will not comment on the remaining three markers as they deviated too much from clock like behaviour (estimates above 1.5) in both parameters in all analyses (Suppl. Table 4.2).



**Figure IV.II-4.** Marker rate variation (mean rate and ucld.mean) among the different Beast analises, when compared to the IP LogN Beast run. Mean rate = number of substitutions per site divided by tree length, ucld.mean = mean of branch rates.

Differences in substitution rates for the four different markers, both in mean and ucld.mean rates, were consistent between the different Beast analyses (Fig. 3, Suppl. Table 4.2). The results for the UNI 1 hypothesis were very similar to those for the IP LogN (Fig. 3), with the IP Exp analysis giving slightly higher values, although not consistently. Both Cyp Exp and Mesobuthus Beast runs gave lower substitution rates values, around 60% of those for the IP LogN (Fig. 3).



**Figure IV.II-5.** Marker rate variation (mean rate and ucld.mean) among the Beast analises, when compared to the 16S mtDNA marker. Mean rate = number of substitutions per site divided by tree length, ucld.mean = mean of branch rates.

The fastest evolving markers in all Beast runs were those of the mtDNA, with the 16S marker evolving, on average, 20% faster (mean rate) than the *cox1* marker (Fig. 4, Suppl. Table 4.2). The nuclear markers were found to evolve at just 20% (c5070) to 6% (PK) of the 16S mtDNA mean substitution rate (Fig. 4), on average. These values were very similar for the ucld.mean estimates, although these gave on average slightly faster estimates (Fig. 4, Suppl. Table 4.2).

## 3.2.3 Divergence time estimates – different Beast calibrations comparisons

As expected we obtained very different divergence estimates when comparing four of the six calibration models used. We selected 10 nodes to compare the results (Table 8). For placement of the nodes on the tree see Fig. 7.

**Table IV.II-8.** Selected 10 nodes of the tree depicted in Fig. 7, with explanation on their placement within the tree. Age – Node age represented in the tree; 95% HPD – 95% highest posterior density interval; Mean Age – Mean of the node age estimated from the 95% HPD.

Explanation	Node	Age	Mean Age	95% HPD
Buthus / Androctonus split	1	9.746	11.981	(8.781–15.18)
(Buthus): crown age	2	6.701	7.943	(6.351–9.534)
( <i>Buthus</i> ): rochati / mardochei groups split	3	4.560	5.238	(3.810–6.666)
(rochati): B. bonito / B. draa split	4	2.705	3.284	(2.250-4.319)
( <i>tunetanus</i> ): Algerian / all other samples split	5	5.486	5.805	(4.625–6.985)
( <i>tunetanus</i> ): Tunisia / East samples split	6	3.850	3.321	(2.418–4.225)
( <i>tunetanus</i> ): Egypt / (Israel+Cyprus) split	7	2.280	2.775	(1.888–3.662)
(tunetanus): Israel / Cyprus split	8	1.234	1.244	(0.664–1.823)
( <i>occitanus</i> ): Iberian / Moroccan samples split	9	5.373	6.008	(5.291–6.725)
(occitanus): <i>B. confluens /</i> other Moroccan samples split	10	4.489	4.885	(4.128–5.641)

We obtained similar estimates with both Iberian Peninsula calibrations (Exp giving 4% younger divergences in mean estimated ages comparing to the LogN, after removing nodes 7 and 8 that were much younger in the first) (Fig. 5A, Table 9). The UNI 1 calibration gave also similar results, with on average 9% older divergence times (Fig. 5A, Table 9). The other three calibrations gave much older branching's in the trees, with *Mesobuthus* producing consistently the oldest branching's (110% older on average than the LogN), followed by the Cyp Exp (60% older on average than the LogN) and UNI2 (30% older on average than the LogN) (Fig. 5A, Table 9).

Also, when analysing the 95% highest posterior density (HPD) interval, the IP LogN calibration obtained the least uncertain mean age estimations, consistently producing the smallest 95% HPD intervals per node (Fig. 5B, Tables 8 and 9). The analysis that produced the most unreliable estimates was that of the Mesobuthus calibration (Fig. 5B, Table 9).



**Figure IV.II-6.** Selected Node age estimates according to the six scenarios ran in Beast. A – Mean node age (in Ma), B – Variance in node age estimation represented by the 95% highest posterior density interval estimate. To represent all Beast analyses in B, we plotted a single point value for each 95% HPD interval estimate that represents the maximum time span for each node placement (in My), resulting from subtracting the maximum from the minimum value within the 95% interval estimate. For the placement of the nodes on the tree see Fig. 7.

**Table IV.II-9.** Node age values obtained with the different Beast analyses using the other 5 calibrations. Represented is the Mean age and the 95% highest posterior density interval. n.a. – node not recovered in the analysis; \* – node with BPP < 95%.

		<b>,</b> ,			
Node	IP Exp	UNI 1	UNI 2	Сур Ехр	Mesobuthus
1	13.384 (8.582-18.186)	12.82 (6.056-19.584)	15.948 (6.860-25.035)	20.016 (13.256-26.775)	24.948 (10.511-39.385)
2	8.377 (6.127-10.626)	8.567 (4.249-12.885)	10.708 (5.114-16.301)	12.544 (8.968-16.120)	16.532 (7.139-25.926)
3	4.332 (2.577-6.087)	5.643 (2.531-8.754)	6.736 (2.988-10.484)	8.244 (4.930-11.557)	11.03 (4.630–17.429)
4	2.961 (1.673-4.249)	3.653 (1.650-5.656)	4.251 (1.802-6.699)	5.158 (3.070-7.246)	7.010 (2.800–11.219)
5	5.174 (3.893-6.455)	6.397 (3.119-9.674)	7.533 (3.574–11.492)	9.356 (7.050-11.661)	12.335 (5.371-19.300)
6	n.a.	3.599 (1.639-5.560)	4.779 (2.247-7.311)	n.a.	6.932 (2.871-10.992)
7	1.325 (0.779–1.871)	3.016 (1.295-4.736)	n.a.	n.a.	5.883 (2.377-9.389)
8	0.721 (0.351-1.091)	1.376 (0.502-2.250)	2.969* (1.232-4.706)	5.420* (5.330-5.510)	2.695 (0.962-4.428)
9	6.200 (5.321-7.078)	6.384 (3.175-9.592)	7.510 (3.477–11.543)	8.96 (6.255-11.665)	11.767 (5.083-18.451)
10	4.686 (3.786-5.586)	5.442 (2.657-8.227)	6.575 (3.085-10.065)	7.838 (5.279-10.396)	10.437 (4.437-16.436)

#### 3.2.4 Buthus diversification - timings and biogeographic scenarios

For the reasons explained above we will focus mainly on the results from the Beast analysis with the IP LogN calibration.

Buthus diverged from Androctonus during the Miocene epoch, sometime between the middle to later Miocene. The divergence between the five main Buthus groups occurred not much later, during the Tortonian or early Messinian. It appears to have been a fast process, although the uncertainty in the placement of the boumalenii group precludes a clearer assessment. Species divergence within each group probably started during the Pliocene, for both the occitanus and tunetanus groups, and later, Pleistocene splitting's for the mardochei and rochati groups. Nevertheless, the latter groups have a smaller sampling which make comparisons difficult. With this calibration, the colonization of the Cyprus Island, at around 1.2 Ma, implies a dispersal through water event, which might have happened even later as we have a poor sampling of the Levant region (Israel, Palestine, Lebanon), were Buthus are known to occur.



**Figure IV.II-7.** Ultrametric Beast tree of the IP LogN calibration. Well supported clades are marked with a black circle. Node age estimates are given and the 95% HPD intervals are represented as blue bars. Global climate change from Miocene to the present, based on deep-sea benthic foraminiferal oxygen-isotope, is represented below the time chart (modified from Zachos et al., 2008). Significant Tectonic and Climatic events are also represented: Aridification? – putative start of arid conditions in Western Palearctic; MCOp – Miocene Climatic Optimum; MMCtr – Middle Miocene Climatic Transition; LMcol – Late Miocene cooling; MSC – Messinian Salinity Crisis; Medit. Climate – putative onset of Mediterranean climate in the Mediterranean basin.

Both Beast analyses with the Cyp Exp and Mesobuthus calibrations arrive at much older splitting times, as stated previously. Both analyses predict a *Buthus/Androctonus* split that occurred early in the Miocene or even in the later Eocene. In the case of the

*Mesobuthus* calibration, it requires two dispersal over water events, one for the colonization of Europe and the other for Cyprus, as neither of these dispersal events coincide with known land-bridges that could have connected Africa with Europe or Cyprus with mainland Anatolia.

The same conclusion holds for the colonization of Europe in the Cyp Exp calibration. However, as this Beast analysis required *a priori* that the colonization of Cyprus occurred during the MSC, the resulting tree, unique to this analysis, placed the Cyprus clade at the root of the Tunisian plus Eastern *Buthus* samples (not shown).

## 4. Discussion

#### 4.1. Divergence time estimates

As explained in the Methods section, we followed the easy and quick approach of Peng et al. (2006) to test how clock-like the molecular markers we were using behaved along the reconstructed phylogeny, using the values of both ucld.stdev and CofV of preliminary relaxed clock model analyses. Although none was found to exhibit a strict molecular clock behaviour, both mtDNA markers and two nuclear markers behaviour could be modelled with a relaxed molecular clock (Suppl. Table 4.2).

**Table IV.II-10.** Divergence time estimates of the four molecular markers found to have a relaxed molecular clock behaviour, for the six differently calibrated Beast analyses. Values are given per million years (My<sup>-1</sup>) divergences.

	16S	cox1	c5070	PK
IP LogN	3.22%	2.70%	0.68%	0.21%
IP Exp	3.74%	2.96%	0.64%	0.22%
UNI 1	3.34%	2.82%	0.71%	0.22%
UNI 2	2.90%	2.36%	0.58%	0.18%
Сур Ехр	2.02%	1.71%	0.42%	0.13%
Mesobuthus	1.82%	1.50%	0.38%	0.12%

The 16S marker was found to be on average 20% faster than the *cox1* (16S: 3.22% My<sup>-1</sup>; *cox1*: 2.70% My<sup>-1</sup>) (Fig. 3, Table 10), and remarkably similar results had already been reported for the Western Mediterranean *Buthus* by Gantenbein and Largiadèr (2003) (16S: 3.1% My<sup>-1</sup>; *cox1*: 2.7% My<sup>-1</sup>). We opted not to use this estimate because it was based on the same time calibration, the MSC, which we were keen to use with our extended dataset. These results differ markedly from other estimates, which have found on average faster *cox1* than 16S divergence times (e.g. Papadopoulou et al., 2010: 16S – 1.06% My-1; cox1 – 3.54% My-1). We have no clear explanation for these differences,

but perhaps differential trimming of poorly aligned regions of the 16S marker might explain these differences. Moreover, both nuclear markers were found to evolve much slower (Table 10), with the protein coding PK marker, as expected, having the slowest divergence time estimated (Fig. 3, Table 10). We had previously determined that the PK marker is much more variable in *Mesobuthus* (Sousa et al., in prep 1) and will provide further biogeographical context below.

Furthermore, from our analyses it was also clear that placing a prior on the stem of the tmrca (Beast analyses IP LogN and IP Exp) produced more informative node age estimates (with smaller 95% HPD intervals per node, Fig. 5B and Tables 8, 9), when compared to those were the prior was used at the mtDNA markers substitution rates (Beast analyses UNI1, UNI 2 and *Mesobuthus*). The only exception was that of Beast Cyp Exp analysis, which despite having a tmrca prior, produced 95% HPD intervals per node comparable to the three Beast analyses that gave the largest intervals.

## 4.2 Mesobuthus substitution-rate calibration

The calibration of the Beast analyses with both the Cyp Exp and *Mesobuthus* calibration wielded much slower mean rates of variation, almost 50% slower (Fig. 2), and thus much lower divergence times (Table 10). This difference has been addressed by others (Gantenbein and Largiader, 2003; Parmakelis et al., 2006a; b), albeit with different reasoning's. We believe it is important to readdress this here because what we have called the Mesobuthus clock (Gantenbein and Largiadèr, 2002; Gantenbein et al., 2005) (Divergence times: 16S - 1.03% My<sup>-1</sup>; cox1 - 1.14% My<sup>-1</sup>, Table 3) is the most used mtDNA substitution rate to generate time-calibrated phylogenies within Scorpiones when no other information is available (Bryson Jr et al., 2013, 2016, Graham et al., 2013a; b; Luna-Ramirez et al., 2017) on the assumption that it is a robust calibration based in four different points. The issue was raised by Gantenbein and Largiadèr (2003), given the differences we reported above, the authors gave as possible explanations differences in effective population size, or that the separation of the Iberian lineages pre-dated the MSC. Parmakelis et al. (2006b), studying again Aegean Mesobuthus, stated that this rate was derived partially from an incorrectly calibrated divergence between Crete and South Anatolian Mesobuthus. These authors reasoned that the possible last connection between these two areas was during the formation of the mid-Aegean trench (12 - 9)Ma), in concordance with other works (e.g. Papadopoulou et al., 2010; Lymberakis and Poulakakis, 2010; Poulakakis et al., 2015). Although the MSC, used to calibrate the Mesobuthus mtDNA rate could indeed, at least theoretically, have promoted a land connection between both areas (Papadopoulou et al., 2010), it required the crossing of large Evaporite deposits (see Fig. 1 of Poulakakis et al., 2015). Nevertheless this calibration was actually not used by Gantenbein and Largiadèr (2002), as the genetic distance between Crete and South Anatolian did not agree with the three other calibration points these authors used (Fig. 3 of their work), which they attributed to homoplasy. Parmakelis et al. (2006b) produced a different, time-calibrated phylogeny, although difficult to interpret because their calibrated nodes lack support. Nevertheless the authors indeed found two well supported divergent clades in each side of the mid-Aegean trench, which does support an earlier split before the MSC. Furthermore, the authors placed the split between *M. gibbosus* and *M. cyprius* to have occurred around 15 Ma, while Shi et al. (2013), using a Central Asia centred dataset, placed *M. cyprius* inside a paraphyletic *M. gibbosus* clade which started diverging only 6.94 Ma (95% HPD: 5.34 - 8.68), although these authors did use a second calibration constrain at the root of the *Mesobuthus* genus that again renders conclusion difficult. It would be interesting to use the separation of Cyprus as a second calibration point for the data of Parmakelis et al. (2006b), together with modern Bayesian inference tools to test if divergence times would still conform with ours.

Largely ignored is the fact that Gantenbein et al. (2003), using precisely the separation of Cyprus from Anatolia did reach a divergence time for *Mesobuthus* much faster than they had previously reported (16S - 3.40% My<sup>-1</sup>). For these reasons we emphasize that researchers should use caution when employing what we called the *Mesobuthus* calibration rate, which yields much older divergence dates. We will further explain why this older divergence time does not fit the biogeographical scenario for *Buthus* diversification below.

#### 4.3 Biogeographic scenario for Buthus diversification – timings and drivers

The biogeographic scenario that best explains our multilocus *Buthus* phylogeny places the centre of origin of the genus in Western North Africa, namely around the Atlas Mountains System that extend East-West from Morocco to Tunisia. It is probable that this centre was actually located in present day Morocco. We will expand this scenario below.

#### 4.3.1 Most parsimonious scenario for Buthus Mediterranean phylogeny

The present distribution of *Buthus* species (South-western Europe, North-Africa, Middle East, Cyprus and possibly Sicily) is difficult to explain without invoking dispersal across water, over the Mediterranean Sea. The only time when these regions were connect during the last 20 My was during the MSC, an event that lasted at most 600 Ky, from 5.971 to 5.33 Ma (Manzi et al., 2013; Roveri et al., 2014). During this event the

Mediterranean dried out almost completely, leaving large gypsum and other salt evaporites, along with other arid areas that would still be difficult to cross, especially over large distances. The route to arrive to South-western Europe opened precisely with the closing of the two "Betico-Rifian Portals" (Blanc, 2000) that connected the Mediterranean with the Atlantic Ocean. Furthermore, the route to Cyprus appears to have been through a northern connection with the Anatolian Peninsula (Plötner et al., 2010), and not through a connection with Palestine. For *Buthus* species to have used this single event to reach both South-western Europe and Cyprus during this limited time-window would require that the genus was already present in both North Morocco and Anatolia during this time.

Given the differences in pairwise distances between *Buthus* representatives of both sides of these two regions (Table 6), and given the existence of just a 2.9% uncorrected pairwise distance between *B. kunti* and *Buthus* sp. from Israel, we concluded that both colonization events could not be explained by the MSC without invoking unprecedented differences in mutation rates in the different *Buthus* species. As such we need to contrast the predictions of both our Beast analyses, for the arrival to Iberia or to Cyprus during the MSC. The slower substitution rates of the Cyprus hypothesis have the same problems that we have already identified for the *Mesobuthus* clock hypothesis. Furthermore, the tree of the Cyp Ex required this branch to be sister clade to the remaining *tunetanus* group, an unlikely biogeographical scenario. This is further exacerbated by the fact that *Buthus* has never been recorded north of Lebanon, much less in Anatolia, except for some erroneous past claims of its existence in Greece, almost certainly misidentifications of *Mesobuthus* or mislabelling of collection localities (Sousa et al., submited). As such we eliminate the hypothesis of dispersal over land for the colonization of Cyprus.

The remaining hypothesis, of a colonization of the Iberian Peninsula over land during the MSC, requires a colonization of the island of Cyprus to have occurred across water, over the Mediterranean Sea, during the Pleistocene at 1.23 Ma (95% HPD: 0.664 to – 1.823 Ma) (Fig. 9). During the Pleistocene Mediterranean Sea levels did fall when glaciers peaked, thereby diminishing the distance to the mainland. The colonization of Cyprus by several animal taxa has been reviewed in Poulakakis et al. (2013), and our date of colonization fits well with those given in this work for what they have called "young colonizers", including species of both reptiles (*Acanthodactylus*) and amphibians (*Pelophylax* and *Hyla*), which were estimated to have reached Cyprus between 0.85 to 1.65 Ma. Moreover, the earlier arrival of *Buthus* to Cyprus, when compared to *Mesobuthus* that appears to have arrived during the MSC, agrees with the centres of origin of both genera, as *Mesobuthus* seems to be have originated earlier in Central Asia, and thus would have been able to be present in the Anatolia Peninsula during the MSC.

Such a feat seems unlikely for a younger *Buthus* genus, originating in western North-Africa. Further evidence for an older origin of *Mesobuthus* was presented by Sousa et al. (Paper 2). While comparing the variability found for the PK marker (a nuclear protein coding marker) they found it to be almost four times more variable in *Mesobuthus* than in *Buthus*. The alternative hypothesis would require a greatly accelerated rate of mutation in *Mesobuthus*.

#### 4.3.2 Buthus origins

There is still no resolved Buthidae phylogeny, but nevertheless some hypotheses have been presented to explain the distribution and morphological patterns observed in the largest and most widespread scorpion family. The current prefered hypothesis has been put forward by Fet et al. (2005) based on morphological characters, following the work of Vachon (1974, 1975). Fet et al. (2005) divided the Buthidae into six buthoid groups of genera, the Buthus' group being the largest in number of genera (39), composed "predominantly of arid-adapted Palearctic genera" with a very old origin, Laurasian to Pangean. The phylogenomic study of Sharma et al. (2015) provided strong support for most groups (although generic level sampling was scarce). Within group relationships are even less well understood. The molecular phylogeny of the Buthidae estimated by Fet et al. (2003), encompassing 17 genera and as such the largest to date, indicated a clade formed by Buthus, Androctonus and Leiurus, although support was low. This gave at least partial support to Vachon's (1952) morphological hypothesis of a close relationship between Buthus and Androctonus. Androctonus species' occur over most of North Africa (several desert adapted species), Middle East and reach the Indian sub-continent (Lourenço, 2005; Turiel, 2013), although eastern species appear adapted to more mesic type habitats. *Leiurus* has a similar distribution (though not reaching Iran), most known species are true desert specialist and the majority have been described from the Arabian Peninsula (Lowe et al., 2014). These authors suggested that Leiurus originated in North-eastern Africa, at a time when Arabia was still part of the continent. The origins of Androctonus are harder to infer, although the mtDNA phylogeny of several Androctonus species found them to be well supported even if inter-species relationships were unresolved (Coelho et al., 2014). Furthermore, of the 28 known species (Rein 2016), 66% occur in Africa and several Asian species are morphologically uniform (e.g. Kovařík and Ahmed 2013), all of which also suggests an African origin for the genus. In contrast to both genera, Buthus species have a clear preference to mesic habitats, only two taxa are known to be desert dwellers, its diversity extends much further south of the Sahel area in Africa and the genus has never been found east of Iraq.

Although we did not explicitly tested this hypothesis because we lacked a Leiurus terminal in our phylogeny, we did recovered, as predicted, a clade formed by Buthus and Androctonus with the two other genera, Mesobuthus (East Palearctic) and Compsobuthus, forming the root clade. The time interval for the split between Androctonus and Buthus in our time-calibrated phylogeny is wide, occurring any time between the Langhian to Tortonian epochs of the Miocene (Table 8, Fig. 7). Its upper limit fits well into what is known as the Middle Miocene Climatic Transition (MMCtr) (≈ 14.2 to 13.8 Ma at the end of the Langhian, Fig. 7). The MMCtr marked a trend towards a cooler climate, after a very long period of warmer, stable climate (Zachos et al., 2001; Hamon et al., 2013), and could have promoted precisely the origin of a more mesic adapted genus like Buthus. Alternatively the split from a common ancestor could have been promoted by vicariance related to tectonic changes happening in Morocco around this period, namely with the second stage of upheaval of the Atlas Mountains System (Missenard et al., 2006; Barbero et al., 2007). However relating these events with our phylogeny remains difficult because the timing of the uplift but also the mechanism that drove it remain open, with this second stage of uplift variously proposed to have started 20 Ma (Barbero et al., 2007, 2011), 15 Ma (Missenard et al., 2006; Babault et al., 2008) or even only 3 Ma (de Lamotte et al., 2008).

## 4.3.3 Buthus diversification, radiation-like?

Our multilocus *Buthus* phylogeny recovered the same five main groups that had already been identified with mtDNA alone (Table 5) (Sousa et al., 2012; Pedroso et al., 2013), although the relationships between the groups still remain partially unresolved.

Both ML and MrBayes BI recovered the *boumalenii* group as sister clade to the remaining *Buthus* groups, although that position was not recovered in any of the Beast analyses, perhaps due to the amount of missing data in the single terminal of the group in the phylogeny (Table 1). The placement of the *boumalenii* group has strong implication for the reconstruction of the biogeographic scenario of *Buthus* initial diversification (Fig. 7, 8). If it is indeed sister clade to all other groups, that would place the centre of origin of the genus further south, but more importantly it would imply first an East-West (*boumalenii* versus all other groups) split and then a secondary North-South split (*occitanus + tunetanus versus mardochei + rochati*), that would then suffer a tertiary East-West split again. An alternative explanation, recovered but without strong supported by the Beast analyses, involves a first North-South split (North groups: *occitanus + tunetanus*; South groups: *boumalenii + mardochei + rochati*) and then independent East-West splits within the North and South groups (Fig. 8).



**Figure IV.II-8.** Reconstructed scenario for the early phylogeny of the genus *Buthus*. We have highlighted the main geographical features discussed in the text. In this map the known distribution overlap (dashed areas) of the *occitanus* and *mardochei* groups is given with greater accuracy than in Fig. 1 and 2. Legend in accordance to Fig. 1.

Our results do suggest that the five groups originated during the Messinian (Fig. 6), an epoch also characterized by marked changes in global climate. These changes appear to have started at least by late Tortonian, and implied a lowering of global temperatures (Herbert et al., 2016). At around 7 Ma the aridification of North Africa appears to have also started, with the onset of the Sahara Desert (Schuster et al., 2006; Zhang et al., 2014) aided by the earlier shrinkage of the Tethys Sea exacerbated by the closure of the eastern seaway to the Indian Ocean (Hamon et al., 2013), and the early formation of glaciers in the Northern Hemisphere (Herbert et al., 2016). Nevertheless the onset of the Sahara is not consensual, with several authors advocating a much later date, at around 2.5 Ma (Kröpelin and Swezey, 2006). Vegetation reconstructions also present this period of Late Tortonian to Messian as an age when vegetation in North Africa changed from tropical to a prominence of grasslands and savannahs (Micheels et al., 2007) or xeric shrublands (Pound et al., 2012), with patches of desert, which fit in the habitat preferences of the majority of present day Buthus species. The uplift of the Atlas Mountains, although its timing is still surrounded by uncertain as explained previously, may have helped to promote the vicariant events that isolated the five *Buthus* groups,

and the High-Atlas could have functioned as an early barrier, either promoting a pattern of climatic vicariance or functioning as an earlier barrier once the split was produced, but they do not fit a scenario in which the High-Atlas was the primary agent of vicariance

There is no clear geographic barrier that could explain the early split between the *occitanus* – *tunetanus* groups (Fig. 1, 8), although they could be the western and eastern extant representatives of an ancestral population that extended to the entire northern mountains of the Atlas System, from the Middle-Atlas to the Tell-Atlas and the Aurès Mountains. This ancestral range could have contracted due to climatic changes surviving only in the mild climates provided by the mountains. This would also explain the early split between the Algerian samples from the rest of the *tunetanus* group, but it does not explain the lack of connection between both portions of the Tell-Atlas.

The same type of scenario could be applied to the other three groups, *boumalenii*, *mardochei* and *rochati* (Fig. 8). They would once have been part of a single ancient population that was separated with the worsening climatic conditions, which would have remained suitable only in mountainous areas. Moreover, the Anti-Atlas appears to be pivotal in the relationships between these three groups, and the region East of Jbel Sirwa, which connects the High and Anti-Atlas, could represent either a secondary contact point between the three groups, or an ancestral area of persistence. Moreover, if this initial diversification did occur fast in a geological time scale, resembling a radiation, this could help explaining the difficulties in reconstructing the deep phylogeny of the genus.

This scenario is not consistent with other published predictions (Lourenço and Duhem, 2009; Lourenço, 2013). These authors claim that Buthus is one of several scorpion genera that compose an assemblage, the "central compartment, which are distributed in the "core Saharan region", together with Androctonus, Buthacus Birula, 1908, Buthiscus Birula, 1905 and Leiurus. And these are in opposition with other genera that form an assemblage with a "peri-Saharan zone of distribution", of which the authors say Butheoloides Hirst, 1925 represent the better example. We argue that Buthus has clearly such a peri-Saharan distribution (Fig. 2), and although also present in arid habitats are much more common in mesic environments with a mediterranean climate. Vachon (1952, page 377) says much the same "Il semble donc que, pour ce genre [Buthus] tout au moins, les causes de la répartition spécifique soient différentes de celles qui ont provoqué la distribution des espèces dans le genre Androctonus". Furthermore, although we cannot exclude that it was present in North Africa during the last 10-15 My (Lourenço and Duhem, 2009; Lourenço, 2013), our work implies that Buthus diversification started later, during the Messinian (10 to 6 Ma, Fig. 7). Several speciation events in *Buthus* appear to be even younger (Fig. 7), during the Pliocene. Some of these events are Zanclean in age, when aridification seems to have been progressing at least in Morocco (Feddi et al., 2011) together with tectonic upheaval (de Lamotte et al., 2008). Several other speciation events appear to have occurred in the Piacenzian (3.6 to 2.58 Ma), an age when the cyclic patterns of drier summers and humid winters characteristic of the mediterranean climate were established (Suc, 1984; Jiménez-Moreno et al., 2010).

Moreover, our multilocus dataset allowed us to confirm not three (Gantenbein and Largiadèr, 2003) but five main groups (Pedroso et al., 2013) within Buthus with robust support. Additionally, it allowed us to understand for the first time the relationships between the five groups, as an old split appears to exist between the northern and southern groups, probably connected with the Atlas Mountains System. However our reconstructed timings imply that climatic changes and not geographic barriers (as implied by Habel et al., 2012), were the primary drivers of allopatric diversification and speciation in the genus. Notwithstanding, geographic barriers most certainly have acted to reinforce and probably provided more time for genetic divergence to ascertain itself thus reinforcing this pattern of allopatric speciation (Habel et al., 2012). Our limited sampling of the mardochei group does not allow us to discuss many of the conclusions in Husemann et al (2012) about diversification within B. elmoutaouakili. However B. elmoutaouakili is likely to be younger than 5 My as this is the time of splitting between the rochati and mardochei groups, and several other species compose the mardochei group. If any of these species were included in the analyses of Husemann et al (2012), and they did included animals from the northern slopes of the High-Atlas (their cluster E) where the species has never been reported before and would possibly imply a trans Atlas dispersal, then the inclusion of other species could explain the amount of genetic divergence they uncovered within what these authors considered as solely B. elmoutaouakili.

#### 4.3.4 Other biogeographic patterns within the Buthus mtDNA groups

The small range of the *boumalenii* group might result from the lack of free available habitat to the West, but does not explain its apparent lack of distribution east of the Oued Todgha, although in this case climatic conditions might be too arid for *Buthus* scorpions, a prediction that can be tested with Environmental niche-modelling. Furthermore, this seems to imply that for *Buthus* scorpions, rivers are not geographic barriers but are rather humid dispersal corridors through dry areas.

The southern distribution of the *occitanus* group in Morocco might also reflect an earlier adaptation to more humid habitats in the North of Morocco, which would explain their expansion to the High-Atlas in detriment of the *boumalenii* group but not of the

*mardochei* group, and also south along the Moroccan coast until Agadir, where they appear to avoid competition with the *mardochei* group adopting a psammophilous life style (*B. atlantis*). However few samples have been sequenced in the plain south of the Oum-er-Rbia River, so the actual contact area of the *occitanus* and *mardochei* groups is poorly understood (Fig. 8).

This observation also strengthens the prediction that the *mardochei* group species occupy the most mesic and humid habitats of the three southern *Buthus* groups, as the Souss River is hydrologically much more stable along its course than the Dra River (Babault et al., 2012). The distribution of this group gives further evidence that rivers do not act as barrier to *Buthus* scorpions.

As for the Sub-Saharan *Buthus* species phylogeny, it remains essentially unknown (except for *B. awashensis*), which connects it with the *tunetanus* group. Nevertheless there is morphological and geographical evidence that predicts that the *rochati* group phylogeny should also expand to that region of Africa. The latter group has the southernmost distribution in Morocco of the five, and some of its species share morphological traits with species found further south. For example *B. draa* shares the same coloration pattern, a darkened fifth metasoma segment and telson, with *B. tassili* and *B. nigrovesiculosus*, which is partially shared also by *B. mariefranceae*. However several of the Sub-Saharan *Buthus* species occur in much more humid habitats than those were we find species that certainly belong to the *rochati* group.

Clearly, sampling the western Sub-Saharan distribution of the genus to include in an enlarged molecular phylogeny is necessary to test these hypotheses.

## 4.3.5 Eastern Mediterranean dispersal

Our phylogeny suggests that this clade dispersed through the Mediterranean coast of North Africa (Fig. 7), possibly from Tunisian ancestrals, having crossed to the Sinai Peninsula and Palestine during the Pleistocene, 2.28 Ma (95% HPD: 1.888 to 3.662 Ma) (Fig. 9), at a time when the Suez Isthmus (5 My) was already in place (Bosworth et al., 2005).

The lack of sampling and support does not allow for a precise understanding of this clade (Fig. 7). Nevertheless, given that it was not responsible for the colonization of the rest of the Middle East, we might propose that specimens moved from Ethiopia to Jordan and not the other way around (Fig. 2). However this might imply a dispersal across water over the Red sea, given that the Bab al Mandeb Strait opened around 5 Ma, marking the end of the land connection between Arabia and Africa through this area (Bosworth et al., 2005; Fernandes et al., 2006). We have already established that *Buthus* must have

reached Cyprus over the Mediterranean Sea, so it is possible. Alternatively, a second wave of *Buthus* may have reached the Middle East through the Suez Isthmus, this time through the Red Sea coast of Egypt. This hypotheses can be tested when more samples are available; the first hypothesis predicts that *Buthus* from Yemen should be intermediary between Jordan and Ethiopian taxa, the second hypothesis implies that Jordan samples should be intermediary between Yemen and Ethiopian taxa. Nevertheless a crossing before the opening of the Bab al Mandeb Strait is not excluded by our analysis.



**Figure IV.II-9.** Map of *Buthus* sampled localities in the Eastern Western Mediterranean, with the *tunetanus* group distribution highlighted. For a full overview of the sampling see Fig. 2. Sampling numbers correspond to Table 1. Legend in accordance to Fig. 1.

Furthermore, the deep branching of the Jordan plus Ethiopia clade inside the *tunetanus* group suggests an early divergence from the remaining of the group. At this time (Messinian) the Sahara Desert was already present (but see above), and although its extent is unknown, periodical green Sahara phases have been identified dating back at least to 8 Ma (Larrasoaña et al., 2013). These green Sahara periods would have facilitated dispersal through the Sahara area from North Africa to what is now the Sahel region. Recently a large paleo-watershed, the Tamanrasett (Paillou et al., 2015), was identified that made a north-south connection through the Sahara area. Such systems would certainly have facilitated the dispersal of *Buthus*, and thus the colonization of the Sahel could have happened without the need of a circum-Sahara dispersal. Furthermore, several species of *Buthus* have persisted throughout the Sahara Desert in continental islands: Tassili n'Ajjer and the Hoggar Mountains in Algeria, the Ennedi Plateau in Chad

and Djebel Meidob in South Sudan. These species, if sampled, would provide invaluable information about the timing of dispersal over the Saharan area.

## 4.4 Insights for Buthus morphological diversification

One interesting result, supported strongly with our phylogeny, is that size changes in *Buthus* have happened independently several times in the past. There are several species of *Buthus* with small adult sizes, three of which occur in Morocco: *B. malhmomeni* (at least the males), *B. mariefranceae* and *B. rochati*. In our phylogeny the cladogenesis of the first species is clearly different form the latter two, and should therefore be explained by convergent evolution, possibly to prey items as the climate is very different in these species ranges. Although we have no sample of *B. mariefranceae* in our multilocus phylogeny, Pedroso et al. (2013) demonstrated that *B. mariefranceae* belongs to the same clade as *B. draa* even if their relationship is not completely understood. We did included samples of *B. draa* in our tree, which might imply that also within the *rochati* group, small size *Buthus* arose twice (*B. mariefranceae* and *B. rochati*), although this hypothesis need further testing.

## 4.5. Buthus Taxonomy

ABGD could not find a barcoding gap in our mtDNA Dt1, but we do not except this negative result to have been cause by the small number of sequences per species, the reason given by Puillandre et al. (2011) for the failure of ABGD to find a barcoding gap in the Agrodiaetus butterfly dataset they tested. Our 315 sequence dataset should include around 42 Buthus species (known to occur north of the Sahara Desert), giving an average of 7.5 sequences per species, well above the minimum number of five sequences they estimated with simulations. Another possibility is that this ABGD failure results from the amount of missing data, a compromise we assumed given the different origins of the Genbank sequences used, that were obtained using different cox1 primers. Nevertheless, a trimmed dataset of 556bp, well above the minimum 250bp proposed by Meusnier et al., (2008) to achieve a success rate of 95%, was also analysed in Mega v6.06 (with uncorrected pairwise, Kimura 2 parameter and Maximum Composite Likelihood distances, results not shown), also revealed no barcoding gap. In our opinion, the most parsimonious conclusion is that there is no barcoding gap in the Buthus cox1 fragment we tested, a result already suggested before in other groups (e.g. Meier et al., 2006; Wiemers and Fiedler, 2007), were the within species divergence overlaps with between species divergence (see Fontaneto et al., 2015 for a review).

The analyses of the reduced mtDNA Dt3 dataset proved more informative. Three "species" pairs comparisons are particularly problematic: *bonito-rochati*, *elmoutaouakili*-

parroti and kunti-"Israel", as they are all below the 3% divergence. Interestingly, all pairs have geographical close distributions. The lowest divergence (0.4%) was found for the first pair, which is even more meaningful because these species are among the few Buthus species that have clear morphological diagnostic traits (Sousa et al., Paper 1. For the second pair, it is possible that we might have misidentified *B. parroti*; although little is known about this species distribution it clearly overlaps with B. elmoutaouakili (Vachon, 1952). The third pair has been reviewed above. Two species were found to exhibit high levels of within intraspecific variability: B. lienhardi and B. mariefranceae. We further divided B. lienhardi sequences in two groups, east and west of the type locality (Oukaimeden, in the High-Atlas Mountains), and found that these two groups had a divergence of 9.6%, suggesting the existence of two divergent mtDNA lineages within B. lienhardi. High levels of divergence between High-Atlas taxa have been reported before (e.g. Barata et al., 2012; Harris et al., 2017). The case of *B. mariefranceae* is even more complex, as identified before (Sousa et al., 2012), because it also involves B. draa (7.1% divergence), again two of the few Buthus species with clear morphological diagnostic traits (Sousa et al., Paper 1. Nevertheless, we also divided the 3 sequences in two groups (again east-west) and again obtained a divergence of 8.6%, again highlighting the potential existence of cryptic diversity within *B. mariefranceae*, although we cannot at this point identify a clear geographical pattern to it.

We found another unexpected result in our reduced dataset analysis, with the pair B. malhommei-B. maroccanus. We had originally included two sequences (Sc0180 and Sc2757, both collected close to the species' type locality) as B. malhommei, again a species easy to identify due to its small male adult size, sturdy pedipalp pincers and distribution along much of the Oum Er-Rbia river basin (Touloun, 2012). Nevertheless their divergence was high (6.5%), especially when we compared them with the sole B. maroccanus representative (Sc0180/Sc0174, 7% and Sc2757/Sc0174, 1.9%). B. maroccanus although superficially different from B. malhommei, with adult representatives of normal size and a body coloration that can be totally black, shares other important resemblances with *B. malhommei*. Both species share the most sturdy pedipalp pincers of all Buthus species, with no appreciable sexual dimorphism (pedipalp chela length to width ratio less than 2.5 in males and less than 2.7 in females in both species, following measurement data from the species original descriptions, Fig. 10), without clear sexual dimorphism in this character (see Sousa et al., submitted for a review on Buthus diagnostic characters). Their distributions, although apparently not overlapping, are close, as *B. maroccanus* occurs around Rabat. This led us to conclude that specimen Sc2757 is probably a juvenile of the lighter colour morph *B. maroccanus*,

which raises the hypothesis that this species has been overlooked and might indeed have a wider distribution, one that actually overlaps with *B. malhommei*.



**Figure IV.II-10.** Relation between Pedipalp Chela length to width ratio and adult body size. We present data from species descriptions, for a total of 30 species for which data for both females and male is available. In each chart species were ordered by size (for more details on pooled data see Sousa *et al.*, Paper 1.

We do not propose the 8% break (Fig. 3B) as a workable barcoding gap because this value is likely too high to have biological meaning given that we are working within a recent, homogenous genus, and is probably a result of the compromise with dataset size in order to have probable species identification. The 8% drop is probably influenced by the reduced amount of data used. Furthermore our results do not support the hypotheses of the 10X rule proposed by Hebert et al., (2004), because we do not observe a gap that according to this rule should be around the 0.85%-1% threshold for the intraspecific divergence (assuming a interspecific divergence around 8.5% to 10%), and we have several homogeneous species with around 3% intraspecific divergence (Table 6).

## 5. Conclusions

Our *cox1* analysis raises questions about the validity of *Buthus bonito*, as it is genetically very similar to *B. rochati*, and is a paraphyletic clade regarding the placement of the latter species (Fig. 7). These species form an old split within the *rochati* clade, but present a very shallow recent cladogenesis. *B. lienhardi* taxonomy is also problematic, with two divergent mtDNA groups occupying the known distribution of this species (Fig. 8).

It is possible that mitochondrial introgression might play a role in some of the unexpected patterns observed. This has been discovered in other species where the mitochondria seems to confer selective advantages related to temperature energetics (Alves et al., 2006; Toews and Brelsford, 2012) and the upper portions of the High-Atlas are a climatic challenge for *Buthus* scorpions. We now have the tools, several variable nuclear markers, to investigate this possibility.

The same North – South of the Atlas Mountains pattern of divergence has been uncovered many times (e.g. Fritz et al., 2005; Terrab et al., 2006), although in several examples this pattern was observed within older splits either in invertebrates such as *Ummidia* (Opatova et al., 2016) and *Trechus* (Faille et al., 2014) and in vertebrates including *Tarentola* (Rato et al., 2012) and *Acanthodactylus* (Tamar et al., 2016a). Nevertheless approximately similar divergence times (early diversification and posterior speciation) have also been reported in *Potomida* (Froufe *et al.*, 2016) and *Lycosa* (Planas et al., 2013) for example.

Regarding specific patterns within the *tunetanus* group, several concordant biogeographical histories have also been published. Dispersal through the Sahara has also been found in other taxa, also uncovering multiple dispersal events through the area in *Acanthodactylus* (Tamar et al., 2016a), *Chalcides* (Carranza et al., 2008) and *Stenodactylus* (Metallinou et al., 2012). Rato et al. (2007) also found a similar pattern where Algerian samples of *Psammophis* snakes were divergent from the rest of the species diversity. Furthermore the timings of a possible route predating the opening of the Bab al Mandeb Strait for *Buthus* to reach the Arabian Peninsula would be similar to what has been uncovered recently for several reptile taxa including *Phoenicolacerta* (Tamar et al., 2015), *Pseudotrapelus* (Tamar et al., 2016b), *Stenodactylus* (Metallinou et al., 2012) and *Trachydactylus* (de Pous et al., 2016), although in these cases older dispersals were also uncovered linked to older persistence times in the east area of North-Africa.

The biogeographic scenario that we uncovered fits particularly well the one published by Carranza et al. (2008) for *Chalcides* skinks. Our results support the MSC splitting of Iberia from North Morocco *Buthus* scorpions, and a much younger dispersal over water of *B. kunti* (our it's ancestrals) to Cyprus from Palestine. The latter is not uncommon in several animal and even plant taxa, but is certainly a remarkable achievement for animals that have always been regarded as having low dispersal capabities (Polis et al., 1985).

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# Chapter V IBERIAN BUTHUS - DIVERSITY,

# **DISTRIBUTION AND PHYLOGEOGRAPHY**

#### Paper 5

Sousa, P., Harris, D. J., Vale, C., Alves, P. C. and Arnedo, M. A. (in preparation). Pliocene tectonics and not Pleistocene glacial oscillations as the main driver of Iberian *Buthus* speciation (Buthidae, Scorpiones) [This page intentionally left blank]

PAPER 5: Pliocene tectonics and not Pleistocene glacial oscillations as the main driver of Iberian *Buthus* speciation (Buthidae, Scorpiones)

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# Abstract

We assessed the Iberian Peninsula Buthus scorpion Systematics using a multilocus approach (2 mtDNA and 5 nuDNA markers). We used the Messinian Salinity Crisis as a biogeographical calibration in a Bayesian Inference framework, as it proved the most parsimonious vicariant event to separate the Iberian clade from the North-African Buthus. Our calibrated phylogeny allowed us to confidently confirm that the Iberian Peninsula was colonised only once from North Morocco. We used a discovery/validation approach to species delimitation. The multispecies coalescent model applied in BP&P recovered seven well supported species, two of which are new. This species all originated during the Pliocene, suggesting that tectonic and orogenic events were the main drivers of speciation. Our extensive sampling of the Iberian Peninsula uncovered a complex phylogeographic pattern mostly originated during the Pleistocene. The South-eastern Iberian region was found to be the most diverse, possibly reflecting the complex geological history of the region since similar patterns have been observed in many other taxa from this area. The persistency patterns uncovered with ENMs provide some support for the involvement of Quaternary glaciations in shaping intraspecific diversification. Current taxonomic keys are of limited use in identifying evolutionary relevant lineages, and a complete morphological reassessment is needed prior to a full formal taxonomic revision. However we can already propose, based on well supported genetic evidence and on limited morphological evidence, the removal of Buthus halius (C. L. Koch, 1839) (n. stat.) from synonymy with *B. ibericus* Lourenço & Vachon, 2004, and, consequently, to have the nomen oblitum status of the former annulled.

# Keywords:

Phylogeny, Phylogeography, Beast, Messinian Salinity Crisis, Calibration, multilocus, Species delimitation, E.N.M., L.I.G., L.G.M., *Buthus* halius, new status

# 1. Introduction

Species are the fundamental units of Biology. Yet defining species have remained for long a seemingly intractable problem, prone to multiple and heatedly debated solutions. The problem seems to have subsided in recent years, probably as a result of the decoupling between the discussion on the theoretical definition of species and the operational criteria used to delimit them in nature. Under the modern evolutionary definition of species, *i.e.* independently evolving metapopulations (de Queiroz, 2007; Mayden, 1997), the plethora of species concepts available in the literature are better viewed as operational criteria to establish the species boundaries (de Queiroz, 2007; Mayden, 1997; Zachos, 2016). These criteria relate to the important and practical problems of species delimitation and how speciation works (The Marie Curie Speciation Network et al., 2012), and probably explain why this field has gone through a renaissance in the past decade (Camargo and Sites, 2013; Carstens et al., 2013; Fontaneto et al., 2015; Rannala, 2015). As part of the renewed interest for quantitative delimitation of species, new methods have been developed for facilitating and automatizing species identification, based on single-locus, *i.e.* DNA Barcoding (Frézal and Leblois, 2008; Hebert et al., 2003). However, the promise of Taxonomy made easy (Hebert et al., 2004) also came with pitfalls and shortcomings (Moritz and Cicero, 2004; Rubinoff et al., 2006). Recently Flot (2015) offered a review of the currently available species delimitation methods (SDM), grouping them into three categories: distance-based, tree-based, and allele sharing-based, although recognising that some methods are difficult to fit into any single category. The recent development of multiple-species coalescent algorithms have greatly facilitated the integration of information from multiple molecular markers for the statistical assessment of species limits (e.g. Jones et al., 2015; Rannala and Yang, 2017; Yang and Rannala, 2010; but see Sukumaran and Knowles, 2017). Moreover, several authors have advocated for an integrative approach to species delimitation that combines multiple lines of evidence and not only molecular data to gain more resolving power (e.g. Barrett and Freudenstein, 2011; Fujita et al., 2012; Miralles and Vences, 2013; Padial et al., 2010).

The Iberian Peninsula is a hotspot for European biodiversity (Mittermeier et al., 2004; Myers et al., 2000), having acted as a refugium during the Pleistocene glaciations (e.g. Miraldo et al. 2011; Hewitt, 2011). Indeed, a complex model of "refugia within refugia "(Gómez and Lunt, 2007) driven by Pleistocene glaciations has been proposed to explain the complex patterns of genetic variation found in Iberian species. The complex geology of south-east Iberia, including Miocene connections with North Africa (Husemann et al., 2014), and the orogeny of the Betic mountain range, a region of

particularly high biodiversity (Rodríguez-Sánchez et al., 2008), has further contributed to the generation of such complex patterns.

Molecular tools and dating methods allow discerning between alternative evolutionary scenarios and have been widely applied across organisms to untangle the relative role of, for example, Pleistocene climate oscillations and Miocene tectonics in driving diversification of Mediterranean organisms (Husemann et al., 2014; Médail and Diadema, 2009).

Scorpions are ancient animals, with a fossil record that dates back to the late Silurian (428 Ma) (Whitfield, 1885), which combined with their poor dispersal ability, makes them good models for exploring biogeographic patterns and evolutionary history. Scorpions have been used as model organisms to investigate diversity patterns within the Iberian Peninsula and identify connections to North Africa. The Iberian Peninsula scorpion fauna comprises three scorpion genera, namely *Belisarius* Simon, 1879 a genus with a relictual distribution (Lourenço, 2015), *Euscorpius* Thorell, 1876, a widespread genus in Europe but with a single representative in Iberia, and the diverse genus *Buthus* Leach 1815 (Sousa et al., Paper 1.

Buthus is a member of the Buthidae C.L.Koch, 1837, the largest scorpion family. Knowledge of the diversity and distribution of the genus has improved remarkably in recent years, with more than three quarters of the 52 known species described in the past dozen years (Sousa et al., Paper 1. Although the bulk of the diversity, 30 species, is circumscribed in the Western Mediterranean area, the genus can be found in semiarid areas across North Africa, the Middle East, and the Iberian Peninsula extending up to southern France. Two island endemic species are known, one from Cyprus (Yağmur et al., 2011) and another from Sicily (Lourenço and Rossi, 2013). In North Africa, Buthus distribution can be roughly defined as peri-Saharan, although many species are only known from their type locality. Notably, some species have been found south of the Sahel region (e.*g. B. jianxinae* Lourenço, 2005; *B. prudenti* Lourenço & Leguin, 2012). Furthermore, although they can be locally abundant, the ecology of most species of the genus remains essentially unknown (but see Skutelsky 1995; Piñero, Tenorio & García 2013). Buthus scorpions are of medical importance, some North African species are highly venomous and are responsible for serious medical conditions (Abourazzak et al., 2009). In the Iberian Peninsula, the genus has recently undergone major taxonomic changes. For more than two centuries only one species was known to occur, B. occitanus (Amoreux, 1789), but in the last years three new species have been described. B. ibericus Lourenço & Vachon, 2004, and B. montanus Lourenço & Vachon, 2004 were described solely based on morphological characters. Gantenbein and Largiader (2003) using mitochondrial (mtDNA) and nuclear DNA (nuDNA) markers, identified three clades

in the Western Mediterranean: a mostly European, an Atlas (Moroccan) and a Tell-Atlas (Tunisian). The European clade could be further divided into three groups, based on allozyme data (Gantenbein, 2004). Sousa et al. (2010) using a broader geographic sampling of the Iberian Peninsula and cox1 mtDNA data, uncovered two additional clades, revealing a more complex evolutionary history than previously reported. In 2012 another Iberian endemic, B. elongatus Rossi, 2012 was described, based on morphological characters but referring to the mitochondrial phylogeographic assessment of Sousa et al (2010) as additional evidence. Sousa et al. (2012), again based on the cox1 marker, but relying on a wider sampling across the Maghreb, uncovered a much higher level of genetic diversity in Morocco. Sousa et al. (2012) identified four main groups within the *Buthus* genus, three of which were exclusive to Morocco. The fourth group joined individuals from the Iberian Peninsula, Algeria and Tunisia with Moroccan clades north of the High-Atlas Mountains, thus revealing a new biogeographic pattern in the genus. A similar relationship was observed by Habel et al. (2012), although these authors focused their geographic sampling in the central region of Morocco. All recent authors reached a similar conclusion regarding the current taxonomy of the genus, indicating that it requires a thorough revision since the existing identification keys are difficult to use, have been rendered incomplete with the pace of species description, and there seems to be a lack of diagnostic traits for some forms. Different scenarios for the colonisation of Iberia from North Africa have been proposed (Habel et al., 2012; Sousa et al., 2012) based on mtDNA alone.

In the present study we combined mitochondrial and nuclear DNA sequence information from an extensive sampling over the entire distribution range of *Buthus* in the Iberian Peninsula and Ecological niche-based models to reveal the evolutionary history of the group and identify the drivers of diversification. Specifically, we aim to: i) test former phylogeographical hypothesis based on mtDNA; ii) infer a timeframe for the diversification of the genus in the region; iii) test the current taxonomy of the Iberian *Buthus*; iv) reconstruct the number of colonization event(s) that brought *Buthus* to Europe; v) evaluate the role of Pleistocene glaciations in driving phylogeographic structure of Iberian *Buthus* and vi) improve the knowledge on the distribution of Iberian species.

# 2. Materials and methods

## 2.1. Data collection

We sampled 121 new locations from the known range of *Buthus* in Europe: the Iberian Peninsula and the South of France, for a total of 239 specimens (Map 1; Table 1). Our sampling included the type locality of *B. ibericus* and *B. elongatus* and nearby localities to the topolocality of *B. montanus*. We also sampled eight new locations in the Tingitana Peninsula and the Rif Mountains in the North of Morocco, including the type locality of *B. confluens*, for a total of 14 specimens (Fig 1; Table 1). Additional mtDNA sequences from scorpions from the Iberian Peninsula and the North of Morocco (39 and seven sequences, respectively) available in GenBank from the works of Gantenbein and Largiadèr (2003), Froufe et al. (2008), Sousa et al. (2012, 2011, 2010) and Pedroso et al. (2013) were also included. We used 3 new *Buthus* specimens from Morocco belonging to mtDNA lineages *mardochei* and *occitanus* (sensu Sousa et al., Paper 1 as outgroups (Table 1).



**Figure V-1.** Map of *Buthus* sampled localities in the present study. Localities numbered in the main dataset according to Table 1. Specimens Sc1548 and Sc1568 from the Anti-Atlas region of Morocco are not represented on the map. Details on all other localities can be found in the Suppl. Table 4.1. The general distribution of the lineages identified in the mtDNA tree here inferred, was constructed using smoothed minimum convex polygons in ArcGIS 10.1,

Although challenging (*e.g.* Habel et al. 2012; Sousa et al. 2012), we made an effort to identified (by PS) all specimens to species level following available keys (Lourenço, 2003; Lourenço et al., 2012; Lourenço and Vachon, 2004; Rossi, 2012; Teruel and Melic, 2015; Vachon, 1952), All specimens are deposited at the collection of CIBIO-UP, Centro de Investigação em Biodiversidade e Recursos Genéticos, Vairão, Universidade do Porto, Vila do Conde, Portugal.

**Table V-1.** List of specimens sequenced for the main dataset used. Countries are listed as Fr. – France, Mo. – Morocco, Pt. – Portugal, and Sp. – Spain. Locality number (Lc.) correspond to the codes used in Figure 1. Geographic coordinates are in the WGS84. GenBank accession codes are organized as follows: cox1, 16S, PK, c0971, c0061, c5070, and 28S.

Code	Lineage	Ctr.	Locality	Lc.	Lat.	Long.	Alt.	GenBank
Sc1752	B. elongatus	Sp.	Igualeja	1	36,66	-5,10	1096	-
Sc2100	B. elongatus	Sp.	Marbella	2	36,53	-4,90	150	
Sc2427	B. elongatus	Sp.	Estepona	3	36,44	-5,16	65	
Sc1100	B. ibericus	Sp.	Darkata Dara	4	36,39	-5,65	50	
Sc1101	B. ibericus	Sp.	Barbale Dam	4	36,39	-5,65	50	
Sc1103	B. ibericus	Sp.	Alcalá de los Gazules	5	36,52	-5,66	403	
Sc1107	B. ibericus	Sp.	Ubrique	6	36,68	-5,42	730	
Sc1110	B. ibericus	Sp.	Antequera	7	36,98	-4,66	554	
Sc1722	B. ibericus	Sp.	Guadalcacín Dam	8	36,62	-5,67	185	
Sc1732	B. ibericus	Sp.	Arcos de la Frontera	9	36,76	-5,81	137	
Sc1125	B. montanus	Sp.	Francisco Abellán Dam	10	37,28	-3,25	1080	
Sc1127	B. montanus	Sp.		11	37,18	-3,06	1235	
Sc1129	B. montanus	Sp.	La Calahorra	11	37,18	-3,06	1235	
Sc1653	B. montanus	Sp.		11	37,18	-3,06	1235	
Sc1590	B. montanus	Sp.	Postero Alto shelter	12	37,13	-3,21	2135	
Sc1591	B. montanus	Sp.		12	37,13	-3,21	2135	
Sc1597	B. montanus	Sp.	Puerto de la Ragua	13	37,11	-3,00	2194	
Sc1612	B. montanus	Sp.	Laguna Seca	14	37,09	-2,97	2270	
Sc1771	B. montanus	Sp.	Baza semi-desert	16	37,54	-2,70	746	
Sc1795	B. montanus	Sp.	Los Navarros	17	37,13	-2,59	810	
Sc1799	B. montanus	Sp.	Aulago	18	37,17	-2,63	1156	
Sc1843	B. occitanus	Sp.	Millars river mouth	19	39,92	-0,02	1	
Sc1844	B. occitanus	Sp.		19	39,92	-0,02	1	
Sc2001	B. occitanus	Sp.	Avinyó Nou	20	41,36	1,79	304	
Sc2017	B. occitanus	Sp.	L'Aldea	21	40,77	0,59	83	
Sc2026	B. occitanus	Sp.	Calamocha	22	40,95	-1,30	880	
Sc2058	B. occitanus	Sp.	Landete	23	39,88	-1,34	986	
Sc2062	B. occitanus	Sp.	Buñol	24	39,42	-0,80	400	
Sc2358	B. occitanus	Sp.	Las Bardenas	25	42,18	-1,53	319	
Sc2365	B. occitanus	Fr.	Abbaye de Valmagne	26	43,49	3,56	122	
Sc1601	Granada	Sp.	Poqueira shelter	27	37,02	-3,32	2535	

Code	Lineage	Ctr.	Locality	Lc.	Lat.	Long.	Alt.	GenBank
Sc1759	Granada	Sp.	Padul	28	37,05	-3,64	916	
Sc1816	Jucar	Sp.	Aledo	29	37,82	-1,58	703	
Sc1834	Jucar	Sp.	. Villena		38,64	-0,86	579	
Sc2074	Jucar	Sp.	Caudete	31	38,68	-1,02	651	
Sc2079	Jucar	Sp.	Jumilla	32	38,49	-1,36	588	
Sc2081	Jucar	Sp.	Hellín	33	38,50	-1,64	494	
Sc1096	B. halius	Sp.	Aracona	34	37,89	-6,56	685	
Sc1097	B. halius	Sp.	Alacena	34	37,89	-6,56	685	
Sc1614	B. halius	Pt.	Alportol	35	37,19	-7,91	280	
Sc1615	B. halius	Pt.	Alporter	35	37,19	-7,91	280	
Sc1616	B. halius	Pt.	Valverde	36	38,53	-8,00	240	
Sc1689	B. halius	Sp.	Zafra	37	38,41	-6,44	598	
Sc2082	B. halius	Sp.	Rihornos	38	38,28	-2,72	723	
Sc2111	B. halius	Sp.	Cañada de Calatrava	39	38,84	-4,03	675	
Sc2127	B. halius	Pt.	Lagarelhos	40	41,67	-7,47	783	
Sc2326	B. halius	Sp.	Hontanar	41	39,61	-4,51	926	
Sc2342	B. halius	Sp.	La Aliseda de Tormes	42	40,33	-5,42	1130	
Sc2344	B. halius	Sp.	Hontoria	43	40,90	-4,14	1030	
Sc2371	B. halius	Pt.	Sapiões	44	41,36	-7,81	800	
Sc2406	B. confluens	Mo.	Lafraraa		34,30	-5,29	100	
Sc2419	B. confluens	Mo.	Bni Moussa		35,40	-5,37	380	
Sc2423	B. confluens	Mo.	Lakherachefa	47	35,31	-6,03	160	
Sc1548	<i>B. sp.</i> 1	Mo.	Ighil	48	30,16	-8,48	1506	
Sc1568	B. sp.2	Mo.	Tagezn	49	29,77	-9,14	1232	
Sc2410	B. sp.3	Mo.	ESE Khemisset	50	33,76	-5,95	180	

## 2.2. DNA extraction, PCR and Sequencing

Whole genomic DNA was extracted from freshly preserved (96% ethanol) muscle tissue (leg) using the SPEEDTOOLS Tissue DNA Extraction Kit (BIOTOOLS). Five gene regions were amplified, two fragments of the mitochondria, namely *cox*1 and 16S, and five nuclear fragments, a Protein Kinase-like (PK) gene fragment, three Anonymous Nuclear Markers (ANM), c0061, c0971 and c5070 and the 28S rDNA large subunit domain D3 (28S). Mitochondrial primers (Folmer et al., 1994; Gantenbein et al., 1999; Palumbi, 1996; Simon et al., 1994) and Polymerase chain reactions (PCR) conditions follow the methodology described in Sousa et al. (in prep 2). Nuclear primers (Nunn *et al.*, 1996; Gantenbein *et al.*, 2003; Sousa *et al.*, Paper 2) and PCR conditions follow Sousa *et al.* (Paper 2).

DNA sequences were edited and assembled using Geneious software v.6.1.8 (Kearse et al., 2012). All multiple sequence alignments were subsequently transformed in ALTER (Glez-Peña et al., 2010) into the specific format preferred by the different

software used. All new sequences obtained in this study are made available in GenBank (Table 1, Supplement Table 1).

### 2.3. Alignment, best-fitting nucleotide model selection

The two protein-coding genes were aligned with Muscle (Edgar, 2004) and no indel were found. The remaining genes were aligned with the MAFFT (v7.017) method G-INSi (Katoh et al., 2002; Katoh and Standley, 2013) in Geneious except for the 16S gene, which was aligned using the online version of MAFFT v7, applying the method Q-INS-I (Katoh and Toh, 2008). Protein coding sequences were translated to amino acids and no stop codons were present.

For the nuclear genes, the haplotype phases were resolved using a two-step procedure: 1) for sequences that were heterozygous for insertions or deletions (all ANM fragments), we used Champuru software online v1.0 (Flot, 2007), that implements the method described by Flot et al. (2006); 2) the Bayesian algorithm implemented in PHASE (Stephens et al., 2001), using the known phases of haplotypes determined with Champuru, when available. Phase was run five times per dataset, and the most probable pair of alleles for each heterozygous individual was retained for the downstream analyses (Bryson Jr et al., 2014), although the majority of positions were resolved with a posterior probability higher than 0.7. Remaining positions were coded as missing data (N). We used Gblocks V.0.91b (Castresana, 2000; Talavera and Castresana, 2007) online software to eliminate poorly aligned positions from the genes with alignments gaps (the 16S and the three anonymous loci).

#### 2.4. Genetic diversity, neutrality tests and pairwise distances

For both mtDNA and nuDNA, genetic diversity indices were estimated using DnaSP v.5.10.01 (Librado and Rozas, 2009). We calculated the number of segregation sites, the number and diversity of haplotypes and the nucleotide diversity  $\pi$ . To test for non-neutral evolution we calculated Tajima's D (Tajima, 1989) and to detect demographic expansions we calculated F's (Fu, 1997). The significance of the test results was assessed using 10,000 coalescent simulations. Uncorrected genetic p-distances between and within mtDNA *cox1* lineages were estimated with MEGA v6.06 (Tamura et al., 2013). The standard error was assessed conducting 1000 bootstraps.

#### 2.5. Molecular delimitation: discovery and validation

As *Buthus* species are difficult to identify using morphological characters, we implemented a molecular-based discovery and validation approach (Carstens et al.,

2013; Rannala, 2015) to generate candidate species and confirm their allocation, or not, to nominal species.

First, we generated an initial assignment to discrete groups and, subsequently, we validated groupings using a multispecies coalescent model in a Bayesian framework (Rannala, 2015).

Preliminary assignment was conducted with three different methods, two based on a single marker approach (i.e. DNA barcoding) using mtDNA data, and one multilocus, using nuclear data. The Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2011) (wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html), using several estimates of genetic distances (uncorrected pairwise, Kimura 2 parameter and Maximum Composite Likelihood distances) calculated using MEGA v6.06 (Tamura et al., 2013), and adjusting the relative gap width to identify a barcoding gap in our distances data. The General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough, 2013; Monaghan et al., 2009; Pons et al., 2006) (http://species.h-its.org/gmyc/) requires an ultrametric tree to identify coalescent clusters. This method assumes that there will be a mixture of a Yule branching process for the species portion of the tree and allele coalescence within populations in the terminal portion of the tree (Rannala, 2015). The GMYC assumes that the coalescence process is far commoner than the speciation and extinction processes within the tree and tries to find a threshold (or multiple depending on the GMYC method) that reflects the transition(s) between both (Carstens et al., 2013). A tree was constructed with MrBayes (v3.2.6) (Ronquist et al., 2012) (see below for details), and then made ultrameric with the help of PATHd8 (Britton et al., 2007), arbitrarily fixing the root age to 1. We removed the out-group before the GMYC analysis. Finally, we used the Bayesian clustering analysis software Structure v2.3.4 (Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000) that uses multilocus genotyped data. Structure assigns samples that share the same allele frequencies at each locus to a predefined number of populations (the K parameter) (Pritchard et al., 2000). Samples can also be found to be admixed, in which case they will be assigned to two or more populations. Although Structure was developed for Population Genetics, it has been used in species delimitation (e.g. Brunes et al., 2014; Satler et al., 2013) because the K parameter that best fits the data can be statistically assessed a posterior. Structure was run with the nuDNA markers. Resolved alleles were converted to allele frequency data through the program xmfa2struct (http://www.xavierdidelot.xtreemhost.com/clonalframe.htm). K was allowed to vary between 2 and 12 (three times the number of known Iberian Buthus species and more than twice the number of known lineages). Each K was run for 10 iterations, with a burn-in length of 50,000 steps, followed by a full-length run of 500,000 using the ancient Admixture model with both allele frequency models, Correlated and

Independent. Alpha was estimated in preliminary analyses and set to 0.3 in the Independent runs. STRUCTURE HARVESTER v.0.6.94 (Earl and vonHoldt, 2012) was used to monitor the estimated log posterior probability of the data [LnP(X/D)] (Pritchard et al., 2000) and the second order rate of change of the likelihood function (Delta K) (Evanno et al., 2005) to assess the best K. Finally, the results of the 10 independent runs were assembled in the program CLUMPAK (beta version) (Kopelman et al., 2015) and checked for biologically meaningful clusters.

The validation step was conducted with the program BP&P v3.3 (Yang, 2015). BP&P use a multispecies coalescent model to compare different models of species delimitation and species phylogeny in a Bayesian framework (analysis A11), accounting for incomplete lineage sorting due to ancestral polymorphism and gene tree - species tree conflicts (Rannala and Yang, 2013; Yang and Rannala, 2014, 2010). As recommend by the author, we performed several independent runs, varying the parameters and algorithms, to test the convergence of the results. We defined the heredity setting to separate the mtDNA genes from the nuclear DNA and allowed for the estimation of each locus rate. We assigned two different population size parameters (Theta) to see the impact these could have on the results, one run with the gamma prior G(2, 2000), and another with a gamma prior G(2, 200). For the divergence time at the root of the species tree (Tau), we assigned the gamma prior G(8, 1000) while the other divergence time parameters are assigned the Dirichlet prior (Yang and Rannala, 2010: equation 2). We tested both algorithms of the species delimitation model, and within each algorithm, we tested prior 1 and 3 for the "speciesmodelprior" The length of each run was set to 200,000, with a burn-in of 4,000 and a sampling frequency of 2. We set BP&P to automatically fine tune the MCMC parameters.

## 2.6. Phylogenetic analyses and haplotype networks

Haplotype networks were constructed with the program TCS v1.21 (Clement et al., 2000), using 30 steps as maximum connection length and treating gaps as a fifth state. Results were visualised with tcsBU (Santos et al., 2015) and used to investigate the congruence of the different nuclear markers used for phylogenetic reconstruction and the mtDNA data.

The best-fitting nucleotide substitution model for each marker was determined using jModelTest2 (v2.1.6) (Darriba et al., 2012; Guindon and Gascuel, 2003) under the Bayesian information criterion (BIC) (Schwarz, 1978) run on CIPRES (Miller et al., 2010), for each marker that were run as independent partitions in all analyses.

Phylogeny reconstruction was performed using Maximum Likelihood and Bayesian inference methods. Maximum Likelihood reconstruction was performed with RAxML-

HPC2 Workflow (v8.1.11) (Stamatakis, 2014) run on CIPRES (Miller et al., 2010), for which we conducted 20 runs to find the best ML tree topology using the best-fitting partition model under GTRGamma. A final optimisation of branch lengths and model parameters was then performed. We conducted a thorough nonparametric bootstrap with 1000 replicates. Bayesian inference was performed with MrBayes (v3.2.6) (Ronquist et al., 2012) run on CIPRES (Miller et al., 2010). MrBayes was analyses was conducted with two runs, each with 8 chains and with a lowered temperature of 0.05 for better chain swapping concluded after preliminary analyses, for 100M generations, sampling trees every 10k generations (and calculating a consensus tree after omitting 25% of the trees). Convergence was evaluated with the PSRF+ parameter, the ESS values calculated in Tracer v1.6 (Rambaut et al., 2014) and with AWTY (Nylander et al., 2008).

#### 2.7 Estimation of divergence times

Divergence times were estimated with Beast v1.7.5 (Drummond et al., 2012) on CIPRES (Miller et al., 2010). We performed several preliminary runs to best tune the parameters for the program. A strict molecular clock was applied for the cox1, PK, C0061, and 28S partitions, while 16S, c0971 and c5070 ANM partitions were run under an uncorrelated relaxed lognormal clock, because a strict clock-like behaviour was rejected for the latter markers by the standard deviation (SD) and coefficient of variation values found (Peng et al., 2006). The Yule process was selected as a tree prior. To avoid incorporating both species and coalescent processes, thus violating the prior assumptions, we used a single representative from each coalescent cluster identified with the GMYC analysis. We did a final 200 million run and checked for convergence with the ESS values calculated in Tracer v1.6 (Rambaut et al., 2012), was also used to estimate divergence times, again run on CIPRES (Miller et al., 2010).

#### 2.8 Modelling of past species' distributions

The past potential distribution ranges of Iberian *Buthus* species predicted using the maximum entropy approach, implemented in the program MaxEnt 3.3.3k (Elith et al., 2011; Phillips et al., 2006, 2004), which predictive performance consistently ranks well when compared to other similar methods (Elith et al., 2006). MaxEnt was also chosen because it requires as input presence-data only, which is the only accurate data available for these species.

Ecological niche-based models (ENM) (Buisson et al., 2010; Guisan and Thuiller, 2005) were built for present conditions (1950-2000 CE) and further projected for two time periods, the Last Inter-Glacial (LIG, 130ka) (Otto-Bliesner et al., 2006), and the Last

Glacial Maximum (LGM, 22ka) (Hijmans et al., 2005), to evaluate the potential effects of Pleistocene glaciations on current phylogeographic patterns of Iberian *Buthus* species. Present and past Bioclimatic variables (BIOV) were obtained from the WorldClim database v1.4 (www.worldclim.org) For the LGM we used three different Global Climate Models (GCM: CCSM4, MIROC-ESM, and MPI-ESM-P). Layers were downscaled to a resolution of 1 x 1 km (30-arc-second). The study area was designed creating a buffer area with the ecoregions (Olson et al., 2001) that contained all our points of Iberian *Buthus* species, including those from the *Buthus* collection at the Madrid Natural History Museum and those from the private collection of Antonio Melic, both of which we were kindly allowed to study.

Iberian *Buthus* species occurrence points were randomly divided into two datasets, one for training and another for validating the models. In both datasets, observations clustering was decreased by randomly removing localities that were clustered according to the Nearest Neighbour Index (NNI) calculated with the "Average Nearest Neighbour Distance" tool in ArcGIS v10.1 (ESRI, Redlands, CA, USA). From the 19 available BIOV, five were chosen to model past *Buthus* distribution in the study area (Table 2). These uncorrelated BIOVs (Pearson  $r \le 0.8$ ) were assumed to be important in shaping *Buthus* species' climatic niche.

**Table V-2.** Range (minimum and maximum) and units of the bioclimatic variables used for modelling the distribution of the Iberian *Buthus* species in the study area. Variables represented as coefficients of variation are expressed as the standard deviation of a percentage of the weekly means, and as such are adimensional (n.a.).

Variable	Description	Unito	Drocont		116		
variable	Description	Units	Fresent	CCSM4	MIROC	MPI	L.I.G.
TMAXWM	Max Temperature of Warmest Month	°C x 10	92 to 365	38 to 297	71 to 290	47 to 304	144 to 409
TMEANDQ	Mean Temperature of Driest Quarter	°C x 10	-89 to 133	-94 to 110	-102 to 108	-122 to 109	-110 to 95
ANUALP	Annual Precipitation	mm	215 to 1792	232 to 1703	219 to 1361	311 to 1763	344 to 1775
TSEASON	Temperature Seasonality (coefficient of variation)	n.a.	2677 to 7033	3046 to 5570	2473 to 6620	3596 to 6327	5122 to 9333
PSEASON	Precipitation Seasonality (coefficient of variation)	n.a.	10 to 78	14 to 83	9 to 81	10 to 65	15 to 68

The number of replicates built for each model type was dependent on the number of records per species, with 20% of test data chosen by bootstrap with a random seed, auto features and logistic output as the options select for the MaxEnt runs (Phillips et al., 2006). We accepted as a measure of model fitness the average area under the curve (AUC) of the receiver operating characteristic (ROC) plot (Fielding and Bell, 1997). We determined the relationship between species' occurrence and BIOV by visual examination of response curves profiles from univariate models. The replicates were averaged to generate a forecast of species presence probability (Marmion et al., 2009). The three GCM used to model the LGM period were averaged to obtain a better forecast

(Araújo and New, 2007). Only those with full concordance between the three GCM were accepted as presence pixels in the LGM model of the species' distributions.

MaxEnt outputs probabilistic maps that need to be reclassified into binary presence/absence maps for downstream analyses. This requires a threshold, adjusted to the prevalence of the training data (Lobo et al., 2008), to accept species' presences. As such we used the minimum training presence threshold (MTP) which forces all training data to be considered as predicted. MTP was calculated according to Vale et al. (2014) and used to produce the binary maps. These were used to evaluate model performance for identifying suitable areas for species occurrence, calculating correct classification rates of data (CCR) (Vale et al., 2014).

Finally, for each species, we compared their two past projections to their present model of distribution to infer overlapping areas in which the species could have persisted throughout the three time periods analysed.

# 3. Results

#### 3.1. mtDNA phylogeography

The mtDNA dataset had a total of 354 *cox1 Buthus* sequences with a total of 658bp: 255 from Iberia (Fig 2, and Supplement Table 1 for the non-multilocus samples), 21 from the North-West of Morocco, and 78 representing the five main groups of *Buthus* diversity (*sensu* Sousa et al., Paper 1. Must of the latter sequences were retrieved from Genbank. We used this dataset to explore the distribution of the mtDNA lineages of *Buthus* in the Iberian Peninsula. We anchored the identity of the main lineages on our sampling of the species' type localities, due to the already mentioned inherent difficulties in *Buthus* morphological identifications. Therefore, each mtDNA lineage in Fig 1 represents the most inclusive supported clade in the phylogeny that included the type locality samples of a nominal species. The *B. montanus* lineage was identified on the basis of the proximity of some of our samples to the presumed type locality (the trail from Puerto de la Ragua to Cerro Pelado in the Sierra Nevada (Lourenço and Vachon, 2004) (Fig 1).

The mtDNA phylogeny supported seven lineages containing all Iberian samples, yet the monophyly of the Iberian samples was not recovered and the relationships between the Iberian lineages (including a concatenated dataset of *cox1* plus 16S, not shown) were not supported (Fig 2A). Four of the seven lineages matched each of the nominal species, namely *B. ibericus*, *B. elongatus*, *B. montanus*, and *B. occitanus*. A fifth lineage corresponded to *B. halius*, a species currently regarded as a synonym to *B. ibericus* (Sousa et al., Paper 1, and two lineages, one from and area close to Granada and a

second one around the Jucar river valley hereafter referred as the Granada and the Jucar lineages, respectively, which may constitute new species.



**Figure V-2.** Results from the mtDNA phylogeny. A – Tree produced with MrBayes, in which the Iberian samples plus those from *B. conflues* (Morocco) are highlighted with colours, according to those used in Figure 1. B and C – Maps of the sampled area, Iberian Peninsula and Tingitana Peninsula of Morocco. In both maps, the sub-lineages within each lineage of the tree in Fig 2A, identified with support (BP  $\ge$  95%) are plotted using the same technic used in Figure 1. Dashed line represent unsupported sub-lineages.

The *B. halius* lineage has the widest distribution, occupying all of Portugal to the west, and reaching the Iberian System to the East, where it probably contacts with *B. occitanus*. We do not have enough data to determine how far north it reaches in the Peninsula, although it clearly surpasses Gredos and Guadarrama Mountains in the centre of the Peninsula and reaches as far North as the south of Galicia (Teruel. & Pérez-Bote, 2005). To the south, it extends to the Guadalquivir depression and along the coast until Barbate, thus effectively surrounding *B. ibericus*. It also encroaches as far south as the Torcal de Antequera, between the ranges of *B. ibericus* and the Granada lineage. *B. occitanus* also exhibits a wide range, although restricted by the Pyrenees, where it extends to its western and southern slopes, and along the south coast of France, reaching east of Marseille (Dupré et al., 2008). *B. occitanus* also occupies the Ebro depression, as far north as La Rioja, and to the south, it reaches the Iberian Mountain

System, at least until the Martés Mountains and along these areas it may meet the Jucar lineage. The Jucar lineage occupies must of Murcia, the East of the Castilla-La Macha region and contiguous areas of the Comunitat Valenciana. It reaches south to the Espuña mountain range, where it comes close to the area of occurrence of *B. montanus*. *B. montanus* occupies a wide altitudinal range, from the sea level at Cabo de Gata to above 2,200m in Sierra Nevada. The species extends north to a portion of the valleys that constitute the Intrabetic Basin, the Baza and Guadix Depression. The Granada lineage also shows a wide elevation range, from the Granada depression up to 2,500m in the southern slope of the Mulhacén Peak of Sierra Nevada. *B. ibericus* was described near the shore of the Guadalcacín reservoir, and its distribution extends from the south tip of Tarifa, across the Alcornoques and Sierra de Grazalema Natural Parks, part of the Serranías de Ronda, and reaches east to Sierra del Torcal. *B. elongatus* has a very small distribution, almost locked to the north by the Serranías de Ronda mountains, including Sierra de las Nieves, and to the south by the Mediterranean Sea. It is also confined between the Guadalhorce and Guadiaro river valleys, east and west respectively.

Overall, we found high levels of within lineage haplotype and nucleotide diversity (Table 3). The *B. elongates* lineage showed smaller values, probably due to smaller sample size, while *B. occitanus* reported smaller haplotype diversity (Table 3). Tajima's D was not significant for any of the eight analysed lineages (including *B. confluens* from north Morocco). However, Fu's FS was significant for *B. halius*, with a negative value suggesting population growth for this lineage (Table 3).

**Table V-3.** Genetic diversity and demographic measures calculated for the cox1 mtDNA gene fragment for the Iberian lineages identified in Fig 2A. *B. confluens* was included for comparison. N, number of individuals; S, number of segregating sites; H, number of haplotypes; Hd, haplotype diversity;  $\pi$ , nucleotide diversity. Neutrality test: D, Tajima (1989). Population growth test: FS, Fu (1997). \*\* Statistical significant results from 10,000 coalescent simulations, for p<0.01. <sup>a</sup> cox1 length reduced from 658bp to remove ambiguities; <sup>b</sup> Iberian lineage N reduced from 255 to 228 to minimise ambiguities; both reductions were done to allow for the use of DNAsp v.5.10.01.

mtDNA Lineage	Lenght	Ν	S	Н	Hd	π	D	Fs
Iberian	629 <sup>a</sup>	228 <sup>b</sup>	179	157	0,995	0,079	0,805	55.173**
B. halius	629	93	120	67	0,991	0,042	-0,154	20.379**
B. occitanus	629	31	43	12	0,847	0,020	0,369	3,261
Jucar	629	17	54	13	0,971	0,023	-0,616	-0,865
B. montanus	629	49	92	36	0,986	0,042	0,618	-4,586
B. ibericus	629	21	49	16	0,971	0,025	0,393	-1,471
B. elongatus	629	6	23	4	0,867	0,013	-1,334	2,092
Granada	629	11	34	9	0,945	0,020	0,289	-0,324
B. confluens (Moroccan)	629	14	51	. 11	0,956	0,023	-0,558	-0,391

We also found high levels of genetic divergence (uncorrected p-distances, Table 4) both between lineages (values between 7.5% and 10.8%) and also to some extent within lineages (min 1.9% and max. 4.2%).

**Table V-4.** Mean *cox1* mtDNA sequence divergence (uncorrected p-distances, values in percentage) of the Iberian *Buthus* lineages identified in Fig 2A. *B. confluens* was again included for comparison. The same 228 sequences from Iberian animals from Table 3 were used here. Below the diagonal are between species distances. Above the diagonal is the standard error calculated with a 1,000 bootstraps. Within species distances are represented in the diagonal. N – number of sequences per species. Calculated in Mega 6.06.

		Ν	1	2	3	4	5	6	7	8
B. confluens	(1)	14	<b>2.02</b> (0.33)	1.1	0.9	0.9	1	1.1	1.1	1
Jucar	(2)	19	10.1	<b>2.21</b> (0.33)	1	1.1	1	1.2	1.1	1.1
B. montanus	(3)	52	8.5	10	<b>4.13</b> (0.52)	1	0.9	1	1	1
B. ibericus	(4)	23	8.8	10.8	9.1	<b>2.55</b> (0.39)	0.8	1	1	0.9
B. halius	(5)	97	10	10.4	9.4	7.5	<b>4.26</b> (0.49)	1	0.9	1
B. occitanus	(6)	30	10	11	10.1	9.5	9.1	<b>1.94</b> (0.33)	1	1
Granada	(7)	13	10.4	10.4	8.8	8.2	9	8.7	<b>2.03</b> (0.35)	1
B. elongatus	(8)	6	8.4	10.2	8.6	8.2	9	8.7	7.7	<b>1.87</b> (0.37)

## 3.2. Molecular delimitation of Buthus species

In both ABGD and GMYC analyses we used a dataset with 255 *cox1* mtDNA sequences from Iberian *Buthus*, with a maximum length of 658bp (Suppl Table 1). The ABGD analysis did not return any univocal barcoding gap (results not shown). The GMYC analysis revealed very high levels of population structuring. It resolved 32 ML clusters and 192 singletons (85%) for a total of 224 ML entities. GMYC has been found to give robust results when no more than 10 to 20% of singletons are recovered (but see Talavera et al., 2013). Because of the large number of cluster recovered, the GMYC were deemed as uninformative regarding the identification of evolutionary relevant lineages.

The Structure analyses were based on five nuclear markers (2,080 bp, 110 variable positions) sequenced for 58 specimens (Table 1). The best K value was 8 (Fig 3). Structure clusters closely matched mtDNA lineages, including the East-West sub structuring within *B. montanus*. Some individuals univocally included in mtDNA lineages show similar probabilities of discordant assignment, which may hint to the existence of introgression between the mtDNA lineages, specifically between *B. elongatus* and *B. ibericus* lineages, and between *B. occitanus* and the Jucar lineage. Furthermore, two



scorpions collected in the North of Portugal, which should correspond to the *B. halius* lineage present signs of admixture with the Jucar lineage (Fig 3).

**Figure V-3.** Population structure estimate with K = 8, considering an admixture model with an alpha of 0.3 and allele frequencies correlated. Each individual is represented by a vertical line. Species assignment and colour codes corresponds to mtDNA lineages (Fig 1). The black vertical divisions separate individuals from mtDNA lineages to reveal cases of organelle/nuclear discordance (individual codes are given bellow bars).

For the BP&P analyses, we used an extended version of the Structure dataset, as the number of sequences per marker does not need to be equal since the program implements the multispecies coalescent. The dataset was composed of 80 and 79 specimens (one sequence each) for the *cox1* and 16S mtDNA markers, respectively. For the nuDNA markers, we used two-phased alleles per marker per specimen, with a total of 62 specimens for PK, 46 for c5070, 56 for c0971, 61 for c0061 and 24 for the 28S marker (Table 1 and Fig 1). Individuals were assigned to 8 candidate species, according to the mtDNA lineages and Structure results. The BP&P analysis confirmed, with 100% Bayesian posterior probabilities (PP), seven out of the 8 candidate species (Fig 4), after lumping together the East and West lineages of *B. montanus*. However, BP&P did not resolve a well-supported species-tree and as such no inference about the relationships between species could be inferred (not shown).

#### 3.3. Phylogenetic analyses and haplotype/allele networks

The specimens, locality data, and GeneBank accession codes of the sequences analysed in this study are listed in Table 1. The mapping of the localities sampled in South-western Europe and Morocco can be seen in Fig 1.

Our main dataset was composed of 51 specimens (including six Moroccan *Buthus* that were used as outgroups) and the six molecular fragments concatenated (cox1, 16S, PKinase, c0061, c0971 and c5070) for a combined length of 2,828 bp. A general overview of markers length and variability is summarized in Table 5. Gaps were treated as missing data in the phylogenetic analyses. The best-fitting models of sequence evolution under the AIC obtained with jModelTest2 were GTR + Gamma ( $\Gamma$ ) + Invariant sites (I) for the *cox1* and 16S, GTR +  $\Gamma$  for the c5070, GTR for the c0061, SYM +  $\Gamma$  for

the c0971, and HKY +  $\Gamma$  for the PK. We choose not to use the 28S marker due to its low coverage in some lineages. Further reduction in the number of individuals used in the Structure and BP&P datasets was due to the inclusion of a single specimen per GMYC cluster and the removal of presumed hybrid animals according to the Structure analysis and the observation of the TCS networks produced (Suppl. Figs 5.1 to 5.10).

**Table V-5.** Main dataset information. N, number of individuals; Length range – variation in the number of sites due to missing data (mtDNA) and indels (nucDNA); Var – number of variable sites; S – number of segregating sites; in %, numbers of Var and S per 100bp. a no information for individuals: Sc1548, Sc1568, Sc2410; b individuals with a 250bp gap: Sc1834, Sc2074, Sc2079; Sc1100 missing 241bp from the beginning.

Marker	Ν	Lenght	Length range	Var	Var (%)	S	S (%)
Complete dataset	112	2828	2559-2828	637	22,5	563	19,9
cox1	56	658	641-658	191	29,0	191	29,0
16S	56	382	380-382	119	31,2	119	31,2
PK	112	362	n.a.	16	4,4	14	3,9
c0971	112	432	417-432	92	21,3	76	17,6
c0061	106 <sup>a</sup>	221	218-221	47	21,3	40	18,1
c5070	112 <sup>b</sup>	773	523-773	172	22,3	123	15,9

Both ML and BI analyses of the concatenated mtDNA and nuDNA produced trees with very similar topologies, and we observed no differences in the nodes with high bootstrap support (BS  $\geq$  80) and high values of Bayesian posterior probability (PP  $\geq$  95%) (Fig 4). The Iberian lineage was supported as monophyletic, however, we did not recover the *B. confluens* lineage as its sister clade. The latter species, which seems to be widespread in North-western Morocco, from the slopes of the Rif Mountains to the Tingitana Peninsula, grouped with other Moroccan samples of the *occitanus* mtDNA group (Sousa et al., Paper 4), that were the sister clade to the Iberian one.

Both analyses recovered Iberian clades (Fig 4, see also Fig. 1) that matched the main mtDNA lineages defined above (Fig 2) and subsequently confirmed by BP&P analyses. The *B. ibericus and B. elongatus* species, and the Granada lineage form a well-supported clade, hereafter referred as the South-western clade (SW Clade). *B. ibericus* and *B. elongatus* were always recovered as sister species albeit with low support. The *B. occitanus* and *B. halius* species, and the Jucar lineage also formed a supported clade, hereafter referred as the Northern Clade (N Clade). *B. occitanus* and *B. halius* were always recovered as sister species albeit. The *B. occitanus* and *B. halius* species, and the Jucar lineage also formed a supported clade, hereafter referred as the Northern Clade (N Clade). *B. occitanus* and *B. halius* were always recovered as sister species albeit with low support. The phylogenetic position of *B. montanus* remain unresolved, BI analysis suggest a closer relationship with the N Clade albeit with low support.

The same overall relationships were recovered in the BI analysis of the concatenated nuDNA alone (Suppl. Fig. 4.8), with the Iberian samples again forming a well-supported clade. The main discrepancies were the low support for the N Clade and the paraphyly of *B. ibericus* and *B. occitanus*, which included *B. elongatus* and the Jucar lineage, respectively.

The concatenated analyses also confirmed the deep geographic structure within the Iberian lineages already revealed by the mtDNA phylogeography (Fig 4).

#### 3.4. Divergence times

The well-supported monophyly of the Iberian *Buthus* allowed us to use the Messinian Salinity Crisis (MSC) as a biogeographical calibration point for the split of the Iberian and Moroccan *Buthus*. Therefore, we applied an informative prior on the stem of the most recent common ancestor (tmrca) of the Iberian clade. We used an exponential prior ( $\mu$  =0.5, offset =5.3) (Andújar et al., 2012b), to include a 95% interval within the 5.96 to 5.33 Ma range, which is the estimated length of the MSC (Krijgsman et al. 1999).

The Beast tree was concordant with the concatenated BI and ML and the mtDNA trees (Figs 4A, 2). The estimated divergence times were three to ten times faster for the mtDNA markers than the nuDNA markers (Table 6).

Marker	Divergence time (My <sup>-1</sup> )	substitutions/site/My
16S	2.80%	0.014 ± 0.0054
c5070	0.91%	$0.004 \pm 0.0017$
PK	0.27%	$0.001 \pm 0.0003$

Table V-6. Estimated divergence times according to the Beast analysis.

The divergence time estimates for the split of the three main Iberian groups (SW, N Clades and *B. montanus*) fall within the beginning of the Pliocene, during the Zanclean Age, less than one My after the end of the MSC (Suppl. Table 5.2, Fig 4B, nodes three and four), although the 95% HPD time interval for the first split does not exclude a separation during the MSC event itself. The estimates for the split of the seven Iberian lineages ranges from 4.80 to 3.32 Ma, during the Zanclean Age, although the three later events could have taken place already during the Piacenzian Age (Suppl. Table 5.2, Fig 4B, nodes three to eight). The main internal divergence in *B. montanus* and *B. halius* lineages can be traced to the Gelasian, at around 2.3 Ma (Suppl. Table 5.2, Fig 4B, nodes nine and ten), although, again, the 95% HPD time interval includes the Pliocene-Pleistocene Epochs boundary. The remaining splits occurred during the Calabrian Age, already in the Pleistocene (Suppl. Table 5.2, Fig 4B).

The species-tree reconstructed with \*Beast did not provide support for the relationships between the seven Iberian lineages (Suppl. Fig. 5.9).

### 3.5. Ecological niche-based models

For the ENMs we used all the presence points from the animals used for the cox1 mtDNA phylogeographic study, which already included presence points from previous genetic studies (Gantenbein and Largiadèr, 2003; Sousa et al., 2010). We added all bibliographic points (Armas and González-Moliné, 2009; Cardoso, 2004; Dupré et al., 2008; Rossi, 2012; Teruel and Pérez-Bote, 2005) that we had knowledge of and that could be confidently mapped at the resolution (1 x 1 km) that we were modelling. Only those points that could be unambiguously attributed to a single species, as defined by the cox1 phylogeographic distributions were used, giving a combined total of 206 presence-points, of which 190 were used for constructing the ENMs (Table 8, and Suppl. Table 5.3). From the seven species that we have predicted to exist in Europe, two, B. elongatus and Granada, could not be modelled due to low sample size (n <10) (Vale et al., 2016). Two other species, B. ibericus and Jucar, were on that threshold and to improve their ENMs we used a leave-one-out (loo) approach (Vale et al., 2016). This approach requires an additional step, in which N MaxEnt runs are made per species, being N the number of presence points. In each run, N-1 points were used, removing a different point in each run.





Figure V-4. Iberian Buthus phylogenies base on the multilocus dataset. A -Phylogram from the MrBayes analysis. Support values in the nodes are given for both BI and ML phylogenies, left and right half's of circle the respectively.

Black: PP  $\ge$  95%; BS  $\ge$  80; Grey: both below the given thresholds but above 50. B – Ultrametric tree from the Beast analysis. 95% HPD time intervals for The point removed in each run of the loo approach was subsequently used as the validation data for that particular run. Runs were averaged and then reclassified as explained in the Methods section (Suppl. Fig. 5.10). For the three GCM that were used to create an average for the LGM, GCMs were first individually reclassified and only then combined.

Overall, the quality of the models was high for both training and test data, with the AUC's values varying for each phase from 85% to 99% and from 82% to 99%, respectively (Table 7). The CCR of the validation dataset was also high, ranging from 83.3 to 100% (Table 7), including the iterative loo approach for both *B. ibericus* and Jucar species.

**Table V-7.** Total number of presence points of each *Buthus* species used to train (N Train), test (N Test), and validate (N Valid) the maximum entropy models. Average and standard deviation (in brackets) of training and test Areas Under the Curve (AUC) of each species' model for the Present. Percentage of Correct Classification Rates (CCR) of the data of the species according to the maximum entropy model using the minimum training presence thresholds. loo – leave-one-out approach, in which N-1 samples were used

Species	Ν	N Train	N Test	N Valid	AUC Train	AUC Test	CCR (%)
B. ibericus <sup>100</sup>	10	8	1	1	0.99 (0.002)	0.99 (0.005)	90.0
B. halius	96	52	12	32	0.85 (0.037)	0.82 (0.051)	100.0
B. occitanus	48	28	7	13	0.93 (0.045)	0.89 (0.055)	100.0
B. montanus	25	16	3	6	0.98 (0.035)	0.94 (0.053)	83.3
Jucar <sup>loo</sup>	11	9	1	1	0.87 (0.019)	0.87 (0.046)	90.9

For four of the five modelled species, PSEASON was found to be most important BIOV, albeit contributing differently per species (Table 8). For two species, ANUALP was found to be equally or more important than PSEASON (Table 8).

**Table V-8.** Measures of the contribution of the bioclimatic variables to the ENMs of the Iberian *Buthus* species derived from the maximum entropy models. Explanation of variable codes is given in Table 2. loo – species modelled with the leave-one-out approach.

Spacing		Percenta	age of co	ntribution		Permutation importance					
Species	TMAXWM	TMEANDQ	ANUALP	TSEASON	PSEASON	TMAXWM	TMEANDQ	ANUALP	TSEASON	PSEASON	
B. ibericus <sup>100</sup>	0.15	0.92	3.28	0.73	94.92	0.72	1.36	1.07	0.45	96.40	
B. halius	9.41	2.56	4.09	9.75	74.19	9.57	5.72	4.44	9.56	70.71	
B. occitanus	14.13	17.87	14.14	16.55	37.31	14.80	23.47	6.83	21.42	33.48	
B. montanus	8.55	3.37	49.18	7.41	31.49	7.22	4.75	22.12	6.70	59.20	
Jucar <sup>loo</sup>	0.23	0.94	89.99	0.42	8.42	3.01	6.43	65.50	0.04	25.02	

Two BIOVs contributed with at least 75% of the total information used in the construction of four of the five species ENMs. Although both were related to precipitation, they were found to be only slightly negatively correlated (Pearson r of -0.106). Only *B*.

occitanus diverged from this pattern, as for this species all BIOVs contributed more equally, even if PSEASON still contributed with almost 40%, reflecting perhaps the more mesic habitat requirements of this species. Although PSEASON was found to be important for modelling four of the five species, each had a different RC for this BIOV. *B. occitanus*, *B. montanus*, and *B. ibericus* ENMs had a strong response to a small range of this variable (Fig. 5A), with the first two species having a bell-shaped response at increasing levels of PSEASON and the latter species responding only to high levels of PSEASON. *B. halius* ENM' had a broader response range (Fig. 5A). In contrast, *B. montanus* and Jucar ENMs responses to ANUALP were very similar (Fig. 5B).



**Figure V-5.** Response curves (RC) for the two Bioclimatic variables most related to the distribution of the target species in each Maxent model. The curves show how the logistic prediction changes as each environmental variable is varied, keeping all other environmental variables at their average sample value and can be hard to interpret for strongly correlated variables.

WE found two distinct patterns in the predicted areas of persistence for the five species modelled (Fig 6). For both *B. ibericus* and *B. halius*, no difference was observed for the three time periods modelled (Fig 6, Suppl. Fig. 5.11), having both species persisted *in situ* throughout the analysed time period, although the projected LIG model had the largest predicted area of occurrence (Suppl. Fig. 5.11). For *B. occitanus* our ENMs suggests no major differences between the modelled present occurrence area and past projected area of occurrence during the LIG. However, for the LIG projection the species would be absent from the Ebro River valley and the southern areas of occurrence (Suppl. Fig. 5.12). Our LGM projection does predict a strong range contraction, whit only the coastal areas of Catalonia occupied. The predicted persistence area only includes a small portion of the Northern clade range of this species (Fig 6). The ENM for the Jucar lineage suggests a smaller projected area of occurrence throughout the LIG and LGM, when compared to the modelled present area of occurrence (Suppl. Fig. 5.12). There should have been two disjoint areas of persistence

for this species, one in an area ranging from Almeria to Alicante and the other around Zaragoza, in the lower portion of the Ebro River (Fig 6, Suppl. Fig. 5.12). The latter persistence area does not match the present distribution of this lineage. For *B. montanus* the modelling uncover almost no concordance for the three time periods modelled. For the present a disjunct second area of occurrence was modelled roughly in the southern portion of the Castile and León community, far from the present range of the species. Furthermore, for the LIG past projection the predicted occurrence area was mostly reduced to the northern portion of the Castile and León community, while for the LGM past projection two disjunct areas of occurrence were also predicted, one in the northeastern Andalusian coast and the other south of the Madrid community (Suppl. Fig. 5.12). No persistence area was predicted during the three time periods modelled (Fig 6).

# 4. Discussion

This study represents the first attempt to understand the origins, diversification and distribution of *Buthus* scorpions in the Iberian peninsula, using an integrative and quantitative approach, and hence represent a major improvement over previous, mostly discursive hypothesis on the group (Lourenço and Vachon, 2004; Rossi, 2012; Sousa et al., 2010).

## 4.1. Phylogeographical patterns in Iberian Buthus

The extended sampling carried out in the Iberian Peninsula confirmed and expanded the main findings in Sousa et al. (2010) where five mtDNA lineages were reported, already an improvement over the identification of three lineages by Gantenbein and Largiadèr (2003). Most importantly, the thorough sampling of our study rejects some preliminary findings of former studies, which had placed Tunisian *Buthus* samples closer to Iberian *Buthus*, even when using nuclear information from the 18S/ITS-1 fragment (Gantenbein and Largiadèr, 2003). Instead, our *cox1* mtDNA dataset did recover a lineage including the Iberian and *B. confluens* and other samples from North Africa (Fig 2A). The subsequent addition of nuclear gene fragments supports the monophyly of the Iberian samples. Unfortunately, despite the use of multiple nuclear gene fragments, we could not fully resolve the relationships between the three main lineages of Iberian *Buthus*. This may result either from insufficient information in the genetic dataset used to recover these relationships (*i.e.* "soft polytomy") or, alternatively, from the rapid diversification of these lineages, which would not have left enough time to accumulate informative changes (*i.e.* "hard polytomy").



**Figure V-6.** Maps of the predicted persistence throughout the LIG (130 kya), LGM (22 kya) and the Present for the five Iberian *Buthus* species modelled. In the first column, the range of persistence throughout the entire study area is presented. In the second column, the results from the cox1 mtDNA phylogeography of Figure 2 have been superimposed so as to observe if any correspondence between both exists.

The general pattern of diversity for the Iberian *Buthus* agrees with what has been found across several animal genera, with highest levels of diversity found in the southern Iberian Peninsula (e.g. *Timon lepidus*, Miraldo et al. 2011; *Podarcis*, Kaliontzopoulou et al., 2011; *Mesocarabus*, Andújar et al. 2012a; *Tarentola*, Rato et al., 2012; *Vipera*, Velo-Antón et al., 2012). All three main clades and six out of the seven lineages can be found in the Betic mountain ranges (Figs 1, 2B, C).

Although B. ibericus, B. elongatus and the Granada lineage have narrow distributions, they exhibit population structure (Fig 2B, C). However, the relative sparse sampling prevents any further discussion and calls for future, more intensive sampling of their distribution area. The best sampled lineages show strong phylogeographic structure (Fig 2). B. montanus is composed of two well-supported lineages with an East-West distribution, one from the Sierra Nevada and the other from the area around the Sierra de Baza, although they seem to be in contact on the eastern slope of Sierra Nevada (Fig 2C). The Jucar lineage is composed also of at least two well-supported lineages, with a North-South distribution, a southern lineage confined to the eastern range of the Prebetic System and a North lineage that partially overlaps the former but that extends to the Tagus basin (Figs 2B, C). B. occitanus also has two well-supported lineages, again with a North-South distribution. The southern lineage occurs in the final portion of the Iberian Mountain System while the northern one occupies the rest of the distribution of the lineage. The north lineage is further subdivided by the Ebro River (Fig 2A, B). Although the demographic tests (Table 3) failed to find any signal of population expansion, its star-shaped haplotype networks (Suppl. Figs. 5.1, 5.2) suggests a recent population expansion. B. halius has the most complex phylogeography, as inferred from the cox1 marker. Surprisingly, lineage A (Figs 2A, B), which is sister to the remaining lineages, was not found in the southern Iberian Peninsula as with all other Buthus lineages but in north of the Central Mountain System, an area known to harbour distinct genetic lineages in many endemic Iberian vertebrates (e.g. Lacerta schreiberi Bedriaga, 1878, Godinho, Crespo, and Ferrand 2008; Chioglossa lusitanica Barboza du Bocage, 1864, Sequeira et al., 2008). This is unexpected as the former examples are animals adapted to more humid environments, while Buthus scorpions are generally not. Moreover, Structure did found signs of admixture with Jucar in two animals represented in this lineage, which, based on the geographical distribution of these species, makes the possibility of gene flow difficult to admit. An alternative hypothesis is the retention of ancestral polymorphism, although further analysis with additional loci and samples will be required to disentangle these two mutually exclusive hypotheses, but might explain the unexpected placent of this lineage wihtih B. halius. Lineages B and C are found on each side of the Guadalquivir basin, which has been described as a barrier for other
terrestrial arthropods (Arnedo and Ferrández, 2007), while lineage D can be found to the south of the Guadalquivir river valley. Lineage E is restricted to the south of Portugal, with one individual (Boolb5 from Gantenbein and Largiadèr, 2003) as the only representative on the eastern margin of the Guadiana river. Lineage G is also restricted to the western portion of the Sistema Central Mountains, namely Serra de Gredos in Spain and Serra da Estrela in Portugal. Lineage F occurs between the margins of the Guadalquivir and Guadiana, although some specimens were also found in the western margin of the Guadiana, where they occur in sympatry with lineage E. Lineage H occurs in a wide area of the Iberian Peninsula centre, and in the south of its distribution it overlaps with Lineage F. Unfortunately, there is no support for the relationships between Lineages B, C, D and E. The *B. halius* lineage had a negative and significant result of Fu's FS (Table 3), which suggest recent population growth, although that scenario is not supported by our modelling to the past.##

The mtDNA data clearly indicates a high level of philopatry (Fig 2A), most likely as a result of the low dispersal ability of scorpions (low vagility) (Polis et al., 1985). Deep population structure as a result of limited dispersal has already been suggested as a key factor in *Buthus* evolution (Habel et al., 2012). Strong phylopatry have been observed in other terrestrial arthropods reputed for low vagility such as mygalomorph spiders (Arnedo and Ferrández, 2007; Hamilton et al. 2014). Some specimens defied the expected and for the most part confirmed phylopatry of Iberian *Buthus* (Sc1119 in lineage C, Sc1114 and Sc1115 in lineage B, Sc1716 and Sc1717 in lineage F, Fig 2C). Assuming they are not a mislabelling mistake during manipulation, the most plausible explanation is their accidental relocation by humans. Human-mediated transport has been suggested to occur with other terrestrial invertebrates of similar size to *Buthus*, for example the spider *Macrothele calpeiana* (Bellvert and Arnedo, 2016).

#### 4.2. Biogeographic scenario for the diversification of Iberian Buthus

Our multilocus dataset supported the monophyly of the Iberian lineages and thus rejects former hypothesis based on mtDNA data that either suggested two independent colonization events of the Iberian Peninsula (Habel et al., 2012) from Morocco or a secondary back colonization of North-Africa (Sousa et al., 2012) from the Iberian Peninsula. Based on this result we proposed that the Iberian Peninsula was colonised by Bluthus after the establishment of a land connection with Morocco during the MSC, and that the Iberian lineage split from its Moroccan sister clade because of the subsequent opening of the Gibraltar Strait.

However, there are alternative explanations that may also explain the patterns here recovered. Paulo et al. (2008) enumerated three hypothetical scenarios to explain

genetic connectivity between both sides of the Strait of Gibraltar (SoG). The first scenario is a Post-MSC overseas dispersal (1). An overseas dispersal across the Mediterranean taking place well after the MSC would imply a lack of clear phylogeographical structure due to the reduced amount of time allowed for local diversification. If the dispersal occurred closer to the MSC, then it would be impossible to differentiate it from the next hypothesis, a Post-MSC vicariance (2) – a land dispersal during the MSC, followed by vicariance at the end of the MSC, creating a pattern of two lineages, one in each side of the SoG. It also implies that originally only one of the land masses, Iberia or Morocco, was occupied by the organisms. The third scenario is a Tortonian (pre-MSC) overseas dispersal – an overseas dispersal across the Mediterranean taking place not long before the MSC, following a stepping stone model. These dispersal movements would have taken advantage of the progressive closing of the seaways between the Atlantic and the Mediterranean due to the arching movements of the Betic-Rif terrains and the several islands that formed that connection (Martín et al., 2009). This scenario would also imply a strong phylogeographic pattern between both sides of the SoG, with multiple different lineages evolving in those forming islands. Such a pattern has been inferred for the eastern Iberian clade of Ummidia (Thorell, 1875) spiders (Opatova et al., 2016). However, this hypothesis implies a strong connection between both sides of the SoG, as the Betic-Rif connection was only broke after the MSC. Also assumes that the western area of the Iberian Peninsula was land colonised from the eastern area, as the eastern area should have been colonised before. We could add a fourth alternative scenario, an older pre-MSC overseas dispersal – an overseas dispersal across the Mediterranean taking place long before the MSC (i.e. 21 to 15 Ma), which would allow for enough time to generate a deep phylogeographical structure. Moreover, under this scenario, we would expect other areas that were at the time island in the Alboran Sea to have been also colonised, including among others, the Balearic Islands. This is rooted in the observation that the prevailing surface currents flow from the Atlantic into the Mediterranean (Krijgsman, 2002) and have been so at least since the Serravallian (Hamon et al., 2013). If, alternatively, the surface currents were to flow from the Mediterranean into the Atlantic, then we should expect that coastal Algerian or even Tunisian organisms to be more closely connected to the organisms arriving at the Alboran islands and to the Iberian Peninsula organisms. This type of inflow has been predicted to have existed at least during the Langhian (16 to 13.8 Ma) (de la Vara et al., 2013). Palmer and Cambefort (2000) suggested an earlier, 15Ma land connection between the Iberian Peninsula and North Morocco, however no recent kinematic reconstruction of the western Mediterranean since the Miocene as recovered such land connection (Jolivet et al., 2006; Popov et al., 2004; Rögl, 1998; Rosenbaum et al., 2002).

Our data seems to reject the first scenario, given that Iberian Buthus exhibits a strong and complex phylogeographic structure. Similarly, the fourth scenario may also be refuted by the fact that no Buthus scorpion has ever been recorded in the Balearic Islands, yet many other groups of poorly dispersing organisms are found in these islands. Spider of the families Dysderidae and Nemesiidae have sister groups in the Balearics and the Betics, and it has been suggested that they originated following the break between the two areas during the Tortonian marine transgression (~9 Ma) (Bidegaray-Batista and Arnedo, 2011; Mora et al., 2017). The absence of *Buthus* in the Balearics could be explained by the absence of this lineage also in the Betics during the times the two lands were connected. Hypotheses two and three are harder to disentangle. The existence of several lineages in the south of Iberia seems to favour the third hypothesis, since the distribution of some lineages fit well with the isolated areas in the region during the Serravallian-Tortonian (e.g. Braga et al., 2003). However, the absence of a sister clade to the Iberian lineage in the North portion of Morocco gives stronger refutation to the third hypothesis also. Only a complete understanding of the relationships between the Iberian lineages can polarise a choice between these two scenarios.

#### 4.3. Timeframe for the diversification of Iberian Buthus

Our estimated divergence time of 2.80% My<sup>-1</sup> for the 16S mtDNA marker is similar to other estimates available for *Buthus* (3.1% My-1 - Gantenbein and Largiadèr, 2003; 3.22 My-1 - Sousa et al., Paper 4) This was to be expected, because although those studies had a broader geographical sampling, the same calibration event, the MSC, was used. The divergence times of the nuclear markers were also similar to those reported before (Sousa et al., Paper 4).

Our estimate of the splitting between the seven Iberian *Buthus* lineages all coincide with the Zanclean Stage of the Pliocene (5.333 to 3.6 Ma) (Suppl. Table 5.2, Fig 4B). The climate during this stage seems to have been very similar to the climate at the end of the Messinian (Jiménez-Moreno et al., 2010), which suggests that it was not climate changes that prompted the diversification of the Iberian *Buthus*, and that they diverged before the Mediterranean climate had become dominant in the area. This time was marked by tectonic uplift in the Beatic-Rif Arc (Braga et al., 2003), and this changes in the landscape might have promoted isolation in areas of the southern Iberian Peninsula, which coincides well with the ranges of some of the lineages delimited. This timeframe of diversification fits with those observed in other organisms (*e.g. Dolichopoda* cave crickets, Allegrucci, Trucchi, and Sbordoni 2011; several lineages of *Podarcis*, Kaliontzopoulou et al., 2011; *Cryptocephalus* leaf beetles, Gómez-Zurita et al. 2012). The single exception is the younger split between *B. elongatus* and *B. ibericus*, which at

3.32 Ma (Suppl. Table 5.2, Fig 4B) coincides remarkably with the onset of the Mediterranean climate, which started around 3.4 to 3.2 Ma (Jiménez-Moreno et al., 2010; Suc, 1984). The two early splits within the *B. montanus* and *B. halius* lineages occurred probably during the Gelasian (2.58 to 1.80 Ma), an age when the Glacial and Interglacial climatic oscillations were well established (Haywood et al., 2009; Rodríguez-Sánchez et al., 2008) and drove an increase of steppe like habitats in the south of the Iberian Peninsula (Feddi et al., 2011). All the remaining divergence times that we have estimated occurred during the Calabrian Age of the Pleistocene, during which continued climatic oscillations persisted that have left a strong mark in the genetic structure of several animals and plants (e.g. Hewitt, 2011, 2000). Of potential importance for *Buthus* diversification was the expansion of herbaceous vegetation that occurred during the Calabrian (P. Sousa, pers. obs.). A similar pattern of earlier Pliocene speciation and Pleistocene diversification has been found for *Alytes* Wagler, 1830 toads (Gonçalves et al., 2015; Maia-Carvalho et al., 2014).

#### 4.4. Insights into Buthus diversification from predictions of past distributions

Interestingly, we found that precipitation and not temperature BIOVs contributed the must to the ENM of the Buthus species analysed, perhaps reflecting that precipitation as a greater limiting factor in their distribution, albeit Buthus scorpions being more easily associated with warm regions. Must of the Buthus species studied occur in the southern areas of the Iberian Peninsula, where water is scarcer, although that pattern is less obvious for B. occitanus and B. halius distributions. The ENMs of both B. ibericus and B. halius did not predict a role for the Quaternary glacial cycles in the present patterns of genetic diversity for these species. Instead the data might be suggesting that competition with *B. halius*, rather than climate, can be the factor limiting the distribution of *B. ibericus* (Suppl. Fig. 5.11). As for *B. occitanus*, only a small portion of the Northern lineage would have persisted throughout this time period. Moreover, no area of persistency was predicted for the region occupied by the southern lineage (Fig. 6), which suggests that glaciations did not play a role in shaping the patterns of genetic diversity found in B. occitanus. Similarly, only the Southern lineage of the Jucar lineage would have persisted throughout the time analysed, although the pattern is less clear. Moreover, the predicted area of persistence around Zaragoza is in strong disagreement to the current distribution of both B. occitanus and Jucar, as this area is presently occupied only by the former species, although our sampling was not exhaustive. Finally, the complete lack of any persistence area for *B. montanus* suggests that the current phylogeographic patterns of this species, again, were probably not shaped by glaciations.

#### 4.5. How many Buthus species inhabit the Iberian Peninsula

The number of putative species in Iberian *Buthus* delimited in this study almost double the current number of nominal species, four. Furthermore, given the deep phylogeographic patterns, the hypothesis of seven species of *Buthus* in Iberia might even be an underestimation. Despite the univocal molecular support for seven distinct lineages, finding morphological diagnostic characters for the different lineages is an arduous task. Even identifying the nominal species based on current keys is challenging. Unfortunately, *Buthus* seems to be a genus with a high degree of morphological conservatism and the few variable characters are frequently polymorphic within species, which may require a thorough quantitative morphometric and meristic study to determine if any reliable diagnostics characters can be identified.

Our study calls for an urgent taxonomic revaluation of Iberian *Buthus* that may provide diagnostic morphological characters for identifying the new lineages found and improve the identification of nominal species. There is, however, one taxonomic amendment that we can already fix with the data at hand. Based on well supported genetic evidence and on limited morphological one, *B. halius* is here found to be a subjective synonym of B. *ibericus*. As such, *B. halius* is removed from synonymy with B. *ibericus* and, consequently, its *nomen oblitum* status is annulled (Sousa et al., Paper 1.

#### Buthus halius (C. L. Koch, 1839) (revised status)

*Androctonus halius* C. L. Koch 1839a: 69-70, pl. CLXIII, fig. 383; Gervais 1844a: 43; C. L. Koch 1850: 90; Simon 1879: 96.

*Buthus ibericus* (MIS): Teruel and Pérez-Bote 2005: 273-276, fig. 1; Armas and González-Moliné 2009: 553-554; Sousa et al. 2010: 207; Rossi 2012: 274-275, 277-278; Pedroso et al. 2013: 300; Teruel and Melic 2015: 6-9.

Buthus occitanus (MIS): Berejano and Pérez-Bote 2002: 59.

**Type material:** Portugal, holotype lost according to Fet and Lowe (2000).

**Diagnosis:** for a general diagnose refer to C. L: Koch (1839) original description.

**Diagnostic characters:** *B. halius* and *B. ibericus* can be separated from all other Iberian *Buthus* species because both have a basal lobe (or notch) on the cutting edge of the pedipalp movable finger, at least on females and males of adult size. According to Teruel and Pérez-Bote (2005) *B. halius* can be tentatively separated from *B. ibericus* by having 2 to 3 large lobed terminal granules in the ventral keels of metasomal' segments II-III, a slightly more robust pedipalp *manus*, and by an increased number of pectinal teeth (M: 30-31; F: 23-27). B. ibericus have normal sized terminal granules in the ventral keels of metasomal' segments II-III, a less robust pedipalp *manus*, and a slight smaller average number of pectinal teeth (M: 25-29; F: 22-26).

**Distribution:** This species has a wide distribution over must of western and central Iberian Peninsula (Fig. 1).

**Remarks:** (Simon, 1879) synonymized *Androctonus halius* with *B. occitanus* based on its type locality. Sousa et al. (Paper 1 removed it from synonymy with *B. occitanus*, and based on the existence of a basal lobe in the movable finger of the pedipalp of both *B. halius* and *B. ibericus*, together with the fact that Rossi (2012) had extended the distribution of *B. ibericus* to Portugal, proposed the two species to be considered synonymous. Applying I.C.Z.N. articles 23.9.1 and 23.9.2, in accordance with prevailing usage, Sousa et al. (Paper 1 maintained as valid the junior synonym *B. ibericus*, as a *nomen protectum*, and considered the senior synonym, *B. halius*, a *nomen oblitum*. Teruel and Pérez-Bote (2005) examined material that clearly comes from the distribution of *B. halius* (Plasenzuela, Cáceres), and found some morphological differences. Nevertheless, although the authors said to have considered describing a new subspecies of *B. ibericus*, refrained from doing so because they lacked sufficient comparison material. This work proved that *B. halius* and *B. ibericus* are not synonymous, yet a thorough morphological study is needed to determine which, if any, morphological traits can be used to accurately differentiate both species.

## 5. Conclusions

Our multilocus approach suggests that the Iberian Peninsula was colonised by a single event, which we propose occurred during the land connection between Morocco and the Iberian Peninsula, during the MSC. We identified two new, previously overlooked lineages, and identified a previous synonym to be a good species, raising the number of known Buthus lineages in Iberia to seven. Moreover, the diversification of Iberian Buthus preceded the Pleistocene glaciations, suggesting that tectonic and orogenic events during the Pliocene were the main drivers of speciation. Additionally, our results do suggest that phylogeographic patterns within each species mostly originated during the Pleistocene. The persistency patterns uncovered with ENMs provide weak support for the involvement of Quaternary glaciations in shaping intraspecific diversification, at least for the Eastern Iberian species. These species have strong East-West or North-South phylogeographic patterns, and these were partially corroborated from those species persistence areas throughout the LIG, LGM and the Present. Phylogeographical patterns for the Western Iberian distributed species are less clear and might be better explained by other ecological forces like competition. However, our results must be regarded with caution as there is a limited number of past variables available, lacking, for example, information on past vegetation, which might be misleading and we might not be satisfactorily modelling the micro-habitat requirements of Buthus species.

This work greatly improved our knowledge on the diversity, diversification and distribution of Iberian *Buthus* species, and we hope, will encourage further researchers on this interesting model system. Our results also rise some conservation concerns, since some of the species (e.g. *B. elongatus* and Granada lineage) have very restricted distributions in areas under high human pressure.

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# **Chapter VI GENERAL DISCUSSION**

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The work presented in this thesis focused on the genus *Buthus*, a genus with uncertain affinities within the Buthidae. The phylogeny of the genus was reconstructed for most of its range, and the knowledge on its Mediterranean biogeography, and Western Mediterranean and Iberian phylogeography were greatly increased, particularly in the latter area. In Iberia we also assessed the genus speciation patterns, comparing them with the known taxonomy, updated the latter while at the same time discovering previously unknown lineages. We also assessed the role Pleistocene glaciations might have played in generating the observed Iberian *Buthus* phylogeography.

### 6.1 New Anonymous Nuclear Markers developed

Most molecular phylogenetic studies on scorpions have so far relied on a relatively low number of Loci (1.86 on average), many of which universally conserved markers that give low resolution at the species level (Paper 2).

We developed five ANMs that proved informative at the intra and interspecific level in *Buthus*. Their nucleotide diversity ranged from 2.2% to 5.6% in five Iberian *Buthus* lineages, and average uncorrected sequence divergence between those five lineages ranged from 0.23% to 5.28% (Paper 2). In subsequent studies, we demonstrated their usefulness for inferring the phylogeny of *Buthus*, both in the broader Mediterranean area and in the Iberian Peninsula (Papers 4 and 5).

We also demonstrated for the first time that two of the newly developed ANM, together with two other markers developed for *Mesobuthus* scorpions, cross-amplify in the Buthidae family, at least within the *Buthus* group of genera, and have therefore the potential to reconstruct the phylogeny of the Buthidae, which includes almost half of the

more than 2,200 extant species of scorpions (Suppl. Table 1.1) (Paper 2).

### 6.2 Mediterranean Biogeography and Phylogeography

The Mediterranean has long been understood as an area with high Biodiversity levels. The special role that the southern European Peninsulas (Anatolian, Balkan, Iberian and Italian) have played on the maintenance of that Biodiversity has also a long history and is well understood (Hewitt, 2011a). Special importance has been given to their role as *refugia* during the Pleistocene Glaciations (Gómez and Lunt, 2007; Miraldo et al., 2011). The contribution of North Africa to this Biodiversity patterns has been slower

to emerge, despite its long-standing placement in the Western Palearctic biogeographic region. Much of that lag in knowledge is due to sampling and political biases that are still a barrier (Harris and Froufe, 2005). Nevertheless, the Mediterranean Sea, long viewed as a Biogeographic barrier, is slowly emerging as a porous barrier if not a mediator in the biotic exchanges between Europe and North Africa (Husemann et al., 2014). The last land connection between both occurred at the end of the Miocene, during the great Messinian Salinity Crisis (MSC) (Hsü et al., 1977; Krijgsman et al., 1999). This event promoted an exchange of Biodiversity, yet its extent and prevalent direction are only known becoming clearer, with North Africa acting as a source for European Biodiversity (Husemann et al., 2014). Another example of a polarised interchanged is that of the "Great American Biotic Interchange" when North American mammals were much more successful at invading South America than vice-versa (Webb, 2006).

Our results also contribute to this broad pattern. We have inferred a single colonisation event of Europe from North Africa for *Buthus* scorpions, most probably linked to the MSC (Papers 4 and 5).

We have also provided further evidence for the existence of the Green Sahara oscillations, given that our predicted time of dispersal is posterior to the Sahara onset, and thus require that at some point the Sahara became less arid, allowing the dispersal of *Buthus* (Paper 4). A different scenario has been proposed for example from the case of the elephant shrews (Douady et al., 2003), that were present in all of North Africa before the onset of the Desert conditions.

Several of the areas with high levels of Biodiversity have been linked to areas of stable climate that acted as *refugia* during climatic hardships. It had been so especially during the Pleistocene, both for plants (Médail and Diadema, 2009), and several faunal elements (Husemann et al., 2014). These have also generated areas of hybridization, when previously separated close species regained contact, resulting in different and complex levels of genetic interchange (Hewitt, 2011b).

We also uncovered a complex phylogeographical pattern in North Africa, focused on the Maghreb, and quite probably, on the Moroccan High-Atlas, although this latter hypothesis is less well supported by our data (Paper 4). We uncovered five groups of species within *Buthus*, well supported by the multilocus dataset, and fine-grained with the single locus dataset (Papers 3 and 4). Three groups are mostly found to the south or in the Moroccan High-Atlas:

 boumalenii, broadly distributed in the southeastern slopes of the High-Atlas and northeastern slopes of the Anti-Atlas;

- mardochei, broadly distributed in the Souss River basin, which flows between the southwestern slopes of the High-Atlas and northwestern slopes of the Anti-Atlas. This group does extend north of the western High-Atlas, almost until Rabat, but always far from the Atlantic coast;
- *rochati* (Fig. 1 and 2); broadly distributed to the south and east of the Anti-Atlas;
   And two other groups that are mostly found north of the High-Atlas:
- occitanus, it also has a strictly Western Mediterranean distribution, existing both to the north of the High-Atlas in Morocco and in the Iberian Peninsula. One species occurs south of the High-Atlas, always on the Atlantic coast;
- tunetanus, its connection with the Atlas Mountains System is unclear at present, yet it has the broadest range of the five groups, extending throughout the entire Southern Mediterranean area. We also confirmed that it extends to the south of the Sahara, at least to the East.



**Figure VI-1.** Schematic illustration of the tempo of the tectonic phases and magmatic activity in Morocco, organised left to right from South to North. The blue arrows represent three different timings for the second stage of the Moroccan Atlas Mountains upheaval, which illustrates the uncertainty still associated with this event (modified from Missenard et al. 2006).

Such North and South phylogeographic patterns, centred in the High-Atlas, have been found in other animals (Faille et al., 2014; Fritz et al., 2005; Rato et al., 2012; Tamar et al., 2016; Terrab et al., 2006), although they vary in their timings.

Our calibrated phylogeny inferred a Tortonian to Messinian (6.3 to 9.5 Ma) split for the five groups that we linked to the onset of aridification in North Africa at 7 Ma (Paper 4). We found less support for a direct role of the High-Atlas, especially given the

uncertainty in the tempo and mode of the second stage of the Atlas Mountain System upheaval (Fig. 1).

We found great concordance between our scenario for *Buthus* diversification and expansion in North Africa, with the one uncovered by Carranza et al. (2008) for *Chalcides* skinks. We were more surprised to recover a dispersal over water as an explanation for the colonisation of the island of Cyprus, given the prevalent view of scorpions as low dispersers. Nevertheless, this is a common scenario that has been found in other animals (reviewed in Poulakakis et al. 2013).

#### 6.3 Iberian Peninsula Phylogeography

The Iberian Peninsula has been an ongoing research area for the study of phylogeography, given its complex geological and climatic paleo-history and complex topography, all prone to have left an influence in the genetic structure of species. Furthermore, its role as a source for European Biodiversity was been well established (Hewitt, 2011a). The phylogeographic patterns found in Iberia have been called the *refugia* within *refugia* model (Gómez and Lunt, 2007), with several pockets of stability within the broader Iberia, itself stable when compared to Central Europe during the Glaciations.

Our extensive sampling of the Iberian Peninsula gave us an unprecedented view on the phylogeographic patterns of an Iberian arthropod genus, with 123 new localities added to the 25 already available in Genbank. The overall pattern of greater genetic diversity in the South of the Iberian Peninsula (Paper 5) is concordant with the findings for many animal taxa (e.g. Miraldo *et al.*, 2011; Kaliontzopoulou *et al.*, 2011; Rato *et al.*, 2012; Andújar *et al.*, 2012b), although other phylogeographic patterns are known for taxa with different ecological requirements (e.g. Maia-Carvalho *et al.*, 2014).

We did infer a complex phylogeographical pattern for the matrilineal marker in the seven *Buthus* lineages (Paper 5). Five of them could be split into two well-supported groups, all had at least a 2% intraspecific sequence divergence, and all species pairs had at least a 7.5% sequence divergence. The phylogeography of *B. halius*, the widest spread Iberian species, was found to be particularly complex, with several partially overlapping sub-clades. These geographical patterns of genetic diversity compare remarkably well with the *Podarcis* phylogeography found in Iberia if only less complex in the middle-northern area of the Peninsula. *Podarcis* does have a wider range overall, and more complexity in the northwestern portion of Iberia. Nevertheless, the wall lizards *Podarcis* is the genus with the most complex phylogeographic patterns found until now in Iberia (Carretero, 2008; Harris et al., 2002; Lima et al., 2009), and also one of the most

studied subjects in this field in Europe (Kaliontzopoulou et al., 2011). They have another important difference, *Podarcis* originated in Europe, and *Buthus* is Africa.

Our modelling of Iberian *Buthus* species and projection to the Last Interglacial, which has been predicted as a good proxy to future climate (Lunt et al., 2013), does not raise much concern for the future of the western species, but the same cannot be said of the eastern *Buthus* species modelled. Special attention must be given to *B. montanus* in such a scenario because this species would lose much of its current distribution.

#### 6.4 Species delimitation: a case of success in Iberian Buthus

Cryptic diversity has been recovered recurrently in many molecular phylogenetic studies. A search for "Cryptic diversity" or "Cryptic species" in Google Scholar yielded almost 6,000 results, further demonstrating that this is a hot topic in Biodiversity studies. However, it is still unclear how cryptic this diversity really is. Is it just the result of poorly studied organisms or does it really stems from hidden diversity (Bernardo, 2011; Pfenninger and Schwenk, 2007; Trontelj and Fišer, 2009)? Its overall importance as also been relativized (Eme et al., 2017). The Barcoding approach (Hebert et al., 2003), with more emphases in the molecular and less on the morphology, gives cheaper and faster results, but as the potential to further exacerbate this trend, especially because it directly competes with funding with traditional taxonomers (Will and Rubinoff, 2004).

*Buthus* scorpions were also a possible model candidate for cryptic diversity until recently. Nevertheless, the number of described species in recent years challenges this view. And yet, the diagnostic characters for the identification of *Buthus* species remain difficult to apply, and in many cases have problematic overlapping features. Furthermore, the description of new *Buthus* species based on a reduced amount of specimens, even if in many cases this is the only possible solution given the paucity of available material, raises questions regarding the correct assessment of intraspecific variability. The modern trend of species descriptions is also in stark contrast with the taxonomical practice of Max Vachon regarding this genus. This author had a cautionary approach. In his study of the North-Western African *Buthus* species, he described a great amount of variability, while at the same time also calling attention to the amount of variation that he found within species (Vachon, 1952). Vachon preferred to use the concept of polytypic species, which he used to incorporate these uncertainties. (Vachon, 1952).

We could not find a Barcoding gap in *Buthus*, neither in the genus as a whole (Paper IV) nor in the Iberian Peninsula alone (Paper 5). We had more success applying molecular species delimitation approaches that require multiple loci to the Iberian *Buthus* (Paper 5). We did find two unknown lineages that have clear genetic distinctiveness but

seem otherwise similar to the other described species. But our success stems also from our ability to sample the type localities of the species. This allowed us to correctly pinpoint the described species to specific genetic lineages that would have otherwise been confounded given the above-mentioned difficulties in morphological identification.

## 6.5 Buthus taxonomy

Taxonomical catalogues are useful repositories of accumulated knowledge and offer a valuable tool to guide the interested researcher through the history of a species name and meaning. Online catalogues are becoming common, and represent the next logical step (e.g. World Spider Catalog, http://www.wsc.nmbe.ch/; Fauna Europaea, https://fauna-eu.org/), yet they can all trace back their origins to paper catalogues that preceded them. There is no website with updated information regarding Scorpiones taxonomy. The "The Scorpion Files" webpage (https://www.ntnu.no/ub/scorpion-files/) keeps a very useful and up to date record on currently valid scorpion species names, but no more taxonomical information is available. Fet et al. (2000) "Catalog of the scorpions of the world (1758-1998)", remains an indispensable work for all those interested in doing scorpion's taxonomy. This work compiled more than a 100 taxonomic and faunistic works on Buthus. Nevertheless, the number of Buthus species described has increased tenfold since its publication (from five to 53), and that's why our catalogue brought a much need updated to that groundbreaking work (Paper 1). Even so, the lack of basic knowledge on most species is evident, 25 species are only known from their type locality and one, B. intumescens, in known from a single specimen and has neither type locality nor any other record.

We have promoted the taxonomical reassessment of three species, which now have species status: *Buthus nigrovesiculosus* Hirst, 1925, *Buthus parroti* Vachon, 1949 (Paper 1), and *Buthus halius* (C. L. Koch, 1839) (Paper 5).

#### 6.6 Concluding remarks and future perspectives

The Iberian Peninsula was indeed an engine of speciation (Hewitt, 2011a) for *Buthus* scorpions that first crossed from North Morocco and then diversified *in situ*.

Our work demonstrates the potential of *Buthus* scorpions in unravelling the paleohistory of the Iberia Peninsula, and furthermore, the Middle Miocene to the Present modifications of North African fauna given *Buthus* distribution that borders all of the Sahara Desert, with a few "island" species therein described, remnants of earlier more humid stages of the Sahara.

The application of a molecular species delimitation approach, coupled with the sampling of species type localities should be expanded to the Maghreb, as this would allow an explicit test of *Buthus* taxonomy. If successful, this results would provide meaning to the abundant published distribution data on Moroccan *Buthus* that have uncertain taxonomical placement.

The Iberian *Buthus* morphology must be fully investigated, using modern tools like geometric morphometrics if necessary, in order to describe the two new lineages and to better separate *B. halius* from *B. ibericus*.

If the study of *Buthus* where to be coupled with *Scorpio*, another scorpion with the same broad distribution except for Iberia, we could use a stronger, comparative phylogeography approach to investigate how animals have been evolving in North Africa (Vachon, 1952).

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## APPENDICES



This was my must invaluable "appendice" during fieldwork. Also, a glimpse to the landscape around Almeria can be seen.

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Appendix A: General Introduction

**Supplementary Table I.1.** Extant Scorpiones classification according to Sharma ea (2015). Number of extant genera and species representatives adapted from Rein (2016). The Akravidae Family status remains doubtful as it was synonymized with Typhlochactidae (Fet, Soleglad, and Zonstein 2011)<sup>+</sup>, although the authors did not made any formal taxonomical change. Furthermore the extant/extinct status of its sole species has not been fully ascertained. As such 20 Families are presented. Symbols used: \* – Polyphyletic Family; ? – Family not analysed.

Таха	Genera	Species
Order Scorpiones C. L. Koch, 1850		
Suborder Neoscorpiones Thorell et Li	indström,	1885
Infraorder Orthosterni Pocock, 1911		
Parvoder Buthida Soleglad & Fet 2003	3	
Buthoidea C.L. Koch, 1837		
Buthidae C. L. Koch, 1837	91	1076
Chaeriloidea Pocock, 1893		
Chaerilidae Pocock, 1893	1	42
Pseudochactoidea Gromov, 1998		
Pseudochactidae Gromov, 1998	3	6
Parvoder lurida Soleglad & Fet 2003		
luroidea Thorell, 1876		
luridae Thorell, 1876	4	14
Bothriuroidea Simon, 1880		
Bothriuridae Simon, 1880	16	150
Incertae sedis		
Superstitioniidae Stahnke, 1940	1	1
* Chactoidea Pocock, 1893		
? Akravidae Levy, 2007	1	1
Caraboctonidae Kraepelin, 1905	4	32
* Chactidae Pocock, 1893	16	196
Euscorpiidae Laurie, 1896	3	59
Scorpiopidae Kraepelin, 1905	7	71
Troglotayosicidae Lourenço, 1998	2	5
? Typhlochactidae Mitchell, 1971	5	12
* Vaejovidae Thorell, 1876	23	208
Scorpionoidea Latreille, 1802	10	400
Diplocentridae Karsch, 1880	10	126
2 Hotoroscorpionidae Kraepolin 1005	1	10
* Hormuridae Laurie 1806	י 11	82
* Scorpionidae Latreille 1802	9	158
Urodacidae Pocock. 1893	2	22
Total: 20 Families	211	2282

<sup>+</sup> - Fet, Victor, Michael E. Soleglad, and Sergei L. Zonstein. 2011. "The Genus Akrav Levy, 2007 (Scorpiones: Akravidae) Revisited." Euscorpius 134:1-49.

**Supplementary Table I.2.** Extant Scorpiones Systematics according to Soleglad & Fet (2003). All taxa under the Subfamily rank were omitted for a clearer presentation. 14 Families are accepted.

Parvorder Pseudochactida Soleglad and Fet, 2003 Superfamily Pseudochactoidea Gromov, 1998 Family Pseudochactidae Gromov, 1998 Parvorder Buthida Soleglad and Fet, 2003 Superfamily Buthoidea C.L. Koch, 1837 Family Buthidae C.L. Koch, 1837 Family Microcharmidae Lourenço, 1996 Parvorder Chaerilida Soleglad and Fet, 2003 Superfamily Chaeriloidea Pocock, 1893 Family Chaerilidae Pocock, 1893 Parvorder lurida Soleglad and Fet, 2003 Superfamily Iuroidea Thorell, 1876 Family Caraboctonidae Kraepelin, 1905 Subfamily Caraboctoninae Kraepelin, 1905 Subfamily Hadrurinae Stahnke, 1974 Family luridae Thorell, 1876 Superfamily Scorpionoidea Latreille, 1802 Family Bothriuridae Simon, 1880 Subfamily Bothriurinae Simon, 1880 Subfamily Lisposominae Lawrence, 1928 Family Liochelidae Fet & Bechly, 2001 Subfamily Liochelinae Fet & Bechly, 2001 Subfamily Hemiscorpiinae Pocock, 1893 Family Scorpionidae Latreille, 1802 Subfamily Diplocentrinae Karsch, 1880 Subfamily Scorpioninae Latreille, 1802 Family Urodacidae Pocock, 1893 Subfamily Urodacinae Pocock, 1893 Subfamily Heteroscorpioninae Kraepelin, 1905 Superfamily Chactoidea Pocock, 1893 Family Chactidae Pocock, 1893 Subfamily Chactinae Pocock, 1893 Subfamily Brotheinae Simon, 1879 Subfamily Uroctoninae Mello-Leita<sup>~</sup> o, 1934 Family Euscorpiidae Laurie, 1896 Subfamily Euscorpiinae Laurie, 1896 Subfamily Megacorminae Kraepelin, 1905 Subfamily Scorpiopinae Kraepelin, 1905 Family Superstitioniidae Stahnke, 1940 Subfamily Superstitioniinae Stahnke, 1940 Subfamily Typhlochactinae Mitchell, 1971 Family Vaejovidae Thorell, 1876

**Supplementary Table I.3.** Extant Scorpiones classification according to Prendini & Wheeler (2005). The authors did not included in their paper any phylogeny of the order. All taxa under the Subfamily rank were omitted for a clearer presentation. 18 Families are accepted.

Family Bothriuridae Simon, 1880 Family Buthidae C.L. Koch, 1837 Family Chactidae Pocock, 1893 Family Chaerilidae Pocock, 1893 Family Diplocentridae Karsch, 1880 Subfamily Diplocentrinae Karsch, 1880 Subfamily Nebinae Kraepelin, 1905 Family Euscorpiidae Laurie, 1896 Family Hemiscorpiidae Pocock, 1893 Family Heteroscorpionidae Kraepelin, 1905 Family luridae Thorell, 1876 Subfamily Caraboctoninae Kraepelin, 1905 Subfamily Hadrurinae Stahnke, 1974 Subfamily Iurinae Thorell, 1876 Family Liochelidae Fet & Bechly, 2001 (1879) Family Microcharmidae Lourenço, 1996 Family Pseudochactidae Gromov, 1998 Family Scorpionidae Latreille, 1802 Family Scorpiopidae Kraepelin, 1905 Family Superstitioniidae Stahnke, 1940 Subfamily Superstitioniinae Stahnke, 1940 Subfamily Typhlochactinae Mitchell, 1971 Family Troglotayosicidae Lourenço, 1998 Subfamily Belisariinae Lourenço, 1998 Subfamily Troglotayosicinae Lourenço, 1998 Family Urodacidae Pocock, 1893 Family Vaejovidae Thorell, 1876

Supplementary Table I.4. Extant Scorpiones Systematics according to Fet & Soleglad (2005)\*. All taxa under the Subfamily rank were omitted for a clearer presentation. 13 Families are accepted. Parvorder Pseudochactida Soleglad and Fet, 2003 Superfamily Pseudochactoidea Gromov, 1998 Family Pseudochactidae Gromov, 1998 Parvorder Buthida Soleglad and Fet, 2003 Superfamily Buthoidea C.L. Koch, 1837 Family Buthidae C.L. Koch, 1837 Family Microcharmidae Lourenço, 1996 Parvorder Chaerilida Soleglad and Fet, 2003 Superfamily Chaeriloidea Pocock, 1893 Family Chaerilidae Pocock, 1893 Parvorder lurida Soleglad and Fet, 2003 Superfamily Iuroidea Thorell, 1876 Family Caraboctonidae Kraepelin, 1905 Subfamily Caraboctoninae Kraepelin, 1905 Subfamily Hadrurinae Stahnke, 1974 Family luridae Thorell, 1876 Superfamily Scorpionoidea Latreille, 1802 Family Bothriuridae Simon, 1880 Subfamily Bothriurinae Simon, 1880 Subfamily Lisposominae Lawrence, 1928 Family Hemiscorpiidae Pocock, 1893 Subfamily Hemiscorpiinae Pocock, 1893 Subfamily Heteroscorpioninae Kraepelin, 1905 Subfamily Hormurinae Laurie, 1896 Family Scorpionidae Latreille, 1802 Subfamily Diplocentrinae Karsch, 1880 Subfamily Scorpioninae Latreille, 1802 Subfamily Urodacinae Pocock, 1893 Superfamily Chactoidea Pocock, 1893 Family Chactidae Pocock, 1893 Subfamily Chactinae Pocock, 1893 Subfamily Brotheinae Simon, 1879 Subfamily Uroctoninae Mello-Leita<sup>~</sup> o, 1934 Family Euscorpiidae Laurie, 1896 Subfamily Euscorpiinae Laurie, 1896 Subfamily Megacorminae Kraepelin, 1905 Subfamily Scorpiopinae Kraepelin, 1905 Family Superstitioniidae Stahnke, 1940 Subfamily Superstitioniinae Stahnke, 1940 Subfamily Typhlochactinae Mitchell, 1971 Family Vaejovidae Thorell, 1876

\* - Fet, Victor and Michael E. Soleglad. 2005. "Contributions to Scorpion Systematics. I. On Recent Changes in High-Level Taxonomy." Euscorpius 31:1–13.

Appendix B: Paper 2

**Supplementary Table 2.5.** Overview of the primers used for sequencing the nuclear Loci used in 30 molecular phylogenetic or phylogeographic studies of Scorpiones, below the family rank, ordered chronologically. Primer name, sequence, other Alias name used, and updated reference of the original publication of the primer are given. Complete references are listed after the table.

Markers	Primer	5' to 3' sequence	Alias	Reference
18S rDNA	18d	R CACACCGCCCGTCGCTACTACCGATTG		Hillis and Dixon (1991)
	181	E: GCCTGCGGCTTAATTTGACTCAACACGGG		Hillis and Divon (1991)
	18SAF		18Sai	Wheeler et al. (1993)
	18540	P: TAACCCCAACAACTTTAAT	18585.0	Wheeler et al. (1993)
	1000		10505.0	Wheeler et al. (1993)
	10000	P. GUIGAAATICTIGGACCOIC	105 41.0	Wheeler et al. (1993)
	10005		185 00.5	Wheeler et al. (1993)
	1000F		10002.0	Wheeler et al. (1993)
	185CR	R: GAGICICGIICGIIAICGGA	1850	wheeler et al. (1993)
	CS249	F: TCGTAACAAGGTTTCCG		Schlotterer et al. (1994)
	18S1F	F: TACCIGGIIGAICCIGCCAGIAG		Giribet et al. (1996)
	18S3F	F: GTTCGATTCCGGAGAGGGA		Giribet et al. (1996)
	18S4R	R: GAATTACCGCGGCTGCTGG		Giribet et al. (1996)
	18S5R	R: CTTGGCAAATGCTTTCGC		Giribet et al. (1996)
	18S6F	F: AAACTTAAAGGAAT		Giribet et al. (1996)
	18S8R	R: ACGGGCGGTGTGTAC		Giribet et al. (1996)
	18S9R	R: GATCCTTCCGCAGGTTCACCTAC		Giribet et al. (1996)
	18Sa0.7	ATTAAAGTTGTTGCGGTT		Whiting et al. (1997)
	18Sa0.79	TTAGAGTGCTYAAAGC		Whiting et al. (1997)
	18SB3.0	GACGGTCCAACAATTTCACC		Whiting et al. (1997)
	18SB3.9	TGCTTTRAGCACTCTAA		Whiting et al. (1997)
	18S7F	F: GCAATAACAGGTCTGTGATGCCC		Whiting (2002)
	18S7R	R: GCATCACAGACCTGTTATTGC		Whiting (2002)
	18Sa1.0	GGTGAAATTCTTGGAYCGTC		Whiting (2002)
	18Sa3.5	TGGTGCATGGCCGYTCTTAGT		Whiting (2002)
	18S b2.9	TATCTGATCGCCTTCGAACCTCT	18SB2.9A	Jarvis, Haas & Whiting (2004)
	Pm18S-R	R: CCTACGGAAACCTTGTTACGACTT		Luo et al. (2007)
	unnamed	F: GGCAGTCCGGGAAACAAAGT		Li et al. (2009)
28S	28Sa	F: GACCCGTCTTGAAACACGGA	D3A	Nunn et al. (1996)
	28Sb	R: TCGGAAGGAACCAGCTACTA	D3B	Nunn et al. (1996)
	28S Bout	R: CCCACAGCGCCAGTTCTGCTTACC	28Sbout	Wheeler in Hovmöller et al. (2002)
	unnamed	F: AAACGGCTACCACATCCAAG		Soleglad & Fet (2003)
	unnamed	R: CAACTAAGAACGGCCATGCA		Soleglad & Fet (2003)
	unnamed	F: CGCGAGACCCGACACTACCGT		Li et al. (2009)
	unnamed	R: ACCGCGAAAGCGGGGCCTAT		Li et al. (2009)
	R1S	F: ACCCGCTGAATTTAAGCAT		Arabi et al. (2010)
	R1AS	R: GCTATCCTGAGGGAAACTTC		Arabi et al. (2010)
	R2S	F: CGACCCGTCTTGAAACACGGA		Arabi et al. (2010)
	R2AS	R: CACCTTGGAGACCTGCTGCGGAT		Arabi et al. (2010)
ITS-2	18S-ITS	F: AGAGGAAGTAAAAGTCGTAACAAG		Navajas et al. (1999)
	ITS-28S	R: ATATGCTTAAATTCAGGGGG		Navajas et al. (1999)
	CAS5p8sFc	F: TGAACATCGACATTTYGAACGCACAT		Ji et al. (2003)
	CAS5p8sFt	F: TGAACATCGACATTTYGAACGCATAT		Ji et al. (2003)
	CAS28sB1d	R: TTCTTTTCCTCCSCTTAYTRATATGCTTAA		Ji et al. (2003)
5.8S rDNA	DT421	R: GCTGCGTTCTTCATCG		Schlötterer et al. (1994)
	5.8Sc-Arach	R: TGTGTCCTGCAATTCACACC		Gantenbein & Largiadèr (2003)
	unnamed	F: GGCTGTACTCCCAAACAACCCGACT		Li et al. (2009)
	unnamed	R: CCTGTCTGAGGGTCGGACGAATAAC		Li et al. (2009)
Protein kinase	03B09for	F: TCTGATGTATGGCAGATGGCAATG	PK	Gantenbein, Fet & Gromov (2003)
	03B09rev	R: CGAACTCAAGATCCACTCCTGTACTCG		Gantenbein, Fet & Gromov (2003)
Chaperonin10-Heat shock protein	unnamed	F: ATGGCTGGATTAGGAAGACGTCTTGTC		Gantenbein & Keightley (2004)
	unnamed	R: TAACTTGACCATTTACCTAGAATGTCAC		Gantenbein & Keightley (2004)
Defensin 4kD	unnamed	F: GCCATGAAAGCCGTTGCTATTCT		Gantenbein & Keightley (2004)
	unnamed	R: ACGACACAAATACAGGTGA		Gantenbein & Keightley (2004)
Lysozyme precursor C	unnamed	F: ATGGCTTTCAAGTTTTCATTTTTCG	Lys-C	Gantenbein & Keightley (2004)
	unnamed	R: TAACAGTTGTTATCATTGATAAATTGG		Gantenbein & Keightley (2004)
Methyl transferase	unnamed	F: TGGGTTCCAGCTCGCAGCGGTAACG		Gantenbein & Keightley (2004)
	unnamed	R: AACTTCGTAGTCGGAATACGAATGTTCTC		Gantenbein & Keightley (2004)
Serinproteinase inhibitor	unnamed	F: TGAACAGTTAGCTAAGGC	Spn2	Gantenbein & Keightley (2004)
	unnamed	R: TTAACCCATTGATTAACTTCAT		Gantenbein & Keightley (2004)
Serin-type endopepdidase	unnamed	F: AGTTCTTATTGGTGTTCTTCTTTTGG	STE	Gantenbein & Keightley (2004)
-	unnamed	R: TTATTGTATCCCTATTAGAATCGCAGTTTAAGG		Gantenbein & Keightley (2004)
Thioredoxin1	unnamed	F: CATGTACTGACGCTGGCATTGCC	Trxr-1	Gantenbein & Keightley (2004)
	unnamed	R: ATTGGCGGGATATTACTTGTG		Gantenbein & Keightley (2004)
Unknown protein	unnamed	F: GAGTGTCATGCCAATAGATTACAG		Gantenbein & Keightley (2004)
-	unnamed	R: ATCCACACATCTTCTAAAACGGTTAATTC		Gantenbein & Keightley (2004)
Actin 5C	Actin5C-F-229	F: AAGTATCCNATTGAGCATGGTATTG		Vink et al. (2008)
	Actin5C-R-1057	R: TTNGADATCCACATTTGTTGGAA		Vink et al. (2008)
ANM - Locus 1075	unnamed	F: GAAGGGCAGGTTTTCCTGTT		Yamashita & Rhoads (2013)
	unnamed	R: CATTGCACAAGTTCGTGAGG		Yamashita & Rhoads (2013)
wingless	SpWgF1	F: GYAAATGCCAYGGWATGTCMGG		Blackledge et al. (2009)
	SpWgR1	R: ACTTGRCAACACCARTGAAAWG		Blackledge et al. (2009)

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Samples	Sc1110	Sc1101	Sc1601	Sc1615	TOTAL
MID	ACGCGTCTAGT	ACGAGTAGACT	ACGTACACACT	ACGTACTGTGT	-
RL	RL5	RL6	RL7	RL8	-
Raw number of reads	80,098	217,368	73,402	116,489	487,357
Base pairs	35,523,932	104,031,488	34,976,130	49,647,716	224,179,266
N50	576	594	597	610	594
Pre-processing step					
Filtered reads	48,439	76,028	48,849	65,650	238,966
Base pairs	16,301,228	25,756,971	16,007,978	20,731,237	78,797,414
N50	336	388	382	395	375
Assembly with CAP3					
Assembled reads	37,626	61,382	38,184	24,092	161,284
% Assembled reads	77.7	80.7	78.2	36.7	68
Contigs	1008	3158	1667	3350	9,183
Mapping with BWA (bwa-mem)					
Filtered sequences					
Reads	314	1515	543	1386	3758
Contigs	133	181	208	770	1292
Sequences used					
Reads	48,125	74,513	48,306	64,264	235,208
Contigs	875	2,977	626	2,580	7,058
Mapping statistics					
Reads	16,812	38,248	20,710	20,588	96,358
Coverage	11.39	5.5	6.68	3.66	-
Coverage Stdev	111.81	22.45	48.16	25.24	-

**Supplementary Table 2.6.** Summary statistics of the Next Generation Sequence data from the four *Buthus* specimens.

**Supplementary Table 2.7.** Estimates of Evolutionary Divergence, between Iberian *Buthus* mtDNA lineages, measured as average uncorrected genetic sequence p-distances calculated in MEGA v6.06. L1 to L5 correspond to the mtDNA Iberian *Buthus* lineages identified in Sousa et al. (2012). Moroccan representatives of the genus *Buthus* (*occi. – occitanus*; *mard. – mardochei*) correspond to two of the four major mtDNA groups identified in Western Mediterranean *Buthus* (*sensu* Sousa et al. 2012; Paper 1. All Iberian *Buthus* lineages are part of the *occitanus* group of species. Comparisons to the *mardochei* group are depicted to give context to distances calculated for the c0061 ANM that does not amplify outside the *occitanus* group. Mean distance (Dist.) and Standard error (S.E.) are present as percentage values. S.E. was estimated with a 1,000 bootstrap replicates.

		CO	<b>c1</b>	c00	37	c00	)61	Ме	et T	c09	)71	c50	)70	c01	18	Р	Κ	28	S
		Dist.	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.	Dist	S.E.
L 1	L 2	7.25	0.9	?	?	4.07	1.3	3.68	0.9	3.26	0.8	2.59	0.4	0.27	0.2	0.90	0.4	0.34	0.1
L 1	L 3	8.89	1.0	5.28	1.4	4.08	1.3	3.49	0.9	3.26	0.8	1.44	0.4	2.05	0.7	0.62	0.3	0.34	0.1
L 1	L 4	9.20	1.0	?	?	4.19	1.2	4.33	0.9	3.55	0.8	1.84	0.4	0.55	0.3	0.97	0.5	0.38	0.1
L 1	L 5	8.97	1.0	4.84	1.3	4.75	1.4	1.12	0.5	2.38	0.7	1.95	0.5	1.91	0.5	0.55	0.2	0.34	0.1
L 2	L 3	8.27	1.0	?	?	0.45	0.3	0.85	0.3	0.23	0.1	2.23	0.4	2.05	0.6	0.76	0.4	0.00	0.0
L 2	L 4	8.66	1.0	?	?	4.19	1.3	1.57	0.5	1.50	0.5	2.55	0.4	0.68	0.3	0.62	0.3	0.58	0.3
L 2	L 5	9.24	1.0	?	?	4.75	1.4	3.88	0.9	1.74	0.6	2.83	0.5	2.19	0.6	1.31	0.5	0.28	0.2
L 3	L 4	8.58	1.0	?	?	4.19	1.3	1.71	0.5	1.50	0.5	1.67	0.4	2.46	0.7	0.90	0.4	0.58	0.3
L 3	L 5	8.89	1.1	2.73	1.0	4.76	1.4	3.42	0.9	1.74	0.6	1.84	0.5	2.87	0.7	1.04	0.4	0.28	0.2
L 4	L 5	9.91	1.1	?	?	0.79	0.5	4.66	1.0	2.08	0.6	1.98	0.5	1.91	0.5	1.38	0.5	0.58	0.3
L 1	occi.	12.79	1.2	13.88	2.1	n.a.	n.a.	3.94	0.9	5.35	1.0	3.44	0.6	7.65	1.3	1.06	0.5	0.48	0.2
L 2	occi.	11.93	1.2	?	?	n.a.	n.a.	1.31	0.5	4.41	0.9	4.11	0.6	7.65	1.3	0.99	0.5	0.69	0.3
L 3	occi.	11.00	1.2	12.65	2.0	n.a.	n.a.	1.18	0.5	4.41	0.9	3.41	0.6	6.15	1.1	0.85	0.4	0.69	0.3
L 4	occi.	12.17	1.3	?	?	n.a.	n.a.	1.70	0.6	4.29	0.9	3.54	0.6	7.24	1.2	0.78	0.4	0.17	0.1
L 5	mard.	12.95	1.3	13.18	2.0	n.a.	n.a.	4.00	1.0	4.69	0.9	3.66	0.6	6.83	1.2	0.99	0.5	0.69	0.3
L 1	occi.	9.09	1.0			7.47	1.7												
L 2	occi.	9.71	1.0			6.11	1.6												
L 3	occi.	8.97	1.0			6.12	1.7												
L 4	occi.	10.84	1.1			6.67	1.7												
L 5	occi.	9.09	1.0			7.24	1.8												
mard.	occi.	11.31	1.1			n.a.	n.a.												

Appendix C: Paper 3

**Supplementary Table 1.** This is an enlarged version of Table 1, which besides information on the MtDNA Lineage and Genbank accession code of all the samples used in the study, also provides geographic location and taxonomic identity for all the samples used in the study. Coordinates are in the WGS84 datum, in decimal degrees. Identifications made by Gantenbein & Largiadèr (2003), are indicated by an **a**.

MtDNA	Scorpion Id	Taxonomy	Lat.	Long.	Count.	GenBank Code
inicage	Bom AG1	R mardocheii <sup>a</sup>	31 836	-9 400	Mo	A 1506880
	Bom HA12	B. mardocheii <sup>a</sup>	31 344	-7, <del>1</del> 00	Mo	A 1506807
A1	Boo CR1	B. maraochell B. occitanus <sup>a</sup>	33 57/	-7,743	Mo	A 15060072
	Sc008	B. Occurations	31 204	7 862	Mo	AJ300904
	Bom AG2a	B mardochaji <sup>a</sup>		-7,802		JQ775905
	Bom AG2h	B. mardocheii <sup>a</sup>	31,533	-9,572	Mo	AJ506881
	Bom AG2	B. mardocheii <sup>a</sup>	31,000	0.670	Mo	AJ300882
	Bom AG4	B. mardochell	20,500	-9,079	Mo	AJ500885
A2	S 2007	D. maraochell	21 496	-9,343	Mo	AJ300884
1 14	Sc007	Butnus sp.	31,480	-7,984	Mo	JQ775904
	Sc037	Butnus sp.	30,949	-8,118	Mo	JQ775914
	Sc039	Buthus sp.	32,672	-7,791	Mo	JQ775915
	Sc177	B. malhommei	33,778	-7,233	Mo	JQ775934
	Sc351	Buthus sp.	32,526	-7,863	Mo	JQ775952
	Bom AA1	B. mardocheii <sup>a</sup>	30,484	-8,994	Mo	AJ506873
	Bom AA2a	B. mardocheii <sup>a</sup>	30,400	-8,700	Mo	AJ506874
	Bom AA2b	B. mardocheii <sup>a</sup>			Mo	AJ506875
	Bom AA3	B. mardocheii <sup>a</sup>	29,721	-8,991	Mo	AJ506876
A3	Bom AA4	B. mardocheii <sup>a</sup>	29,754	-9,095	Мо	AJ506877
	Bom AA5b	B. mardocheii <sup>a</sup>	30,098	-8,472	Mo	AJ506879
	Sc065	Buthus sp.	30,783	-8,394	Mo	JQ775928
	Sc178	Buthus sp.	30,058	-9,087	Mo	JQ775935
	Sc191	Buthus sp.	29,743	-8,961	Mo	JQ775942
	Bom AA5a	B. mardocheii <sup>a</sup>	30,098	-8,472	Mo	AJ506878
A4	Bom HA2a	B. mardocheii <sup>a</sup>	31.148	-7.495	Mo	AJ506894
	Bom HA2b	B. mardocheii <sup>a</sup>			Мо	AJ506895
	Bom AS1a	B. mardocheii <sup>a</sup>	30 590	-7 227	Mo	AJ506885
	Bom AS1b	B. mardocheii <sup>a</sup>	50,590	-7,227	Mo	AJ506886
	Bom HA1b	B. mardocheii <sup>a</sup>	31,344	-7,743	Мо	AJ506893
	Bom HA4a	B. mardocheii <sup>a</sup>	21 297	7 291	Мо	AJ506898
	Bom HA4b	B. mardocheii <sup>a</sup>	51,207	-7,381	Mo	AJ506899
A5	Sc013	Buthus sp.	31,101	-7,914	Mo	JQ775909
	Sc043	Buthus sp.	30,942	-8,119	Mo	JQ775916
	Sc052	Buthus sp.	31,201	-7,855	Мо	JQ775921
	Sc055	Buthus sp.	31,094	-7,914	Мо	JQ775923
	Sc061	Buthus sp.	32,671	-5,453	Мо	JQ775925
	Sc064	Buthus sp.	30.953	-8.250	Мо	JO775927

MtDNA lineage	Scorpion Id	Taxonomy	Lat.	Long.	Count.	GenBank Code
	Sc214	Buthus sp.	30,743	-7,610	Мо	JQ775945
	Sc266	Buthus sp.	30,790	-7,586	Mo	JQ775946
A5	Sc277	Buthus sp.	31 291	-7 381	Mo	JQ775947
	Sc278	Buthus sp.	51,271	-7,501	Mo	JQ775948
	Sc331	Buthus sp.	31,112	-7,313	Mo	JQ775951
<b>B1</b>	Sc205	Buthus sp.	30,996	-5,816	Mo	JQ775944
	Bom HA3a	B. mardocheii <sup>a</sup>	30.018	6 024	Mo	AJ506896
	Bom HA3b	B. mardocheii <sup>a</sup>	50,710	-0,724	Mo	AJ506897
B2	Bom HA6a	B. mardocheii <sup>a</sup>	30 943	-7 123	Mo	AJ506901
	Bom HA6b	B. mardocheii <sup>a</sup>	50,745	7,125	Mo	AJ506902
	Sc047	Buthus sp.	30,972	-7,062	Mo	JQ775919
C1	Sc401	B. marieFrae	28,964	-9,999	Mo	JQ775957
<b>C</b> 2	Sc181	Buthus sp.	29,482	-10,087	Mo	JQ775937
C2	Sc185	Buthus sp.	29,512	-9,062	Mo	JQ775940
C3	Sc192	B. marieFrae	28,891	-9,777	Мо	JQ775943
	Bom DR1	B. mardocheii <sup>a</sup>	30,516	-6,905	Mo	AJ506887
	Bom DR2a	B. mardocheii <sup>a</sup>	30 738	-6 638	Mo	AJ506888
	Bom DR2b	B. mardocheii <sup>a</sup>	50,750	-0,050	Mo	AJ506889
	Bom DR3	B. mardocheii <sup>a</sup>	30,328	-5,849	Mo	AJ506890
	Bom DR4	B. mardocheii <sup>a</sup>	29,911	-7,302	Mo	AJ506891
C4	Sc066	B. draa	29,591	-8,001	Мо	JQ775929
	Sc182	B. draa	30 680	-6 425	Mo	JQ775938
	Sc183	B. draa	50,000	0,120	Mo	JQ775939
	Sc187	B. draa 2	28,686	-9,319	Mo	JQ775941
	Sc330	B. draa	30.568	-6.738	Mo	JQ775950
	Sc394	B. draa 2	00,000	0,700	Mo	JQ775956
	Sc370	Buthus sp.	36,832	4,090	Al	JQ775953
D1	Sc373	Buthus sp.	36,472	4,007	Al	JQ775954
	Sc405	Buthus sp.	35,398	1,332	Al	JQ775959
	Bot TA1	B. tunetatus <sup>a</sup>	32,523	8,054	Tu	AJ506916
	Bot TA2	B. tunetatus <sup>a</sup>	32,619	8,135	Tu	AJ506917
	Bot TA3	B. tunetatus <sup>a</sup>	32,618	8,054	Tu	AJ506918
	Bot TU1	B. tunetatus <sup>a</sup>	35,361	10,115	Tu	AJ506915
	Sc402	Buthus sp.	35,582	6,063	Al	JQ775958
	Sc407	Buthus sp.			Tu	JO775960
D2	Sc408	Buthus sp	33,847	10,831	Tu	10775961
	Sc409	Buthus sp.	35.483	8,744	Tu	10775962
	Sc+07	Dunus sp.	35.483	8,744	т.,	10775062
	SC410	Dutnus sp.	22,105		TU T	JU//3903
	Sc411	Buthus sp.	36,212	8,900	Tu	JQ775964
	Sc412	Buthus sp.	04.010	0.000	Tu	JQ775965
	Sc413	Buthus sp.	36,212	8,900	Tu	JO775966

MtDNA lineage	Scorpion Id	Taxonomy	Lat.	Long.	Cntr	GenBank Code
	Ba AC1	B. atlantis	31,508	-9,789	Мо	AJ506869
	Ba AC2a	B. atlantis	30 828	-9 810	Mo	AJ506870
	Ba AC2b	B. atlantis	50,020	9,010	Mo	AJ514323
	Ba AC3	B. atlantis	30,705	-9,849	Mo	AJ506871
	Ba AC4	B. atlantis	30,500	-9,633	Mo	AJ506872
D3	Bom HA5a	B. mardocheii <sup>a</sup>	31,197	-7,438	Mo	AJ506900
	Bom HA5b	B. mardocheii <sup>a</sup>	,	,	Mo	AJ507584
	Sc006	Buthus sp.	34,878	-4,611	Mo	JQ775903
	Sc023	Buthus sp.	34,025	-2,606	Mo	JQ775911
	Sc053	Buthus sp.	33,872	-3,039	Mo	JQ775922
	Sc376	Buthus sp.	35,170	2,217	Al	JQ775955
D4	Sc098	Buthus sp.	36,639	-5,248	Sp	GQ168523
	Sc099	B. occitanus			Sp	GQ168524
	Sc002	Buthus sp.	34,630	-5,538	Mo	JF820096
	Sc004	Buthus sp.	35,471	-6,031	Mo	FJ198056
	Sc010	Buthus sp.	•	•	Mo	JQ775906
D5	Sc011	Buthus sp.			Mo	JQ775907
	Sc012	Buthus sp.			Mo	JQ775908
	Sc014	Buthus sp.			Mo	JQ775910
	Sc050	Buthus sp.	34,053	-4,170	Mo	JQ775920
	Sc096	Buthus sp.	37,740	-2,569	Sp	GQ168522
	Boo IB1a	B. occitanus <sup>a</sup>	43,488	3,558	Fr	AJ506905
	Boo IB1b	B. occitanus <sup>a</sup>			Fr	AJ506906
	Boo IB2	B. occitanus <sup>a</sup>	43,183	3,000	Fr	AJ506907
D6	Boo IB3a	B. occitanus <sup>a</sup>	42,433	3,117	Fr	AJ506908
	Boo IB3b	B. occitanus <sup>a</sup>			Fr	AJ506909
	Boo IB4	B. occitanus <sup>a</sup>	42,050	2,582	Sp	AJ506910
	Boo IB6	B. occitanus <sup>a</sup>	36,831	-2,467	Sp	AJ517296
	EU523755	B. occitanus	<b>:</b>		<b>.</b>	EU523755
~-	Boo IB7a	B. occitanus <sup>a</sup>	36,534	-5,650	Sp	AJ517182
<b>D</b> 7	Boo IB7b	B. occitanus <sup>a</sup>			Sp	AJ517183
	Boo IB8	B. occitanus <sup>a</sup>	36,700	-5,417	Sp	AJ517184
	Boo IB5a	B. occitanus <sup>a</sup>	37,717	-7,600	Pt	AJ506911
	Boo IB5b	B. occitanus <sup>a</sup>	37,717		Pt	AJ506912
	Sc084	B. ibericus	38,130	-7,019	Pt	GQ168519
	Sc089	B. ibericus	38,052	-7,028	Pt	GQ168520
D8	Sc095	Buthus sp.	41,549	-6,231	Pt	GQ168521
	Sc100	B. ibericus	38,074	-7,046	Pt	GQ168525
	Sc104	Buthus sp.	41,439	-6,324	Pt	GQ168526
	Sc105	B. ibericus	10.075	-	Pt	GQ168527
	Sc106	B. ibericus	40,055	-7,193	Pt	GQ168528

MtDNA lineage	Scorpion Id	Taxonomy	Lat.	Long.	Cntr	GenBank Code
	Sc107	B. ibericus			Pt	GQ168529
	Sc108	B. ibericus	39,954	-7,119	Pt	GQ168530
	Sc109	B. ibericus			Pt	GQ168531
	Sc113	B. ibericus	37,055	-8,924	Pt	GQ168533
	Sc114	Buthus sp.	37,022	-8,924	Pt	GQ168534
	Sc115	B. ibericus	38,163	-8,579	Pt	GQ168535
D8	Sc116	B. ibericus	38,685	-8,346	Pt	GQ168536
Do	Sc112	B. ibericus	38 528	-8 004	Pt	GQ168532
	Sc120	B. ibericus	50,520	0,001	Pt	GQ168537
	Sc121	B. ibericus	37,186	-7,914	Pt	GQ168538
	Sc157	B. ibericus	39,433	-7,578	Pt	GQ168539
	Sc158	B. ibericus	39,512	-7,065	Pt	GQ168540
	Sc161	Buthus sp.	39,360	-4,358	Sp	GQ168541
	Sc190	B. ibericus	36,797	-6,378	Sp	GQ168542
<b>D</b> 9	Sc001	Buthus sp.	33,742	-4,832	Mo	FJ198055
D	Sc059	Buthus sp.	34,059	-5,357	Mo	JQ775924
	Bop MA1	B. paris <sup>a</sup>	31,566	-7,686	Mo	AJ506913
	Bop MA2	B. paris <sup>a</sup>	31,738	-7,029	Mo	AJ506914
	Sc029	Buthus sp.	31,660	-6,926	Mo	JQ775912
	Sc030	Buthus sp.			Mo	JQ775913
	Sc044	Buthus sp.	31,645	-7,115	Mo	JQ775917
	Sc045	Buthus sp.	32,219	-6,511	Mo	JQ775918
D10	Sc063	Buthus sp.	31,585	-7,317	Mo	JQ775926
	Sc071	Buthus sp.	34,233	-6,587	Mo	JQ775930
	Sc078	Buthus sp.	32,662	-5,499	Mo	JQ775931
	Sc174	B. marrocanus	33,778	-7,235	Mo	JQ775932
	Sc180	Buthus sp.	32,661	-7,793	Mo	JQ775936
	Sc176	Buthus sp.	34 025	-6717	Мо	JQ775933
	Sc329	Buthus sp.	57,023	0,717	Мо	JQ775949
	AF370829	A. australis				AF370829
	Sc292	A. mauritanicus	32.661	-7.792	Mo	JF820097



Appendix D: Paper 4

**Supplementary Table 4.9.** Sequences of cox1 mtDNA that compose the Dataset 3 (Dt3). They present the available animals that can be most parsimoniously attributed to *Buthus* species. Three criteria were used: 1) morphological identification, for those species that have readily identifiable characters; 2) geographical proximity, for species that were collected close to the species type locality. In areas where different *Buthus* species may occur in sympatry, we required less than 5km of proximity to the type locality, for species from areas where no conspecific have been reported (outside of Morocco), a proximity of up to 20 km was tolerated; and 3) a combination of the previous criteria. GenBank accession codes are given, together with the original source of the sequences when appropriate.

Taxa	ID	Oritorio	Country	mtDNA	Acession	Source	Notes
i axa	U	Criteria	Country	CLADE	Code	Source	Notes
B. atlantis	Ba AC1a*	Morphology? + Geography	Morocco	occitanus	AJ506869	Gantenbein & Largiadèr, 2003	= AC2b
B. atlantis	Ba AC2a	Morphology? + Geography	Morocco	occitanus	AJ506870	Gantenbein & Largiadèr, 2003	
B. atlantis	Ba AC2b	Morphology? + Geography	Morocco	occitanus	AJ514323	Gantenbein & Largiadèr, 2003	
B. atlantis	Ba AC3a	Morphology? + Geography	Morocco	occitanus	AJ506871	Gantenbein & Largiadèr, 2003	
B. atlantis	Ba AC4a	Morphology? + Geography	Morocco	occitanus	AJ506872	Gantenbein & Largiadèr, 2003	
B. aureus	Sc0402	Geography (< 15km)	Algeria	tunetanus	JQ775958	Sousa et al., 2012	
B. aureus	Sc0403	Geography (< 15km)	Algeria	tunetanus	KF824991	Pedroso et al., 2013	
B. awashensis	Sc2379	Morphology	Ethiopia	tunetanus	1	new	
B. bonito	Sc1534	Morphology	Morocco	rochati	1	new	
B. bonito	Sc1535	Morphology	Morocco	rochati	KF824985	Pedroso et al., 2013	
B. bonito	Sc1537	Morphology	Morocco	rochati	1	new	
B. boumalenii	Sc0491	Morphology + Geography	Morocco	boumalenii	KF824993	Pedroso et al., 2013	
B. chambiensis	Sc2497	Geography (< 5km)	Tunisia	tunetanus	1	new	
B. confluens	Sc2414	Morphology + Geography	Morocco	occitanus	1	new	
B. confluens	Sc2419*	Morphology + Geography	Morocco	occitanus	1	new	= Sc2420
B. confluens	Sc2420	Morphology + Geography	Morocco	occitanus	1	new	
B. confluens	Sc2423	Morphology + Geography	Morocco	occitanus	1	new	
B. confluens	Sc2424	Morphology + Geography	Morocco	occitanus	1	new	
B. draa	Sc0182	Morphology <sup>a</sup>	Morocco	rochati	JQ775938	Sousa <i>et al.</i> , 2012	
B. draa	Sc0183	Morphology <sup>a</sup>	Morocco	rochati	JQ775939	Sousa <i>et al.</i> , 2012	
B. draa	Sc0330	Morphology <sup>a</sup>	Morocco	rochati	JQ775950	Sousa <i>et al.</i> , 2012	
B. draa	Sc0394	Morphology <sup>a</sup>	Morocco	rochati	JQ775956	Sousa <i>et al.</i> , 2012	
B. draa	Sc0548	Morphology <sup>a</sup>	Morocco	rochati	KF824997	Pedroso et al., 2013	
B. draa	Sc0557*	Morphology <sup>a</sup>	Morocco	rochati	KF824998	Pedroso et al., 2013	= Sc0548
B. draa	Sc1505	Morphology <sup>a</sup>	Morocco	rochati	KF824981	Pedroso et al., 2013	
B. draa	Sc1506	Morphology <sup>a</sup>	Morocco	rochati	KF824982	Pedroso et al., 2013	
B. elmoutaouakili	09_6_1_BCO	Geography (< 4km)	Morocco	mardochei	JN831995	Husemann <i>et al.</i> , 2012	
B. elmoutaouakili	Sc0178	Geography (< 7km)	Morocco	mardochei	JQ775935	Sousa <i>et al.</i> , 2012	
B. kunti	Sc2569	Morphology	Cyprus	tunetanus	1	new	
B. lienhardi	Bom HA1a	Geography (< 20km)	Morocco	mardochei	AJ506892	Gantenbein & Largiadèr, 2003	East
B. lienhardi	Sc0008	Geography (< 1km)	Morocco	mardochei	JQ775905	Sousa <i>et al.</i> , 2012	East
B. lienhardi	Sc0013	Geography (< 15km)	Morocco	mardochei	JQ775909	Sousa <i>et al.</i> , 2012	West
B. lienhardi	Sc0052	Geography (< 1km)	Morocco	mardochei	JQ775921	Sousa <i>et al.</i> , 2012	West
B. lienhardi	Sc0055	Geography (< 15km)	Morocco	mardochei	JQ775923	Sousa <i>et al.</i> , 2012	West
B. malhommei	Sc0180	Morphology	Morocco	occitanus	JQ775936	Sousa <i>et al.</i> , 2012	
B. malhommei <sup>b</sup>	Sc2757	Morphology <sup>b</sup>	Morocco	occitanus	1	new	used as B. maroccanus
B. mariefranceae	Sc0192	Morphology <sup>c</sup>	Morocco	rochati	JQ775943	Sousa <i>et al.</i> , 2012	lineage 2
B. mariefranceae	Sc0401	Morphology <sup>c</sup>	Morocco	rochati	JQ775957	Sousa <i>et al.</i> , 2012	lineage 1
B. mariefranceae	Sc0778	Morphology <sup>c</sup>	Morocco	rochati	KF825002	Pedroso et al., 2013	lineage 1
B. maroccanus	Sc0174	Morphology <sup>c</sup>	Morocco	occitanus	JQ775932	Sousa <i>et al.</i> , 2012	
B. occitanus	Sc1843	Morphology + Geography	Spain	occitanus	1	new	
B. occitanus	Sc2026	Morphology + Geography	Spain	occitanus	1	new	
B. occitanus	Sc2365	Morphology + Geography	France	occitanus	1	new	
B. parroti	Sc1431	Morphology + Geography	Morocco	mardochei	KF824971	Pedroso et al., 2013	
B. pusillus	Sc0372	Geography (< 20km)	Algeria	tunetanus	1	new	
B. rochati	Sc2388	Morphology	Morocco	rochati	1	new	
B. sp.	Sc0955	n.a.	Egypt	tunetanus	1	new	
B. sp.	Sc0956	n.a.	Egypt	tunetanus	1	new	
B. sp.	Sc2570	n.a.	Jordan	tunetanus	1	new	
B. sp.	Sc2571	n.a.	Jordan	tunetanus	1	new	
B. sp.	Sc2590	n.a.	Israel	tunetanus	1	new	

**Supplementary Table 4.10.** Estimated Substitution Rates per marker in *Buthus*, obtained with Beast using uncorrelated relaxed clocks. Three analyses were calibrated with one biogeographic point (the Messinian Salinity Crisis): using a lognormal prior (IP LogN), an exponential prior (IP Exp), both at the separation of Iberia from North African samples or an exponential prior (Cyp Exp), at the separation of Cyprus from the mainland. The remaining three analyses were calibrated with published estimates of mitochondrial DNA substitutions rates: universal arthropod rates (UNI 1) (Papadopoulou et al., 2010), a universal flat arthropod rate (UNI 2) (Brower, 1994), and Mesobuthus scorpion calibrated rates (Gantenbein and Largiadèr, 2002; Gantenbein et al., 2005). The last three markers deviated from a molecular clock behaviour and were not discussed in the paper. a – Number of substitutions per site divided by tree length; b – Mean of branch rates. c – The SD of the branch rates (according to Papadopoulou et al., 2010).

Marker	Clock	Mean Rate <sup>a</sup>	ucld.mean <sup>b</sup>	ucld.stdev <sup>c</sup>	Coeff. Variance <sup>d</sup>	Covariance <sup>e</sup>
	IP LogN	0.0161 ± 0.0018	0.0156 ± 0.0018	0.2281 ± 0.1395	0.2309 ± 0.1435	-0.0097 ± 0.0674
	IP Exp	0.0187 ± 0.0027	0.0172 ± 0.0026	0.7173 ± 0.1341	0.7650 ± 0.1553	0.0586 ± 0.0747
168	UNI 1	0.0167 ± 0.0043	0.0161 ± 0.0041	0.2351 ± 0.1430	0.2386 ± 0.1475	-0.0093 ± 0.0680
105	UNI 2	0.0145 ± 0.0038	0.0138 ± 0.0035	0.3454 ± 0.1440	0.3552 ± 0.1534	-0.0104 ± 0.0675
	Сур Ехр	0.0101 ± 0.0014	0.0098 ± 0.0014	0.2739 ± 0.1482	0.2791 ± 0.1541	-0.0005 ± 0.0679
	Mesobuthus	0.0091 ± 0.0027	0.0089 ± 0.0026	0.2154 ± 0.1358	0.2178 ± 0.1394	-0.0089 ± 0.0681
	IP LogN	0.0135 ± 0.0014	0.0145 ± 0.0017	0.6409 ± 0.1201	0.6975 ± 0.1497	-0.0624 ± 0.0619
	IP Exp	0.0148 ± 0.0019	0.0154 ± 0.0024	0.8959 ± 0.1371	1.0275 ± 0.1854	-0.0088 ± 0.0701
001	UNI 1	0.0141 ± 0.0036	0.0155 ± 0.0039	0.6427 ± 0.1226	0.6948 ± 0.1502	-0.0623 ± 0.0612
COXI	UNI 2	0.0118 ± 0.0032	0.0127 ± 0.0033	0.6650 ± 0.1194	0.7275 ± 0.1512	-0.0693 ± 0.0598
	Сур Ехр	0.0086 ± 0.0011	0.0093 ± 0.0014	0.7368 ± 0.1200	0.8205 ± 0.1586	-0.0569 ± 0.0582
	Mesobuthus	$0.0075 \pm 0.0022$	0.0082 ± 0.0023	0.6600 ± 0.1200	0.7215 ± 0.1545	-0.0618 ± 0.0606
	IP LogN	0.0034 ± 0.0013	0.0037 ± 0.0011	1.0467 ± 0.2715	1.3151 ± 0.4815	-0.0172 ± 0.0653
	IP Exp	0.0032 ± 0.0010	$0.0034 \pm 0.0009$	0.8606 ± 0.3153	1.0315 ± 0.4831	-0.0206 ± 0.0653
c5070	UNI 1	0.0035 ± 0.0016	0.0039 ± 0.0015	1.0379 ± 0.2722	1.2991 ± 0.4804	-0.0165 ± 0.0649
C3070	UNI 2	0.0029 ± 0.0013	$0.0032 \pm 0.0012$	1.0154 ± 0.2706	1.2613 ± 0.4636	-0.0174 ± 0.0661
	Сур Ехр	0.0021 ± 0.0008	$0.0023 \pm 0.0007$	1.0107 ± 0.2724	1.2489 ± 0.4667	-0.0186 ± 0.0659
	Mesobuthus	$0.0019 \pm 0.0009$	$0.0021 \pm 0.0009$	1.0399 ± 0.2663	1.2969 ± 0.4662	-0.0164 ± 0.0670
	IP LogN	0.0011 ± 0.0002	$0.0016 \pm 0.0008$	1.0501 ± 0.5701	1.4757 ± 0.9924	-0.0013 ± 0.0692
	IP Exp	0.0011 ± 0.0003	$0.0019 \pm 0.0009$	1.3120 ± 0.4480	1.8801 ± 0.8507	-0.0026 ± 0.0694
DK	UNI 1	0.0011 ± 0.0004	0.0017 ± 0.0009	1.0037 ± 0.5916	1.4208 ± 1.0255	-0.0030 ± 0.0684
FN	UNI 2	$0.0009 \pm 0.0003$	$0.0014 \pm 0.0008$	1.1242 ± 0.5666	1.6086 ± 1.0049	-0.0063 ± 0.0649
	Сур Ехр	0.0006 ± 0.0001	$0.0008 \pm 0.0004$	0.6010 ± 0.5315	0.7759 ± 0.8361	-0.0038 ± 0.0673
	Mesobuthus	0.0006 ± 0.0002	$0.0009 \pm 0.0005$	0.9779 ± 0.5684	1.3647 ± 0.9739	-0.0008 ± 0.0683
	IP LogN	$0.0093 \pm 0.0070$	0.0187 ± 0.0058	1.9506 ± 0.1842	2.9815 ± 0.5520	-0.0244 ± 0.0513
	IP Exp	0.0093 ± 0.0056	0.0176 ± 0.0058	1.8956 ± 0.1894	2.8794 ± 0.5818	-0.0212 ± 0.0541
c0971	UNI 1	$0.0097 \pm 0.0080$	0.0194 ± 0.0079	1.9488 ± 0.1828	2.9938 ± 0.5620	-0.0249 ± 0.0496
00371	UNI 2	$0.0080 \pm 0.0058$	0.0160 ± 0.0064	1.9366 ± 0.1797	2.9507 ± 0.5548	-0.0238 ± 0.0537
	Сур Ехр	0.0058 ± 0.0041	0.0113 ± 0.0036	1.9017 ± 0.1842	$2.8900 \pm 0.5429$	-0.0215 ± 0.0516
	Mesobuthus	0.0052 ± 0.0041	0.0105 ± 0.0046	1.9524 ± 0.1891	2.9410 ± 0.5571	-0.0233 ± 0.0533
	IP LogN	0.0105 ± 0.0082	0.0140 ± 0.0045	1.9016 ± 0.2190	3.2996 ± 0.6368	-0.0157 ± 0.0528
	IP Exp	$0.0093 \pm 0.0059$	0.0118 ± 0.0041	1.7132 ± 0.2520	2.5842 ± 0.6430	-0.0450 ± 0.0447
c0061	UNI 1	0.0107 ± 0.0085	0.0145 ± 0.0056	1.9005 ± 0.2113	3.3555 ± 0.6458	-0.0139 ± 0.0514
00001	UNI 2	$0.0089 \pm 0.0081$	0.0120 ± 0.0052	1.8631 ± 0.2308	2.9144 ± 0.6125	-0.0181 ± 0.0556
	Сур Ехр	$0.0063 \pm 0.005$	0.0088 ± 0.0028	1.9095 ± 0.2117	3.3535 ± 0.6252	-0.0193 ± 0.0509
	Mesobuthus	0.0059 ± 0.0049	0.0078 ± 0.0033	1.8997 ± 0.2190	3.2908 ± 0.6426	-0.0150 ± 0.0539
	IP LogN	$0.0015 \pm 0.0008$	0.0015 ± 0.0010	1.7344 ± 0.3994	2.7357 ± 0.9883	0.0040 ± 0.0668
	IP Exp	$0.0015 \pm 0.0007$	0.0014 ± 0.0009	1.6986 ± 0.4100	2.6058 ± 0.9892	-0.0031 ± 0.0642
285	UNI 1	$0.0016 \pm 0.0009$	0.0015 ± 0.0010	1.7086 ± 0.3889	2.6961 ± 0.9616	0.0005 ± 0.0620
200	UNI 2	$0.0013 \pm 0.0007$	$0.0012 \pm 0.0009$	1.7079 ± 0.4103	2.6649 ± 0.9809	0.0023 ± 0.0644
	Сур Ехр	$0.0010 \pm 0.0004$	$0.0009 \pm 0.0005$	1.6958 ± 0.4001	2.6167 ± 0.9807	0.0016 ± 0.0649
	Mesobuthus	$0.0009 \pm 0.0005$	$0.0008 \pm 0.0006$	1.7381 ± 0.4010	2.7418 ± 0.9926	-0.0006 ± 0.0630



0.04

**Supplementary Figure 4.2.** RaxML phylogeny of the concatenated dataset displaying only the different haplotypes used. Support was calculated with 1.000 bootstraps. Highlighted in Yellow is the mardochei group, in Green the rochati group and in blue the Iberian clade.



0.03

**Supplementary Figure 4.3.** Mr Bayes phylogeny of the concatenated dataset. Support as a measure of bpp. Highlighted in Red is the boumalenii group, in Yellow the mardochei group, in Green the rochati group and in blue the Iberian clade. The Ethiopian sample has been highlighted in Salmon and the Cyprus sample in Light Green.

Appendix E: Paper 5

Sc	lineages	Cnt.	mtDNA	Lat	Long	cox1	16S	PK	c0971	c0061	c5070	28S
Sc1751	B. elongatus	Sp	occitanus	36.660	-5.100	yes			•	-		
Sc1752	B. elongatus	Sp	occitanus	36.660	-5.100	yes	yes	•	yes	yes	yes	
Sc2099	B. elongatus	Sp	occitanus	36.530	-4.900	yes	•					
Sc2100	B. elongatus	Sp	occitanus	36.530	-4.900	yes	yes	yes	yes	yes	yes	yes
Sc2101	B. elongatus	Sp	occitanus	36.530	-4.900	yes			-			
Sc2427	B. elongatus	Sp	occitanus	36.440	-5.160	yes	yes	yes	yes	yes	yes	yes
Sc1100r	B. ibericus	Sp	occitanus	36.390	-5.650	yes	yes	yes	yes	yes		
Sc1101	B. ibericus	Sp	occitanus	36.390	-5.650	yes	yes	yes	yes	yes	yes	
Sc1102	B. ibericus	Sp	occitanus	36.390	-5.650	yes						
Sc1103	B. ibericus	Sp	occitanus	36.520	-5.660	yes	yes	yes	yes	yes	yes	yes
Sc1106	B. ibericus	Sp	occitanus	36.520	-5.660	yes						
Sc1107	B. ibericus	Sp	occitanus	36.680	-5.420	yes	yes	yes	yes	yes	yes	yes
Sc1108	B. ibericus	Sp	occitanus	36.680	-5.420	yes					· .	
Sc1110	B. ibericus	Sp	occitanus	36.980	-4.660	yes	yes	yes	yes	yes	yes	yes
Sc1111	B. ibericus	Sp	occitanus	36.980	-4.660	yes						
Sc1722	B. ibericus	Sp	occitanus	36.620	-5.670	yes	yes		yes	yes	yes	
Sc1724	B. ibericus	Sp	occitanus	36.620	-5.670	yes						
Sc1731	B ibericus	Sn	occitanus	36 759	-5 805	yes	yes	yes	yes	yes		yes
Sc1732	B ibericus	Sn	occitanus	36 759	-5 805	ves	ves	ves	ves	ves	ves	
Sc1748	B ibericus	Sn	occitanus	36 920	-5 420	ves	ves	ves	,	ves	ves	
Sc1749	B ibericus	Sn	occitanus	36 920	-5 420	ves	ves	,	•	,	,	
Sc1750	B. ibericus	Sn	occitanus	36 770	-5 280	ves	,		•	· ·	· ·	
Sc2316	B ibericus	Sn	occitanus	36 740	-5.200	ves	•		•	· ·	•	
Sc2377	B ibericus	Sn	occitanus	36.020	-5.590	ves	•		•	· ·	•	
Sc2378	B. ibericus	Sn	occitanus	36.020	-5.500	Ves	•	· ·	•	· ·	· ·	
Sc2400	B. ibericus	Sp	occitanus	36.600	-5.350	ves	•	Ves	•	Ves	•	
Sc2400	B. ibericus	Sp	occitanus	36 760	-5.810	ves	•	yes	•	yes	•	
Sc2401	B ibericus	Sn	occitanus	36 760	-5.810	ves	•	•	•	· ·	· ·	
Sc2402	B ibericus	Sn	occitanus	36 760	-5.810	ves	•	•	•	· ·	· ·	
Sc1927	B. halius	Pt	occitanus	38 210	-8 460	ves			•	•	•	
Sc1996	B halius	Pt	occitanus	38 210	-8 460	ves	-		-			
Sc1896	B halius	Pt	occitanus	38,390	-8 260	ves	· ·		•			
Sc1897	B. halius	Pt	occitanus	38 390	-8 260	ves	· ·		•	· ·	· ·	
Sc1937	B halius	Pt	occitanus	38 510	-8.080	ves	-		-			
Sc1938	B halius	Pt	occitanus	38 510	-8.080	ves			-	-		
Sc1914	B halius	Pt	occitanus	38 440	-8 070	ves	· ·		•			
Sc1915	B halius	Pt	occitanus	38 440	-8 070	ves			-			
Sc1982	B halius	Pt	occitanus	38 520	-8.030	ves						
Sc1983	B halius	Pt	occitanus	38 520	-8 030	ves						
Sc1955	B. halius	Pt	occitanus	38.530	-8.020	ves						
Sc1956	B. halius	Pt	occitanus	38.530	-8.020	yes						
Sc1616	B. halius	Pt	occitanus	38.530	-8.000	yes	yes		yes	yes	yes	yes
Sc1617	B. halius	Pt	occitanus	38.530	-8.000	yes						
Sc1614	B. halius	Pt	occitanus	37.190	-7.910	yes	yes	yes	yes	yes	yes	
Sc1615	B. halius	Pt	occitanus	37.190	-7.910	yes	yes	yes	yes	yes	yes	yes
Sc2371	B. halius	Pt	occitanus	41.360	-7.810	yes	yes	yes	yes	yes	yes	yes
Sc2428	B. halius	Pt	occitanus	41.200	-7.800	yes	· .	•	•	•	· ·	
Sc2429	B. halius	Pt	occitanus	41.200	-7.800	yes						
Sc2126	B. halius	Pt	occitanus	41.670	-7.470	yes	•					
Sc2127	B. halius	Pt	occitanus	41.670	-7.470	yes	yes	yes	yes	yes	yes	yes
Sc1997	B. halius	Pt	occitanus	40.200	-7.430	yes	· .	•	•	•	· ·	
Sc1999	B. halius	Pt	occitanus	40.200	-7.430	yes	•					

Sc	lineages	Cnt.	mtDNA	Lat	Long	cox1	16S	PK	c0971	c0061	c5070	28S
Sc2123	B. halius	Pt	occitanus	40.140	-7.020	yes						
Sc2124	B. halius	Pt	occitanus	40.140	-7.020	yes	•	•				
Sc1690	B. halius	Sp	occitanus	37.470	-6.810	yes			-			
Sc1691	B. halius	Sp	occitanus	37.470	-6.810	yes						
Sc1680	B. halius	Sp	occitanus	40.160	-6.790	yes						
Sc2122	B. halius	Sp	occitanus	40.160	-6.790	yes			-			
Sc1096	B. halius	Sp	occitanus	37.890	-6.560	yes	yes	yes	yes	yes	yes	yes
Sc1097	B. halius	Sp	occitanus	37.890	-6.560	yes	yes	· .	yes	yes	yes	yes
Sc1689	B. halius	Sp	occitanus	38.410	-6.440	yes	yes	yes	yes	yes	yes	yes
Sc2119	B. halius	Sp	occitanus	39.700	-6.410	yes						-
Sc2120	B. halius	Sp	occitanus	39.700	-6.410	yes			-			
Sc2312	B. halius	Sp	occitanus	40.500	-6.360	ves						
Sc2314	B. halius	Sp	occitanus	40.500	-6.360	ves						
Sc1716	B halius	Sp	occitanus	36 790	-6 330	ves						
Sc1717	B halius	Sn	occitanus	36 790	-6.330	ves						
Sc1685	B halius	Sp	occitanus	38,980	-6.330	ves	-		-		-	
Sc2309	B halius	Sn	occitanus	40 900	-6.320	ves			-	-		
Sc2310	B. halius	Sn	occitanus	40.000	-6 320	ves	· ·		•	· ·	•	
Sc1713	B. halius	Sn	occitanus	38 100	-6 230	ves			-	· ·		
Sc1681	B. halius	Sn	occitanus	38 970	-6 230	ves	· ·		•	· ·	· ·	
Sc1683	B. halius	Sn	occitanus	38 970	-6 230	ves	· ·		•	· ·	· ·	
Sc1733	B. halius	Sn	occitanus	36 200	-5 930	ves	ves		•	· ·	· ·	
Sc1734	B. halius	Sn	occitanus	36 200	-5 930	ves	ves		-			
Sc2117n	B. halius	Sn	occitanus	39 460	-5.840	ves	,		•	· ·	· ·	
Sc2118	B. halius	Sn	occitanus	39.460	-5.840	ves	•	•	•	· ·	· ·	
Sc1031n	B. halius	Sn	occitanus	38 250	-5.670	ves	•	· ·	-	· ·	· ·	
Sc1693	B. halius	Sn	occitanus	37 / 00	-5.630	ves	•		•	· ·	•	
Sc1694	B. halius	Sp	occitanus	37.490	-5.030	Ves	•	· ·	-	· ·	· ·	
Sc2317	B. halius	Sn	occitanus	30,880	-5.570	Ves	•	· ·	-	· ·	· ·	
Sc2318	B. halius	Sp	occitanus	30,880	-5.570	ves	•	•	•	•	•	
Sc1665	D. halius	Sp	occitanus	39.000	5.570	Ves	•	•	-	•	•	
Sc1666	B. halius	Sp	occitanus	38 180	-5.500	Ves	•	•	-	•	•	
Sc1000	B. halius	Sp	occitanus	30.100	-5.300	ves	•	•	-	•	•	
Sc2324	B. Italius R. balius	Sp	occitanus	39.400	-5.420	Ves	•	•	-	•	•	
Sc2342	D. Italius P. holius	Sp	occitanus	40 330	-5.420	Ves						
Sc2342	B. Italius R. balius	Sp	occitanus	40.330	-5.420	yes	yes	yes	yes	yes	yes	•
Sc2113	B. Italius R. balius	Sp	occitanus	30 100	-5.420	Ves	•	•	•	•	·	
Sc2114	B. Italius R. balius	Sp	occitanus	30,100	-5.220	Ves	•	•	-	•	•	
Sc2337	B. halius	Sp	occitanus	40 300	-5.220	Ves		Ves	•			
Sc2330	D. halius	Sp	occitanus	40.300	5.010	Ves	ycs	yes	-	ycs	ycs	•
Sc1696	B. Italius R. balius	Sp	occitanus	39 190	-5.010	Ves	•	•	•	•	·	
Sc21030	B. Italius R. balius	Sp	occitanus	37 390	-5.010	Ves	•	•	-	•	•	
Sc2103	B. halius	Sp	occitanus	37.380	-4.090	Ves	•	•	-	•	•	
Sc2334	B. halius	Sp	occitanus	40.300	-4.030	Ves	•	•	-	•	•	
Sc1112n	B. halius	Sn	occitanus	36 060	_4 520	Ves		•	-	•	•	
Sc1114	B. halius	Sp	occitanus	36.020	-4.520	Ves	•	•	-	•	•	
Sc114	B. halius	Sp		36.020	-4.520	Vee	· ·	•	•	· ·	•	
Sc1115	B. halius	Sp Sp	occitarius	30,800	4.520	yes						VOC
Sc2320	B. halius	Sp Sp	occitarius	40 220	4.00	ves	yes	yes	yes	yes	yes	yes
Sc2105	D. nanus R. bolius	Sp Sp	occitarius	40.000	4.400	Vee	· ·	•	•	•	•	
Sc2105	B. halius	Sp		37 270	-4.270	Ves	· ·	•	•	· ·	· ·	
Sc2328	B. halius	Sp	occitanus	30 750	-4.210	Ves	· ·	•	•	•	•	

Sc	lineages	Cnt.	mtDNA	Lat	Long	cox1	16S	PK	c0971	c0061	c5070	28S
Sc2332	B. halius	Sp	occitanus	39.750	-4.210	yes			-	-		
Sc2347	B. halius	Sp	occitanus	40.540	-4.150	yes		•		-	•	
Sc2348	B. halius	Sp	occitanus	40.540	-4.150	yes						
Sc2344	B. halius	Sp	occitanus	40.900	-4.140	yes	yes	yes	yes	yes	yes	
Sc2111	B. halius	Sp	occitanus	38.840	-4.030	yes	yes	yes	yes	yes	yes	
Sc2112	B. halius	Sp	occitanus	38.840	-4.030	yes				-		
Sc1118	B. halius	Sp	occitanus	38.360	-3.760	yes				-		
Sc1119	B. halius	Sp	occitanus	38.360	-3.760	yes						
Sc2095n	B. halius	Sp	occitanus	38.100	-3.730	yes						
Sc2096	B. halius	Sp	occitanus	38.100	-3.730	yes						
Sc2380	B. halius	Sp	occitanus	38.390	-3.490	yes						
Sc2381	B. halius	Sp	occitanus	38.390	-3.490	yes						
Sc2090	B. halius	Sp	occitanus	38.820	-3.340	yes						
Sc2091	B. halius	Sp	occitanus	38.820	-3.340	yes						
Sc2087	B. halius	Sp	occitanus	38,520	-2.780	ves						
Sc2088	B. halius	Sp	occitanus	38.520	-2.780	ves						
Sc2082	B halius	Sp	occitanus	38 280	-2 720	ves	ves	ves	ves	ves	ves	
Sc2085	B halius	Sp	occitanus	38 280	-2 720	ves					,	
Sc2030	B halius	Sn	occitanus	41 170	-2 710	ves						
Sc2031	B halius	Sn	occitanus	41 170	-2 710	ves			-	-	-	
Sc2366	B halius	Pt	occitanus	Bóticas	Bóticas	Ves	· ·			-		
Sc2367	B. halius	Pt	occitanus	Marão	Marão	Ves	•		•	•	•	
Sc1128n	B. montanus	Sn	occitanus	37 180	-3.060	ves	Ves	Ves	Ves	Ves	Ves	
Sc1121	B. montanus	Sn	occitanus	37.100	-3.000	ves	yes	y 00	yco	yes	yes	•
Sc1121	B. montanus	Sp	occitanus	37.290	3 250	Ves						VAS
Sc1125	B. montanus	Sp	occitanus	37 190	3.060	Ves	Ves	yes	Ves	Ves	Ves	Ves
Se1420	B. montonuo	Sp	occitanus	37.100	-3.000	yes	yes		yes	yes	yes	yes
Sc1129	B. montonuo	Sp	occitanus	37.100	-3.000	yes	yes	yes	yes	yes	yes	yes
SC1130	B. montanus	Sp	occitanus	30.020	-3.300	yes		•				
Sc1590	B. montanus	Sp	occitanus	37.130	-3.210	yes	yes		yes	yes	yes	
Sc1591	B. montanus	Sp	occitanus	37.130	-3.210	yes	yes	yes	yes	yes	yes	yes
0.4500	B. montanus	Sp	occitanus	37.130	-3.210	yes	•	•	•	•	· ·	
SC1596	B. montanus	Sp	occitanus	37.110	-3.000	yes	•	•			•	
501597	B. montanus	Sp	occitanus	37.130	-3.210	yes	yes	yes	yes	yes	yes	yes
501598	B. montanus	Sp	occitanus	37.130	-3.210	yes	•	•	•	•	·	
Sc1605	B. montanus	Sp	occitanus	37.110	-3.000	yes	•	•	•	•	•	
SC16100	B. montanus	Sp	occitanus	37.090	-2.970	yes	•	•	•	•	•	
SC1612	B. montanus	Sp	occitanus	37.090	-2.970	yes	•	•	yes	yes	yes	•
Sc1613	B. montanus	Sp	occitanus	37.090	-2.970	yes	•	•	•		•	
Sc1653	B. montanus	Sp	occitanus	37.180	-3.060	yes	yes	yes	yes	yes	yes	•
Sc1654	B. montanus	Sp	occitanus	37.180	-3.060	yes	•	•	•	•	•	
Sc1660	B. montanus	Sp	occitanus	37.590	-1.340	yes	yes	yes	•	yes	•	•
Sc1664	B. montanus	Sp	occitanus	37.590	-1.340	yes	•	•	•	•	•	
Sc1674	B. montanus	Sp	occitanus	36.950	-3.170	yes	•	•	•	•	•	
Sc1676	B. montanus	Sp	occitanus	36.950	-3.170	yes	· .	·	•	•	· ·	
Sc1678	B. montanus	Sp	occitanus	36.950	-3.170	yes	· .	· .	•	•	· ·	
Sc1761	B. montanus	Sp	occitanus	36.960	-3.010	yes	· .	•	·	·	· ·	
Sc1762	B. montanus	Sp	occitanus	36.960	-3.010	yes	· .	•			· ·	
Sc1764	B. montanus	Sp	occitanus	36.950	-3.060	yes		•			· ·	
Sc1766	B. montanus	Sp	occitanus	36.950	-3.060	yes	· ·	•			· ·	
Sc1767	B. montanus	Sp	occitanus	36.950	-3.170	yes	. ·	· ·			· ·	
Sc1770	B. montanus	Sp	occitanus	37.540	-2.700	yes					· ·	
Sc1771	B. montanus	Sp	occitanus	37,540	-2.700	yes	ves	ves	ves	ves	ves	

Sc	lineages	Cnt.	mtDNA	Lat	Long	cox1	16S	PK	c0971	c0061	c5070	28S
Sc1784	B. montanus	Sp	occitanus	37.530	-2.690	yes			•			
Sc1786	B. montanus	Sp	occitanus	37.530	-2.690	yes		•				
Sc1788	B. montanus	Sp	occitanus	37.370	-3.150	yes						
Sc1789	B. montanus	Sp	occitanus	37.370	-3.150	yes						
Sc1791	B. montanus	Sp	occitanus	37.040	-2.410	yes			-			
Sc1792	B. montanus	Sp	occitanus	37.040	-2.410	yes						
Sc1795	B. montanus	Sp	occitanus	37.130	-2.590	yes	yes	yes	yes	yes	yes	
Sc1796	B. montanus	Sp	occitanus	37.130	-2.590	yes						
Sc1799	B. montanus	Sp	occitanus	37.170	-2.630	yes	yes	yes	yes	yes	yes	
Sc1800	B. montanus	Sp	occitanus	37.100	-2.100	yes						
Sc1801	B. montanus	Sp	occitanus	37.100	-2.100	yes						
Sc1805	B. montanus	Sp	occitanus	37.000	-2.070	yes						
Sc1806	B. montanus	Sp	occitanus	37.000	-2.070	ves						
Sc1807	B. montanus	Sp	occitanus	36,980	-1.910	ves						
Sc1808	B montanus	Sn	occitanus	36,980	-1 910	ves						
Sc1811	B montanus	Sn	occitanus	37 530	-1 810	ves						
Sc1812	B montanus	Sn	occitanus	37 530	-1 810	ves			-			
Sc1845	B montanus	Sn	occitanus	Bco Es	Bco Es	ves			•			
Sc1846	B. montanus	Sn	occitanus	Bco Es	Bco Es	ves			•			
Sc1849	B. montanus	Sn	occitanus	Bco Es	Bco Es	Ves	· ·		•	•	•	
Sc1850	B. montanus	Sn	occitanus	Bco Es	Bco Es	Ves	· ·		•	•	•	
Sc0439	Granada	Sn	occitanus	37 100	-3 400	ves	ves	Ves	•	Ves	Ves	Ves
Sc044	Granada	Sp	occitanus	37.100	-3.400	ves	yco	y 00	•	yes	yco	yes
Sc0441	Granada	Sp	occitanus	37.100	-3.400	Ves	•	•	-	•	•	
Sc1040n	Granada	Sp	occitanus	37.370	-3.470	Ves	•	•	-	•	•	
Sc1601	Granada	Sp	occitanus	27.020	-3.470	yes						
Se1602	Granada	Sp	occitanus	27.020	-3.320	Ves	ycs	yes	ycs	ycs	ycs	•
Sc1602	Granada	Sp	occitanus	37.020	-3.320	yes						
Sc1003	Granada	Sp	occitanus	26.000	-3.320	yes	yes	yes	yes	yes	yes	
Sc1754	Granada	Sp	occitanus	26,000	-3.990	yes	yes	yes	•	yes	yes	yes
Sc1755	Granada	Sp	occitanus	27.050	-3.990	yes	•	•	•	•	•	
Sc1750	Granada	Sp	occitarius	37.050	-3.040	yes						
Sc1759	Granada	Sp	occitanus	37.000	-3.040	yes	yes	yes	yes	yes	yes	yes
Sc2097	Granada	Sp	occitarius	37.090	-3.510	yes	•	•	•	•	•	
Sc2090	Granada	Sp	occitanus	37.690	-3.510	yes	·	•	-	•	•	
Sc1030	B. Occitarius	Sp	occitarius	40.110	-0.730	yes	•	•	-	•	· ·	
Sc1039	B. Occitarius	Sp	occitarius	40.110	-0.730	yes						
SC1043	B. occitanus	Sp	occitanus	39.920	-0.020	yes	yes	yes	yes	yes	yes	yes
Sc1044	B. occitanus	Sp	occitanus	39.920	-0.020	yes	yes	yes	yes	yes	yes	yes
Sc2001	B. Occitarius	Sp	occitarius	41.300	1.790	yes	•	yes	yes	yes	yes	•
502002	B. occitanus	Sp	occitanus	41.360	1.790	yes	·	•	-	•	•	
SC2005	B. occitanus	Sp	occitanus	41.140	1.320	yes	·	•	•	•	·	
SC2006	B. occitanus	Sp	occitanus	41.140	1.320	yes	·	•	-	•	•	
502009	B. occitanus	Sp	occitanus	40.970	0.430	yes	·	•	-	•	•	
502010	B. occitanus	Sp	occitanus	40.970	0.430	yes	· ·	•	•	•	· ·	
SC2013	B. occitanus	Sp	occitanus	40.910	0.320	yes	•	•	•	•	·	
Sc2014	B. occitanus	Sp	occitanus	40.910	0.320	yes	•	•	•	•	· ·	
Sc2017	B. occitanus	Sp	occitanus	40.770	0.590	yes	yes	yes	yes	yes	yes	· ·
Sc2019	B. occitanus	Sp	occitanus	40.770	0.590	yes	•	•	-	•	•	
Sc2021	B. occitanus	Sp	occitanus	40.990	-0.050	yes	·	· ·	-	•	•	
Sc2022	B. occitanus	Sp	occitanus	40.990	-0.050	yes	·	•	-	•	•	
Sc2024	B. occitanus	Sp	occitanus	40.810	-0.620	yes	•	•	-	· ·	· .	
Sc2025	B. occitanus	Sp	occitanus	40.810	-0.620	yes	.		-		.	

Sc	lineages	Cnt.	mtDNA	Lat	Long	cox1	16S	PK	c0971	c0061	c5070	28S
Sc2026	B. occitanus	Sp	occitanus	40.950	-1.300	yes	yes	yes	yes	yes	yes	
Sc2029	B. occitanus	Sp	occitanus	40.950	-1.300	yes		•				
Sc2058	B. occitanus	Sp	occitanus	39.880	-1.340	yes	yes	yes	yes	yes	yes	
Sc2059	B. occitanus	Sp	occitanus	39.880	-1.340	yes			•	•		
Sc2061	B. occitanus	Sp	occitanus	39.420	-0.800	yes				-		
Sc2062	B. occitanus	Sp	occitanus	39.420	-0.800	yes	yes	yes	yes	yes	yes	
Sc2349	B. occitanus	Sp	occitanus	42.250	-2.090	yes		•			•	
Sc2358	B. occitanus	Sp	occitanus	42.180	-1.530	yes	yes	yes	yes	yes	yes	
Sc2359	B. occitanus	Sp	occitanus	42.180	-1.530	yes						
Sc2364	B. occitanus	France	occitanus	43.490	3.560	yes						
Sc2365	B. occitanus	France	occitanus	43.490	3.560	yes	yes	yes	yes	yes	yes	yes
Sc2430	B. occitanus	Sp	occitanus	41.510	0.720	yes						
Sc1027	Jucar	Sp	occitanus	38.260	-1.190	yes						
Sc1813	Jucar	Sp	occitanus	37.820	-1.580	yes						
Sc1816	Jucar	Sp	occitanus	37.820	-1.580	yes	yes	yes	yes	yes	yes	
Sc1834	Jucar	Sp	occitanus	38.640	-0.860	yes	yes	yes	yes	yes	yes	yes
Sc1836	Jucar	Sp	occitanus	38.640	-0.860	yes	yes	yes	yes	yes		yes
Sc2052	Jucar	Sp	occitanus	40.230	-2.290	yes				•		
Sc2053	Jucar	Sp	occitanus	40.230	-2.290	yes		•	•	•	•	
Sc2063	Jucar	Sp	occitanus	39.610	-1.240	yes		•	•	•	•	
Sc2064	Jucar	Sp	occitanus	39.610	-1.240	yes		•	•	•	•	
Sc2068	Jucar	Sp	occitanus	39.540	-1.820	yes		•	•		•	
Sc2069	Jucar	Sp	occitanus	39.540	-1.820	yes		•			•	
Sc2072	Jucar	Sp	occitanus	39.090	-1.050	yes		•			•	
Sc2073	Jucar	Sp	occitanus	38.680	-1.020	yes		•			•	
Sc2074	Jucar	Sp	occitanus	38.680	-1.020	yes	yes	yes	yes	yes	yes	
Sc2079	Jucar	Sp	occitanus	38.490	-1.360	yes	yes	yes	yes	yes	yes	•
Sc2080	Jucar	Sp	occitanus	38.490	-1.360	yes		•			•	
Sc2081	Jucar	Sp	occitanus	38.500	-1.640	yes	yes	yes	yes	yes	yes	yes
Sc2092	Jucar	Sp	occitanus	39.120	-2.830	yes	•	•			•	
Sc2094	Jucar	Sp	occitanus	39.120	-2.830	yes	yes	yes	yes	yes	•	•
Sc2406	B. confluens	Мо	occitanus	34.300	-5.290	yes	yes	yes	yes	yes	yes	yes
Sc2413	B. confluens	Мо	occitanus	34.920	-5.540	yes	•	•	•	•	•	
Sc2414	B. confluens	Мо	occitanus	34.920	-5.540	yes	yes	yes	yes	•	yes	yes
Sc2416	B. confluens	Мо	occitanus	35.080	-5.310	yes	•	•	•	•	•	
Sc2418	B. confluens	Мо	occitanus	35.080	-5.310	yes	•	•	•	•	•	
Sc2419	B. confluens	Мо	occitanus	35.400	-5.370	yes	yes	yes	yes	yes	yes	yes
Sc2420	B. confluens	Mo	occitanus	35.400	-5.370	yes	yes	yes	yes	yes	yes	yes
Sc2421	B. confluens	Mo	occitanus	35.520	-5.710	yes	•	•	•	•	•	
Sc2423	B. confluens	Mo	occitanus	35.310	-6.030	yes	yes	yes	yes	yes	yes	yes
Sc2424	B. confluens	Mo	occitanus	35.310	-6.030	yes	yes	yes	yes	yes	yes	yes
Sc2425	B. confluens	Mo	occitanus	35.560	-5.490	yes	•	•	•	•	· ·	
Sc2426	B. confluens	Mo	occitanus	35.560	-5.490	yes	•	•	•	•	· ·	
Sc2580	B. confluens	Mo	occitanus 	35.790	-5.900	yes	•	•	·			
Sc2581	B. confluens	Mo	occitanus	35.790	-5.900	yes	•	•	•	•	•	
Sc1548	В. sp.1	Mo	mardochei	30.160	-8.480	yes	yes	yes	yes	•	yes	yes
Sc1568	в. sp.2	Mo	mardochei	29.770	-9.140	yes	yes	yes	yes	•	yes	yes
Sc2408	Androctonus	Mo	Outgroup			yes	yes	yes	yes	•	· ·	yes
Sc2591	Compobuthus	Oman	Outgroup			yes	yes	yes	yes	•	•	•
502523		Grece	Outgroup			yes	yes		•	•	•	·
502520	Wesoputhus	Grece	Outgroup			yes	yes	yes	•	•	•	·
Sc2405	Scorpio	MO	Outgroup			yes	yes		•	•	· ·	· · ·

**Supplementary Table 5.12.** Node age estimates, Mean and 95% High Posterior Distributions (HPD), obtained in the Beast analysis. Node numbers according to Figure 4. n - Clade without bpp support.

Node	Mean	95% HPD	Age	Epoch
1	5.57	5.3 - 6.44	Messinian	Miocene
<b>2</b> <sup>n</sup>	5.08	4.35 - 5.91	Messinian/Zanclean	Miocene/Pliocene
3	4.8	4.18 - 5.61	Messinian/Zanclean	Miocene/Pliocene
<b>4</b> <sup>n</sup>	4.51	3.88 - 5.27	Zanclean	Pliocene
5	4.16	3.58 - 4.92	Zanclean	Pliocene
6	3.93	3.25 - 4.73	Piacenzian/Zanclean	Pliocene
<b>7</b> <sup>n</sup>	3.87	3.24 - 4.59	Piacenzian/Zanclean	Pliocene
8	3.32	2.73 - 4.05	Piacenzian/ Zanclean	Pliocene
9	2.37	1.88 - 2.94	Gelasian/Piacenzian	Pliocene/Pleistocene
10	2.3	1.87 - 2.81	Gelasian/Piacenzian	Pliocene/Pleistocene
11	1.46	1.11 - 1.86	Calabrian/Gelasian	Pleistocene
12	1.43	1.08 - 1.85	Calabrian/Gelasian	Pleistocene
13	1.33	1.02 - 1.71	Calabrian	Pleistocene
14	1.17	0.85 - 1.53	Calabrian	Pleistocene
15	1.14	0.81 - 1.52	Calabrian	Pleistocene
16	0.96	0.70 - 1.25	Calabrian/Middle Pleist.	Pleistocene
17	0.89	0.63 - 1.22	Calabrian/Middle Pleist.	Pleistocene
18	0.85	0.61 - 1.14	Calabrian/Middle Pleist.	Pleistocene

**Supplementary Table 5.13.** Presence points of European *Buthus* used to construct the Species Distribution Models. We have not differentiated here between training and the validating datasets.

Source	Species	Lat	Long	Source	Species	Lat	Long
Armas & González-Moliné, 2009	B. halius	37.292	-6.738	New, this work	B. halius	38.361	-3.755
OBS - PSousa	B. halius	40.616	-6.424	New, this work	B. halius	38.528	-8.004
Rossi, 2012	B. halius	41.346	-8.320	New, this work	B. halius	38.179	-5.503
Rossi, 2012	B. halius	41.346	-8.320	New, this work	B. halius	40.157	-6.788
Rossi, 2012	B. halius	41.354	-8.344	New, this work	B. halius	38.968	-6.227
Rossi, 2012	B. halius	41.357	-8.344	New, this work	B. halius	38.979	-6.328
Rossi, 2012	B. halius	41.347	-8.319	New, this work	B. halius	38.414	-6.443
Gantenbein and Largiadèr 2003	B. halius	37.717	-7.600	New, this work	B. halius	37.468	-6.807
OBS - PSousa	B. halius	37.200	-7.926	New, this work	B. halius	37.487	-5.625
OBS - Cristiana Marques	B. halius	41.258	-7.780	New, this work	B. halius	37.991	-4.794
Teruel and Pérez-Bote 2005	B. halius	39.409	-5.409	New, this work	B. halius	38.178	-5.007
Cardoso, 2004	B. halius	37.749	-7.800	New, this work	B. halius	38.097	-6.230
Cardoso, 2004	B. halius	37.661	-7.488	New, this work	B. halius	36.794	-6.333
OBS	B. halius	40.540	-7.038	New, this work	B. halius	36.203	-5.931
OBS - PSousa	B. halius	40.188	-7.444	New, this work	B. halius	38.388	-8.261
OBS - PSousa	B. halius	41.201	-7.802	New, this work	B. halius	38.437	-8.067
OBS - PSousa	B. halius	37.095	-8.951	New, this work	B. halius	38.206	-8.456
Sousa et al. 2010	B. halius	38.130	-7.019	New, this work	B. halius	38.515	-8.077
OBS - PSousa	B. halius	40.418	-7.456	New, this work	B. halius	38.528	-8.019
OBS - PSousa	B. halius	40.241	-7.560	New, this work	B. halius	38.518	-8.032
OBS - PSousa	B. halius	41.283	-7.393	New, this work	B. halius	40.199	-7.435
OBS - PSousa	B. halius	37.576	-7.535	New, this work	B. halius	41.174	-2.707
Sousa et al. 2010	B. halius	38.052	-7.028	New, this work	B. halius	38.285	-2.716
OBS - PSousa	B. halius	37.031	-8.952	New, this work	B. halius	38.525	-2.782
OBS - PSousa	B. halius	41.228	-7.419	New, this work	B. halius	38.821	-3.340
OBS - PSousa	B. halius	41.345	-7.281	New, this work	B. halius	38.103	-3.727
Sousa et al. 2010	B. halius	41.549	-6.231	New, this work	B. halius	37.383	-4.694
Sousa et al. 2010	B. halius	38.074	-7.046	New, this work	B. halius	37.368	-4.268
OBS - PSousa	B. halius	39.669	-7.060	New, this work	B. halius	38.844	-4.027
OBS - PSousa	B. halius	39.741	-7.474	New, this work	B. halius	39.185	-5.217
Sousa et al. 2010	B. halius	41.439	-6.324	New, this work	B. halius	39.457	-5.845
Sousa et al. 2010	B. halius	40.055	-7.193	New, this work	B. halius	39.698	-6.409
Sousa et al. 2010	B. halius	39.954	-7.119	New, this work	B. halius	40.143	-7.024
OBS - PSousa	B. halius	37.821	-7.618	New, this work	B. halius	41.674	-7.465
Sousa et al. 2010	B. hallus	37.055	-8.924	New, this work	B. hallus	40.900	-6.319
	B. halius	37.022	-8.924		B. halius	40.504	-6.362
	B. hallus	38.163	-8.579	New, this work	B. hallus	39.884	-5.569
	B. halius	38.685	-8.346	New, this work	B. halius	39.401	-5.424
OBS - PSousa	B. hallus	38.090	-7.280	New, this work	B. hallus	39.605	-4.513
CBS - PSousa	B. nalius	30.021	-0.102	New, this work	B. halius	39.752	-4.215
	B. nalius	37.100	-7.914	New, this work	B. nalius	40.334	-4.402
OBS - FSOUSA	B. Italius	20 165	-0.340	New, this work	B. halius	40.303	-4.013
Sousa et al. 2010	B. halius	30.100	-0.505	New, this work	D. Italius B. balius	40.302	-5.009
	B. halius	30 512	7.065	New, this work	B. halius	40.003	-3.423
	B. halius	30,630	4 666	New, this work	B. halius	40.903	4.157
Sousa et al. 2010	B halius	30 360	-4.358	New this work	B halius	38 301	-3 /07
Sousa et al. 2010	B halive	36 707	-6 378	New this work	B halius	38 301	-3.497
OBS - PSousa	B halive	41 472	-7 624	New this work	B halive	41 201	-7 802
New this work	B halius	38 253	-5.668	Gantenbein and Largiadèr 2004	B ibericue	36 534	-5.650
New this work	B halive	37 888	-6 562	Gantenbein and Largiader 2004	B ibericus	36 700	-5 417
New, this work	B. halius	36,962	-4.519	OBS - Iñigo Sánchez	B. ibericus	36.619	-5.665
New, this work	B. halius	36.984	-4.517	OBS - Iñigo Sánchez	B. ibericus	36.618	-5.664

**Supplemental Table 5.3 (Cont.).** Presence points of European *Buthus* used to construct the Species Distribution Models. We have not differentiated here between training and validating datasets.

Source	Species	Lat	Long	Source	Species	Lat	Long
OBS - Iñigo Sánchez	B. ibericus	36.625	-5.661	Dupré et al. 2008	B. occitanu:	43.908	5.360
New, this work	B. ibericus	36.631	-5.664	Dupré et al. 2008	B. occitanu:	43.138	3.093
New, this work	B. ibericus	36.388	-5.651	Dupré et al. 2008	B. occitanu:	43.342	6.393
New, this work	B. ibericus	36.518	-5.656	Dupré et al. 2008	B. occitanu:	43.932	4.729
New, this work	B. ibericus	36.685	-5.424	Dupré et al. 2008	B. occitanu:	43.446	3.632
New, this work	B. ibericus	36.975	-4.664	Dupré et al. 2008	B. occitanu:	43.232	5.387
New, this work	B. ibericus	36.625	-5.668	Dupré et al. 2008	B. occitanu:	43.631	3.277
New, this work	B. ibericus	36.759	-5.805	Dupré et al. 2008	B. occitanu:	42.735	2.680
New, this work	B. ibericus	36.922	-5.417	Dupré et al. 2008	B. occitanu:	44.225	4.733
New, this work	B. ibericus	36.774	-5.277	Dupré et al. 2008	B. occitanu:	43.624	3.362
New, this work	B. ibericus	36.017	-5.589	Dupré et al. 2008	B. occitanu:	43.814	3.759
New, this work	B. ibericus	36.602	-5.854	Dupré et al. 2008	B. occitanu:	42.739	2.446
New, this work	B. montanus	36.961	-3.005	Dupré et al. 2008	B. occitanu:	43.923	5.485
Sousa et al. 2010	B. montanus	37.740	-2.569	Dupré et al. 2008	B. occitanu:	43.942	5.131
New, this work	B. montanus	37.427	-2.718	New, this work	B. occitanu:	41.282	1.835
New, this work	B. montanus	37.283	-3.252	New, this work	B. occitanu:	40.112	-0.727
New, this work	B. montanus	37.178	-3.056	New, this work	B. occitanu:	39.918	-0.016
New, this work	B. montanus	36.818	-3.299	New, this work	B. occitanu:	41.361	1.787
New, this work	B. montanus	37.127	-3.214	New, this work	B. occitanu:	41.141	1.317
New, this work	B. montanus	37.108	-2.999	New, this work	B. occitanu:	40.971	0.430
New, this work	B. montanus	37.114	-3.188	New, this work	B. occitanu:	40.912	0.320
New, this work	B. montanus	36.760	-2.127	New, this work	B. occitanu:	40.765	0.589
New, this work	B. montanus	37.094	-2.969	New, this work	B. occitanu:	40.993	-0.053
New, this work	B. montanus	37.179	-3.057	New, this work	B. occitanu:	40.810	-0.622
New, this work	B. montanus	37.588	-1.339	New, this work	B. occitanu:	40.947	-1.303
New, this work	B. montanus	36.961	-3.007	New, this work	B. occitanu:	39.877	-1.337
New, this work	B. montanus	36.951	-3.057	New, this work	B. occitanu:	39.419	-0.803
New, this work	B. montanus	36.955	-3.166	New, this work	B. occitanu:	42.249	-2.090
New, this work	B. montanus	37.538	-2.697	New, this work	B. occitanu:	42.180	-1.527
New, this work	B. montanus	37.532	-2.693	New, this work	B. occitanu:	43.490	3.555
New, this work	B. montanus	37.370	-3.153	New, this work	B. occitanu:	41.511	0.721
	B. montanus	37.041	-2.405	OBS - Luis Chueca	B. occitanus	42.078	-1.729
New, this work	B. montanus	37.128	-2.592	OBS - Luis Chueca	B. occitanus	41.962	-1.615
New, this work	B. montanus	37.169	-2.031	Dupré et al. 2008	B. occitanus	43.318	2.562
New, this work	B. montanus	37.099	-2.104	ODS DSauge	B. occitanus	43.807	3.954
New, this work	B. montanus	30.990	-2.000	OBS - PSousa	Jucar	37.020	-1.505
New, this work	B. montanus	27 527	1 906	New, this work	Jucar	20 765	-1.190
New, this work	B. montanus	36 720	-1.000	New the work	Jucar	38 630	-0.850
	B. montanus	12 337	3 002	New, this work	Jucar	40 231	-0.009
	B. occitanus	42.007	3.054	New this work	Jucar	39 607	-1 240
Dupré et al. 2008	B. occitanus	42.436	3.070	New this work	Jucar	39 535	-1.240
Dupré et al. 2008	B. occitanus	43 835	4 611	New this work	Jucar	39.000	-1.053
Dupré et al. 2008	B. occitanus	44 126	5 173	New this work	Jucar	38 677	-1 019
Gantenbein and Largiadèr 2006	B. occitanus	43 488	3 558	New this work	Jucar	38 488	-1.360
Gantenbein and Largiader 2000	B. occitanus	43,183	3.000	New, this work	Jucar	38,497	-1.645
Gantenbein and Largiader 2007	B. occitanus	42 433	3 117	New this work	Jucar	39 121	-2 830
Gantenbein and Largiader 2009	B. occitanus	42.050	2.582		- 4041	50.121	2.000
Dupré et al. 2008	B. occitanus	42,771	2.784				
Dupré et al. 2008	B. occitanus	42,528	2.400				
Dupré et al. 2008	B. occitanus	43,535	6.139				
OBS - Enric Planas	B. occitanus	41.511	0.721				
Dupré et al. 2008	B. occitanus	43.721	4.713				



**Supplementary Figure 5.4.** TCS network of the *cox1* mtDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. The network was visualised with TCSBU.



**Supplementary Figure 5.5.** TCS network of the **16S** mtDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. In red are mutational steps that were omitted for a clear presentation, but that connect the networks. The network was visualised with TCSBU.



**Supplementary Figure 5.6.** TCS network of the **Protein Kinase-like** nuDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. The network was visualised with TCSBU.



**Supplementary Figure 5.7.** TCS network of the **28S** nuDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. The network was visualised with TCSBU.



**Supplementary Figure 5.8.** TCS network of the **c0971** nuDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. In red are mutational steps that were omitted for a clear presentation, but that connect the networks. The network was visualised with TCSBU.



**Supplementary Figure 5.9.** TCS network of the **c0061** nuDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. The network was visualised with TCSBU.



**Supplementary Figure 5.10.** TCS network of the **c5070** nuDNA marker constructed with a user specified maximum connection steps equal to 30, and with Gaps treated as fifth state. The network was visualised with TCSBU.


**Supplementary Figure 5.11.** Phylogram of the **concatenated nuDNA** made with MrBayes. Support is the PP.



Supplementary Figure 5.12. Ultrametric \*Beast tree. Support is the PP.



**Supplementary Figure 5.13.** Maps of the predicted areas of occurrence for the five Iberian *Buthus* species, projected for the three different past Global Climate Models (CCSM4, MIROC-ESM, and MPI-ESM-P), and used to construct the Last Glacial Maximum period projection (L.G.M., 22ka) used in subsequent analyses.



**Supplementary Figure 5.14.** Maps of the predicted areas of occurrence of *B. ibericus* and *B. halius*, modelled for the Present time (1950-2000 CE), and projected for the Last Inter-Glacial (L.I.G., 130ka) and Last Glacial Maximum (L.G.M., 22ka) time periods. The predicted area of persistence throughout the entire period of time modelled is also presented for the entire study area.



**Supplementary Figure 5.15.** Maps of the predicted areas of occurrence of *B. occitanus*, the Jucar lineage and *B. montanus*, modelled for the Present time (1950-2000 CE), and projected for the Last Inter-Glacial (L.I.G., 130ka) and Last Glacial Maximum (L.G.M., 22ka) time periods. The predicted area of persistence throughout the entire period of time modelled is also presented for the entire study area.

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