Integrating ecological, morphological and genetic variability analyses to identify evolutionary units within *Vipera latastei-monticola*

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O Presidente do Júri,

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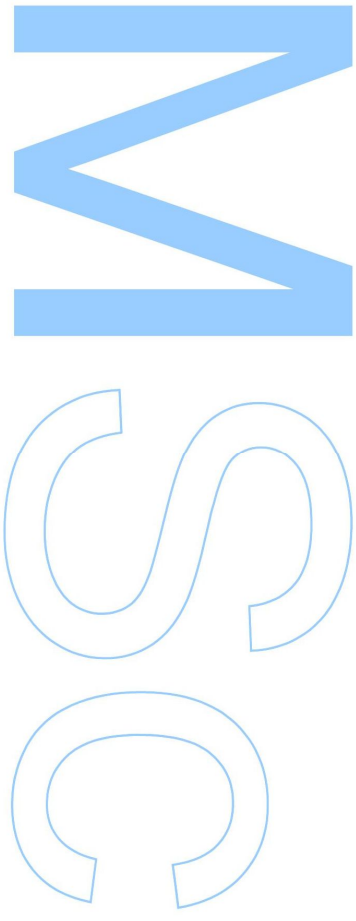

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Resumo

Os eventos paleogeográficos e climáticos que ocorreram no passado moldaram os padrões actuais de biodiversidade da região Mediterrânica ocidental, resultando na elevada variabilidade e diferenciação encontrada em vários taxa. Vários estudos têm usado répteis e anfíbios como modelos para relacionar estes eventos com os padrões de diferenciação genética e morfológica; como tal estes grupos têm sido alvo de importantes revisões taxonómicas.

A víbora-cornuda *Vipera latastei* habita áreas de clima Mediterrânico na Península Ibérica e Norte de África, evidenciando elevada diferenciação genética e morfológica nas duas regiões. No entanto, a taxonomia deste grupo continua por avaliar, impedindo a designação de unidades de conservação nesta espécie vulnerável.

O objetivo deste estudo foi investigar a variabilidade intraespecífica do complexo de espécies *Vipera latastei-monticola* e definir unidades taxonómicas e de conservação recorrendo a uma abordagem integrativa que combina análises da variabilidade genética (análises filogenéticas e de estrutura populacional), morfológica (análises univariadas e multivariadas) e ecológica (modulação de nichos ecológicos e testes de sobreposição de nichos).

Os genes mitocondriais (Cytb and ND4) revelaram um padrão filogeográfico profundo com duas linhagens vicariantes, linhagem Ibérica e Africana, altamente estruturadas, enquanto que os genes nucleares (PRLP and β-fib) revelaram pouca resolução para identificar as mesmas. Também os microssatélites revelaram ser pouco informativos para realizar um estudo a nível intraespecífico nesta espécie, o que impediu investigar o isolamento reprodutivo entre as principais linhagens Ibéricas. Assim, as análises de variação morfológica e ecológica consideraram três níveis de estruturação genética: 1) as duas linhagens mitocondriais principais (Ibérica e Norte Africana), 2) as linhagens mitocondriais identificadas na Península Ibérica e 3) as sublinhagens.

Análises morfológicas univariadas e multivariadas para dez características merísticas revelaram um padrão complexo de variabilidade morfológica entre as populações Ibéricas e Africanas e diferenciação morfológica entre as linhagens da Península Ibérica. Quanto às sublinhagens, as análises indicam diferenciação entre os grupos localizados na região norte e sul da Península sugerindo adaptação a gradientes ambientais.

Modelos de nicho ecológico baseados no príncipio da Máxima Entropia sugerem que as populações de África têm variabilidade ecológica reduzida quando comparadas às populações Ibéricas. Relativamente às linhagens e sublinhagens Ibéricas, os modelos

prevêm extensas áreas de potencial co-ocorrência entre grupos. De acordo com estes resultados, os testes de sobreposição de nichos, baseados na análise de componentes principais da variabilidade ambiental dos registos geográficos e da área de distribuição dos grupos, sugerem uma tendência para conservação de nichos.

Em suma, os resultados deste estudo contribuiram para o conhecimento da história evolutiva e biogeográfica de *V. latastei* e suportam a classificação das duas linhagens vicariantes, Ibérica e Norte Africana, como unidades evolutivas e de conservação independentes. No entanto, para uma avaliação mais precisa da taxonomia da espécie, estudos futuros deverão ser realizados com principal foco no estudo dos padrões contemporâneos de dispersão e fluxo genético entre as diferentes unidades mitocondriais.

Palavras-chave: variabilidade morfológica e ecológica, unidades evolutivas, diferenciação genética, taxonomia, *Vipera latastei-monticola*

Abstract

Past climatic and paleogeographical events have shaped the current biodiversity patterns of the Western Mediterranean region, resulting in high levels of endemism and differentiation for many taxa. Amphibians and reptiles have been frequently used as study models to infer the role of past events in shaping genetic and morphological differentiation patterns; consequently, both groups have been recently subjected to important taxonomic revisions.

The Lataste's viper *Vipera latastei* inhabits areas with Mediterranean climate in the Iberian Peninsula and North Africa with populations displaying strong patterns of genetic and morphological differentiation within both regions. However, the species' taxonomy remains unclear, hampering a coherent designation of conservation units within this vulnerable species.

In this study we investigate the intraspecific variability of the *Vipera latastei-monticola* complex and define coherent taxonomic and conservation units using an integrative approach that combines genetic (phylogenetic and population genetic structure analyses), morphological (univariate and multivariate morphological analyses) and ecological analyses (ecological niche-based modelling and niche overlap tests).

Mitochondrial DNA (Cytb and ND4) recovered a deep phylogeographic pattern with two main vicariant groups, Iberian and North African lineages, highly structured, while the nuclear genes (PRLP and β-fib) provided insufficient resolution to identify the main mitocondrial lineages within the species. In addition, the microssatelite loci proved to be non informative at an intraspecific level in the southern Iberian populations of *V. latastei*, precluding further investigation on the reproductive isolation within the main Iberian mitocondrial lineages.

Therefore, morphological and ecological comparisons were based on three levels of mitochondrial structure: 1) the two main lineages (Iberia and North Africa), 2) Iberian lineages and 3) sublineages.

Morphological univariate and multivariate analyses over ten meristic traits found a complex pattern of variation across the Strait of Gibraltar and clear morphological differences between the main Iberian lineages. At lower levels of genetic structure, morphological differentiation between northern and southern sublineages suggests local adaptation to environmental gradients.

Ecological niche models (ENM) based on the Maximum Entropy approach suggested reduced ecological variability for North African populations when compared to Iberian populations. Within Iberia, ENMs predicted extensive areas of potential co-existence

among main lineages and particularly among sublineages. In concordance, tests of niche overlap, measuring the environmental variability of records and areas of occupancy for lineages and sublineages, suggested a general tendency to niche conservatism within the species.

Overall, this study shed new light into the biogeography and evolutionary history of *V. latastei* and supports the assignment of the two vicariant lineages, North African and Iberian, as independent evolutionary and conservation units. However, for an accurate assessment of this species taxonomy further investigation is needed, with special focus on current gene flow between the different mitochondrial units.

Key-words: ecological and morphological variability, evolutionary units, genetic differentiation, taxonomy, *Vipera latastei-monticola*

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Abbreviationes index

µl - Microliter

bp – base pairs

Cytb – Cytochrome b

DFA – Discriminant function analyses

EGVs – Ecogeographical variables

HCA – Hierarchical cluster analyses

HWE – Hardy Wienberg Equilibrium

Mya – Million years ago

ND4 – Dehydrogenase subunit 4

PCA – Principal Component analyses

PCR – Polymerase chain reaction

PRLP – Prolactine receptor

β-fib – Beta Fibrinogen

I. Introduction

1. Background

Speciation, the process by which populations evolve to become distinct species, has been a major focus in evolutionary biology. Evolutionary forces and mechanisms such as natural selection, genetic drift, gene flow and chance mutation and chromosomal rearrangements are the main drivers of this complex process (Butlin *et al.,* 2012). Gene flow in particular is a central force in this process since the interruption of gene exchange by intrinsic or extrinsic barriers allows the isolation of populations ultimately leading to differentiation into distinct species due to the independent action of evolutionary processes (Slatkin, 1987).

Traditionally, research in evolutionary biology relies upon the biological species concept (Mayr, 1942) that defines species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other groups". Despite commonly adopted in several biological fields, alternative definitions of species are also currently used in biology. Mayden (1997) listed 24 different species concepts including: the ecological concept in which species are defined by their ecological niches (Van Halen, 1976); the evolutionary concept in which a species is a lineage evolving separately from others (Mayden, 1997); the phylogenetic concept that defines species as a diagnosable cluster of organisms within each there is a parental pattern of ancestry and descent (i.e monophyletic) (Mishler, 1985); the phenetic concept that defines species as morphological distinct populations (Sneath & Sokal, 1973); and the biological concept.

Many of these concepts are at least partially incompatible and so they can lead to potential taxonomic conflicts, precluding an accurate species delimitation essential in all biological fields and of critical importance to organize conservation efforts (de Queiroz, 2005, 2007). More recently, the "unified species concept" (de Queiroz, 2005, 2007) was proposed in an attempt to reconcile all species concepts. It is now commonly accepted that species are separately evolving lineages of populations or metapopulations and the different biological properties upon which several of the alternative are based (e.g. intrinsic reproductive isolation, occupation of distinct ecological niches, monophyly, morphological distinctiveness) are characters that

species may or not may acquire during the speciation process (de Queiroz, 2005, 2007; Padial *et al*., 2010).

2. Integrative taxonomy: combining molecular, morphological and ecological methods

Taxonomy, the science that describes and classifies organisms, dates back to the mid-18th century when Linnaeus developed the system of binominal nomenclature and categorization of living organisms. For centuries, the identification of species was predominantly based on morphological data by the observation of external features (DeSalle *et al.,* 2005). More recently, the possibility of sequencing fragments of species´ genomes and the decrease in the costs associated have enabled the use of molecular biology in taxonomy, supporting or replacing traditional methods based on morphological, ecological and behavioral analyses (Padial *et al.,* 2010). The use of genetic methods in taxonomy has been particularly useful to delimit highly divergent lineages that would be impossible to recognize using classical morphological characters due to morphological convergence or parallelism, i.e. cryptic species (e.g. Colborn *et al*., 2001; Hebert *et al*., 2004; Pringle *et al.,* 2005; Kankare *et al*., 2005; Stuart *et al*., 2006; Perera & Harris, 2010; Ahmadzadeh *et* al., 2013; Vod *et al.,* 2015).

Initial phylogenetic and phylogeographic studies relied mostly on mitochondrial DNA to delimit evolutionary lineages and establish phylogenetic relationships among them, revealing only a limited view of the evolutionary history of the species (Wan *et al.,* 2004). The numerous advantages that this marker offers have greatly contributed to its popularity in evolutionary studies (Ballard & Whitlock, 2004). Mitochondrial DNA is a haploid maternal inherited molecule that accumulates nucleotide substitutions several times faster than a single-copy nuclear DNA (scnDNA), and so it is particularly useful to resolve relationships among recently diverged species/populations (Wan *et al*., 2004). However, because mtDNA is maternally inherited, it reflects only the patterns of females gene flow and dispersal which may be specially problematic in case of sex-biased dispersal (Avise, 2004). In contrast, nuclear DNA is bi-parentally inherited and evolves much slower than mtDNA, and thus giving less resolving power for recent divergences (Wan *et al.,* 2004). Nuclear and mitochondrial markers differ strongly in the way they evolve and have different modes of inheritance. Thus, to obtain a complete picture of the evolutionary history of organisms and accurate delimitation of lineages, the use of both markers is required (Godinho *et al.,* 2008). Over the years,

advances in molecular biology have led to the introduction of new types of molecular markers, including the fast evolving markers microsatellites. Microsatellites, also known as short tandem repeats (STR), are highly polymorphic and present high mutation rates, and so they are ideal to infer modern genetic patterns and fine-scale population structure (Wan *et al*., 2004). In addition, they provide information about the extent of gene flow among populations and detect admixtured individuals, which is particularly valuable to delimit species. Nonetheless, due to the high mutation rates, these markers are not appropriate to infer phylogenetic relationships.

Advances in the development of molecular markers and computational methods (e.g. employing of the coalescent theory to produce phylogenetic trees, Fujita *et al*., 2012) have greatly contributed to the increased use of genetic methods to delimit species and study intraspecific diversity (Templeton *et al*., 2001; Hickerson *et al*., 2010).

Recently, the idea of integrative taxonomy has been proposed as holistic tool that combines both classic (morphological, ecological and behavioral data) and modern methods (molecular data) to clarify taxonomic problems (Dayrat, 2005; Padial *et al*., 2010). Both morphological and ecological analyses were greatly improved since the early times of taxonomy. Modern morphology is now based on a broad number of traits, focusing on different anatomical features relevant for addressing the biological questions of interest. For example, pholidotic characters (e.g. different scale counts, presence and absence of particular scales, scale shape and texture) are extensively used in reptiles for taxonomic purposes, presenting high variability at both interspecific and intraspecific levels (e.g. Santos & Pleguezuelos, 2003; Brito *et al.,* 2006; Kaliontzopoulou *et al*., 2012). Variation in these traits is often associated to environmental gradients, reflecting adaptive processes (see Sanders *et al*., 2004; Martínez-Freiría & Brito, 2013). In addition, new morphological tools and statistical analyses have been developed to investigate variation in the morphological characters. For instance, geometric morphometric techniques are now widely used to examine organisms shape (e.g. Kaliontzopoulou *et al.,* 2008) and geostatistics were introduced to examine the geographic patterns of morphological variation (e.g. Brito *et al*., 2008; Martínez-Freiría & Brito, 2013).

The development of ecological niche-based models (ENMs) and the increase availability of topographic (e.g. digital elevation models; http://srtm.csi.cgiar.org/), environmental (e.g. climatic layers; Hijmans *et al*., 2005) and habitat (e.g. Corine Land Cover; http://www.eea.europa.eu/) georeferenced data contributed greatly to the application of ecological methods in research (Kozak *et al*., 2008; Elith, 2009). ENMs

correlate presence/absence species data with environmental variables to determine and map the environmental niche of species (Franklin, 2010). When projected to a different geographic space, ENMs are able to identity suitable areas for the species to occur outside their range. The environmental niche of the species can also be projected to different times (past or future) to, for instance, identify the location of stable climatic areas (i.e. areas acting as climatic refugia; Waltari *et al*., 2007).

Ecological models as well as other multivariate techniques such as environmental principal component analyses can also be applied to examine niche divergence between different evolutionary lineages or species (Warren *et al*., 2008; Broennimann *et al*., 2012; Peterson *et al*., 2011). Statistical tests are essential to interpret the significance of the patterns obtained with these techniques and assess the role of niche divergence in the speciation process (Warren *et al.,* 2008). The implementation of such tests often produced conflicting conclusions, misleading the role of ecological preferences in species differentiation (e.g. Peterson *et al*., 1999; Graham *et al*., 2004). Such inconsistences may be related not only with the different nature of the speciation process itself but also with the use of different methods (Warren *et al*., 2008). New statistical methods recently developed have shown that sister species tend to occupy similar niches but not equivalent (Warren *et al*., 2008), possibly due to phylogenetic niche conservatism (i.e. closely related species are more ecologically similar than expected based on their phylogenetic relationships; Wiens & Graham, 2005; Losos, 2008). Furthermore, ecological segregation might act as a barrier to gene flow among related taxa, promoting species isolation and maintaining their range boundaries (e.g. Tarroso *et al*., 2014).

Studies that combine genetic tools, morphological variation analyses and ecological niche modelling techniques to study intraspecific patterns and identify undescribed taxa are increasing in different groups (e.g Ruiz-Sanchez & Sosa, 2009; Barata *et al.,* 2012; Ahmadzadeh *et al*., 2013). However, incongruences between these methods are found very frequently. Concordant patterns of divergence among different taxonomic characters are more probable to obtain in species that diverged in the distant past than in recently evolved species, since the biological properties acquired along the speciation continuum evolve at different rates (e.g. reproductive isolation, morphological distinctiveness and ecological segregation; Padial *et al*., 2010). Thus, it is possible to support the species status on the basis of a single set of characters if they are functional relevant for the speciation process (Padial *et al*., 2010).

3. Biodiversity patterns in the Mediterranean Basin

The Mediterranean Basin is one of the 25 world biodiversity hotspots (Myers *et al.,* 2000). Past paleogeographical and climatic events have shaped the current biodiversity patterns of this region, resulting in high levels of endemism and strong patterns of differentiation in many taxa.

Tectonic events have deeply affected the Mediterranean basin since the Miocene, shaping biogeographical patterns of biota (Popov *et al.,* 2006). For instance, in the late Miocene the African and Iberian plates became connected, inducing the desiccation of the basin (Messinian salinity crisis, from 5.9 to 5.3 Mya). The emergence of land bridges connecting the two continents allowed for several terrestrial taxa to cross and expand their ranges (Krijgsman *et al.,* 1999; Duggen *et al*., 2003). Posteriorly, the formation of the Strait of Gibraltar and refilling of the Mediterranean sea (ca. 5.3 Mya) induced vicariant processes between sister taxa inhabiting the Iberian Peninsula and North Africa. Accordingly, studies on several taxa highlighted the role of the Strait of Gibraltar as a major barrier to dispersion and gene flow, leading to deep divergences between European and African populations (e.g. Steinfartz *et al*., 2000; Magri *et al*., 2007; Paulo *et al.*, 2008; Jaramillo-Correa *et al*., 2010; Habel *et al*., 2012; Velo-Antón *et al*., 2012). Nonetheless, colonization events after the Strait reopening (mostly during the Pliocene and Pleistocene but also during the Holocene) has also been reported for several taxa, suggesting a permeable barrier to gene flow (e.g. lizards: Kaliontzopoulou *et al*., 2011; turtles: Velo-Antón *et al*., 2015; Veríssimo *et al.*, 2016; snakes: Carranza *et al.,* 2004, 2006; mammals: Cosson *et al.,* 2015).

The alternation of multiple cooling and warming cycles during the Quaternary (2 to 0.2 Mya) induced massive changes in species distribution which deeply affected their patterns of genetic structure (Hewitt, 2000). Species differentially responded to these cycles, accordingly to their ecological requirements and life history traits, as well as, to the geographic position of their range (Hewitt, 2004). During glacial cycles, Mediterranean species (i.e. thermophilic species) retreated due to the expansion of ice sheets from the pole. In contrast, Euro-Siberian species (i.e. temperate species) expanded their distribution ranges during these periods. Southern European peninsulas (Iberian, Italic and Balkans), for both Mediterranean and Euro-Siberian species (Taberlet *et al.,* 1998; Hewitt, 1999, 2000), and the North African region for Mediterranean species (Husemann *et al.,* 2014) constituted major refugia during unfavorable periods, providing suitable conditions due to the heterogeneous climate

and topography. Mountains were extremely important for the survival of many taxa, allowing for range altitudinal shifts as climate conditions changed (Hewitt, 1999). Isolation in multiple refugia and/or adaptation to different environmental conditions led to strong differentiation patterns and speciation processes in several species (Taberlet *et al.*, 1998; Gómez & Lunt, 2007). When suitable conditions were again established, populations sheltered in the southern refugia tended to expand their ranges. This process was characterized by successive founding events and bottlenecks leading to a decrease of genetic diversity (Hewitt, 2004). Thus, after long periods of allopatric isolation in multiple refugia, populations were able to expand and eventually meet, leading to secondary contact with potential hybridization events (Weiss & Ferrand, 2007).

In particular, the Iberian Peninsula and the Maghreb region were important differentiation and speciation centres for Mediterranean taxa during the glacial periods and relevant colonization sources for Europe after climate amelioration (Husemann *et al.*, 2014). This can be implied by the high level of endemism and genetic diversity found in both regions (e.g. Martinez-Solano *et al*., 2006; Velo-Antón *et al*., 2008; Perera & Harris, 2010; Kaliontzopoulou *et al*., 2011; Miraldo *et al*., 2011; Sousa *et al*., 2012). The strong topographic heterogeneity with several mountain ranges and the unique influence of the Atlantic Ocean and Mediterranean Sea induced diverse climate conditions and a variety of microclimates that sustained temperate populations in multiple refugia (i.e. refugia within refugia; Gómez & Lunt, 2007; Husemann *et al*, 2014). However, while providing habitat stability for the species and allowing the preservation of ancestral diversity (i.e. acting both as refugia and sanctuaries; Recuero & García-París, 2011), mountain ranges also represent strong barriers to dispersal. For instance, in North Africa distinct lineages are often found in north or south of the Atlas Mountains (Gonçalves *et al*., 2012; Veríssimo *et al*., 2016).

4. *Vipera latastei-monticola* complex

Vipers (family *Viperidae*) are venomous snakes with sophisticated venom delivery mechanisms that inhabit in all continents except Oceania and Antarctica, and occupy a wide variety of ecosystems. These characteristics have contributed for the use of these organisms as models in biological research, particularly in biogeographical and ecological studies. Despite the wide distribution and adaptive plasticity, vipers display "slow" life history traits (e.g. slow growth and low levels of reproduction) that enhance their vulnerability to isolation and extinction (Maritz *et al.,* 2016).

European vipers (genus *Vipera*) are a monophyletic group of Old World vipers (subfamily *Viperinae*) that differentiated from other Eurasian vipers in the early Miocene, diversified during the Miocene-Pliocene and posteriorly evolved under the Pleistocene climatic oscillations (Lenk *et al*., 2001; Garrigues *et al*., 2005; Wüster *et al.,* 2008; Alencar *et al*., 2016) which induced subsequent levels of genetic structuration within species (e.g. *Vipera berus*: Ursenbacher *et al*., 2006; *Vipera ammodytes*: Ursenbacher *et al*., 2008; *Vipera aspis*: Barbanera *et al*., 2009; *Vipera latastei*: Velo-Antón *et al*., 2012; *Vipera seoanei*: Martínez-Freiría *et al*., 2015). This group mainly distributes across Europe although some species also expanded to Africa (e.g. *Vipera latastei-monticola*) and Asia (e.g. *Vipera berus*) (see Martínez-Freiría *et al*., 2014). Phylogenetic analyses based on mitochondrial DNA recovered three major parapatric clades within this genus (Lenk *et al.,* 2001; Garrigues *et al*., 2005; Wüster *et al*., 2008; Alencar *et al*., 2016): (1) the EuroSiberian *pelias* group (including species such as the widespread *V. berus*, the endemic to Iberia *V. seoanei* or the meadow vipers *V. ursinii*); (2) the Eastern Mediterranean *ammodytes* group (which includes *V. ammodytes* and *V. transcaucasiana*); and (3) the Western Mediterranean *aspis* group (which includes *V. aspis* and *V. latastei-monticola*). Among other European vipers, the Western Mediterranean *V. latastei-monticola* is perhaps the species showing the most complex evolutionary scenario.

Vipera latastei (Boscá, 1878) is distributed across almost all the Iberian Peninsula, Spain and Portugal, and Northern Maghreb, Morocco, Algeria and Tunisia (Martínez-Freiría *et al*., 2014; Fig. 1). Despite its high tolerance to contrasting climatic conditions (occurring in humid, sub-humid and semiarid Mediterranean biotopes), its current distribution is mostly fragmented and restricted to mountain regions, due to human pressure and habitat loss (Santos *et al*., 2006; Brito *et al*., 2011; Martínez-Freiría *et al*., 2014). Furthermore, the species exhibits particular life history traits such as low

dispersal rates, slow generation time and low reproductive success (Brito *et al*., 2003; Pleguezuelos *et al.,* 2007; Santos *et al*., 2007). These factors together, contribute to its high level of vulnerability at global (Vulnerable in the IUCN red list; Miras *et al*., 2009) and regional scales (Vulnerable and Near Threatened in the IUCN red list, for Portugal and Spain respectively; Martínez-Freiría *et al*., 2014).

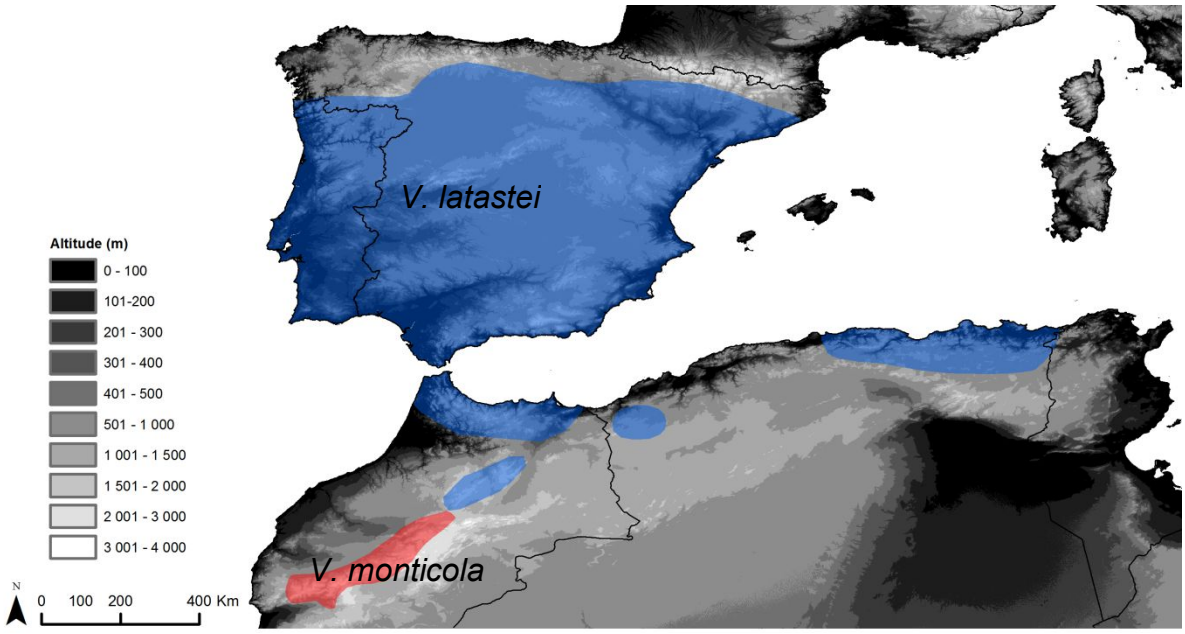

Figure 1 Distributional range of the *Vipera latastei-monticola* complex (from IUCN red list; www.iucnredlist.org).

Late 20th century studies on morphological variability within *V. latastei* lead to the differentiation of two subspecies based on the different number of dorsal and ventral scales and size of cephalic scales: *V. l. latastei* (Boscá, 1878), occurring in northern Portugal and central and eastern areas of Spain and *V. l. gaditana* (Saint-Girons, 1977), located at south-western Iberia and North Africa. Populations of the High Atlas Mountains of Morocco (from 2000 to 2900 m.a.s.l.) were first described as a subspecies of *V. latastei, V. l. monticola* (Saint-Girons, 1954). However, due to relevant morphological differences (i.e. smaller body size, lower number of subcaudal and dorsal scales) and geographic isolation from other North African populations they were later established as full species, *Vipera monticola* (Beerli *et al*., 1986).

Recent studies on morphological variability of the *V. latastei-monticola* complex found clear morphological differences in the African populations (i.e. Rif, High Atlas and Algeria), suggesting that the Algerian populations might represent an undescribed taxon. Regarding the Iberian Peninsula, the results supported the existence of a homogeneous central area and peripheral isolated populations with morphological differentiation (Brito *et al.,* 2006). More recently, morphological and ecological studies

on *V. latastei-monticola* have used geostatistics and ecological niche modelling to predict evolutionary scenarios and to infer ecological niche requirements. These studies have found five morphological groups (Western and Eastern Iberia, Rif/Middle Atlas, Algeria and High Atlas) for which distributional ranges are differentially affected by environmental factors such as precipitation and temperature. Potential areas of secondary contact between Western and Eastern Iberian groups were also identified matching areas of natural barriers such as Trás os Montes-Sanabria, Malcata-Gata Mountains in the Iberian Central System and the valley of Guadalquivir-Jándula rivers in Andalusia (Brito *et al.,* 2008; Fig. 2). Similarly, two major groups, Western and Eastern, were identified in the geographic variability of dorsal pattern shape exhibited within the Iberian *V. latastei* (Santos *et al.,* 2014). Further ecological studies also confirmed geographical differences in the consumption of the two main preys (small mammals and reptiles) explained by climatic variables such as rainfall, temperature and radiation, which suggests high ecological specialization (Santos *et al*., 2008).

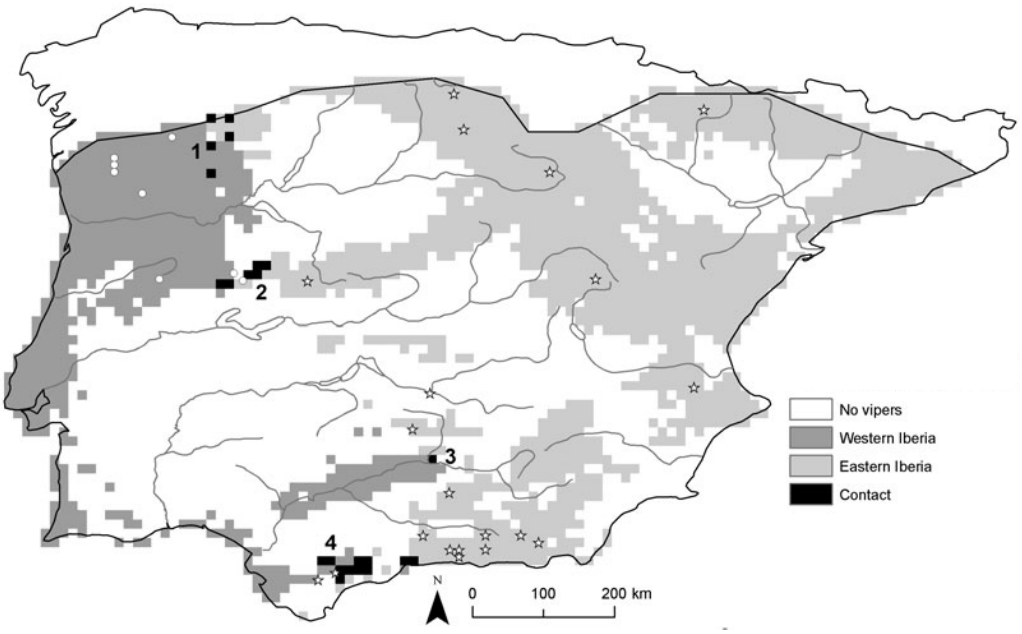

Figure 2 Distribution of the Western and Eastern Iberian groups of *Vipera latastei* and potential contact zones among them at a 10 x 10 Km scale. 1 – Trás os Montes–Sanabria axis; 2 – Malcata–Gata Mountains; 3 – Guadalquivir–Jándula river valleys; 4 – Cádiz–Málaga axis. Adapted from Brito *et al*. (2008).

Phylogenetic analyses based on mitochondrial data identified deep levels of structuration within the *V. latastei-monticola* complex (Velo-Antón *et al.,* 2012). Accordingly, two main lineages can be identified within the species (Fig. 3): one in the Iberian Peninsula divided in three main sublineages (Western, Eastern and Southern) which are further structured in independent groups; and another in North Africa

including three sublineages (Rif/Atlas, Algeria and High Atlas), one of them partially corresponding to *V. monticola* (High Atlas). The authors of this work suggested that *V. latastei* likely colonized North Africa from the Iberian Peninsula during the Messinian salinity crisis and experienced a divergence process coinciding with the reopening of the Strait of Gibraltar that led to the split between the Iberian and African lineages. Posteriorly, climatic events during Pliocene and Pleistocene led to the Iberian and African lineages diversity.

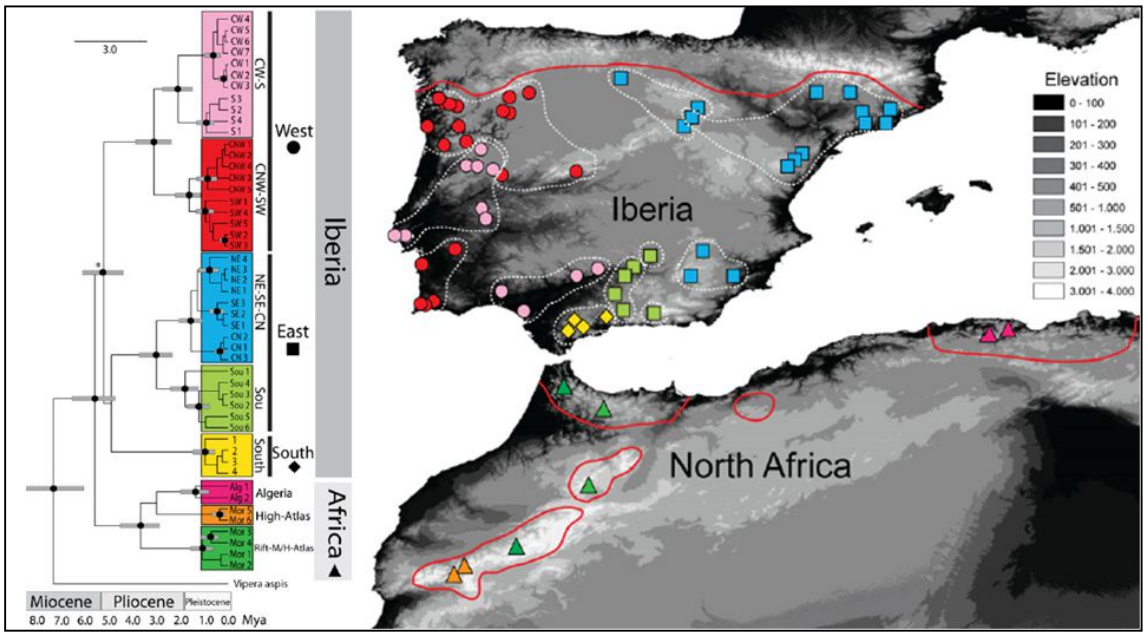

Figure 3 To the left, Bayesian phylogenetic tree based on mitochondrial DNA. Symbols show African and three Iberian lineages and the colors correspond to different sublineages. To the right, geographic distribution of *V. latastei-monticola* samples. Symbols with different colors represent specimens from different lineages and sublineages (concordant with the phylogenetic tree). Adapted from Velo-Antón *et al.* (2012).

When phylogenetic and morphological studies are compared, a high agreement between the levels of phylogenetic structuration and the most recent scenario of morphological variability proposed for this species can be noticed (Brito *et al.*, 2008; Velo-Antón *et al.*, 2012). The only exception is the Southern lineage within Iberia which remained undetected by morphological variability studies. Also, both studies do not support the specific status of *V. monticola* or either the subspecies ranges. However, in the absence of more detailed studies testing the reproductive isolation among lineages and also characterizing morphological and ecological variability of these groups, the current systematics of *V. latastei-monticola* remains unresolved, and thus hampering a coherent designation of conservation units within this highly threatened reptile species.

II. Objectives

In this study we aim to understand the intraspecific variability of the *Vipera latastei-monticola* complex and define coherent taxonomic and conservation units using an integrative approach that combines phylogenetic analyses, population genetic structure analyses, univariate and multivariate morphological analyses and ecological niche-based modelling.

Phylogenetic analyses are conducted to delimit *V. latastei-monticola* lineages and sublineages using an increased sample size in comparison to the previous phylogeographic study by Velo-Antón *et al.* (2012). Population genetic structure analyses are aimed to assess population structure and estimate the extent of gene flow among Iberian main lineages. Morphological and ecological analyses are performed to characterize and compare the morphological variability and the ecological niches of the mitochondrial lineages and sublineages identified in the phylogenetic analyses, focusing on three distinct levels of genetic structure: 1) the two main clades, Iberian Peninsula and North Africa, 2) the Iberian lineages and 3) the Iberian sublineages.

In particular we aim to: I) investigate whether Iberian lineages of *V. latastei-monticola* are reproductive isolated; II) assess whether *V. latastei-monticola* genetic groups are morphologically distinct and occupy different ecological niches; III) examine niche divergence between different evolutionary lineages.

III. Methods

Overview

Analyses of genetic, ecological and morphological variability of the *Vipera latastei-monticola* complex were conducted along the species distributional range, covering the Iberian Peninsula and Northern Magreb.

Comparative analyses of ecological and morphological variation were performed considering the genetic groups recovered in the phylogenetic analyses, with a main focus on the Iberian populations.

1. Analyses of Genetic Variability

1.1. Sampling

The phylogenetic analyses were conducted over 147 tissue samples collected from most of *V. latastei-monticola* distributional range (Fig. 4; Appendix 1). Population genetic analyses were conducted on a smaller number of samples (n = 42) in an area restricted to the Southern third of the Iberian Peninsula, as this region harbours the three main lineages and the five sublineages identified in Velo-Antón *et al*. (2012) for the Iberian Peninsula (Fig. 5; Appendix 1).

Samples consisted of tissue portions (<1 cm of the tip of the tail in both alive specimens and road-killed specimens) and/or saliva swabs collected from live-captured, as well as specimens stored in ethanol from the following museum collections: Museo Nacional de Ciencias Naturales (MNCN, Madrid; 3 samples), Estación Biológica de Doñana (EBD, Sevilla; 22 samples) and Departamento de Biología Animal y Ecología (DBAG, Granada University; 4 samples) and Departamento de Biología Animal, Barcelona University, Spain (DBAUB; 4 samples).

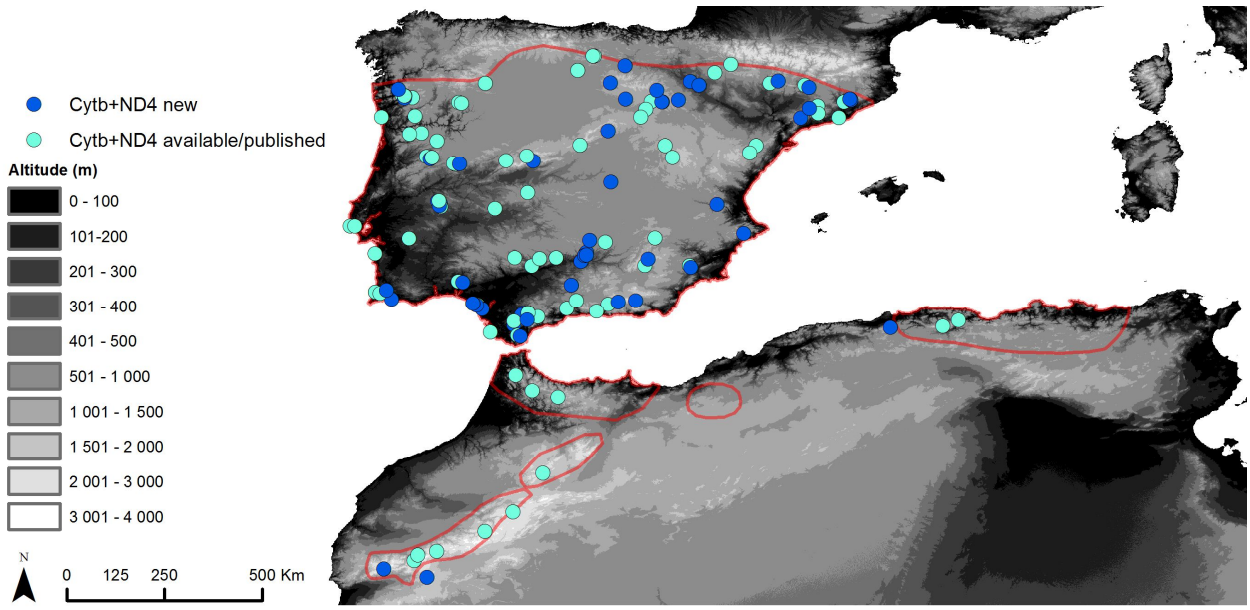

Figure 4 Distribution of *Vipera latastei-monticola* samples considered in the phylogenetic analyses.

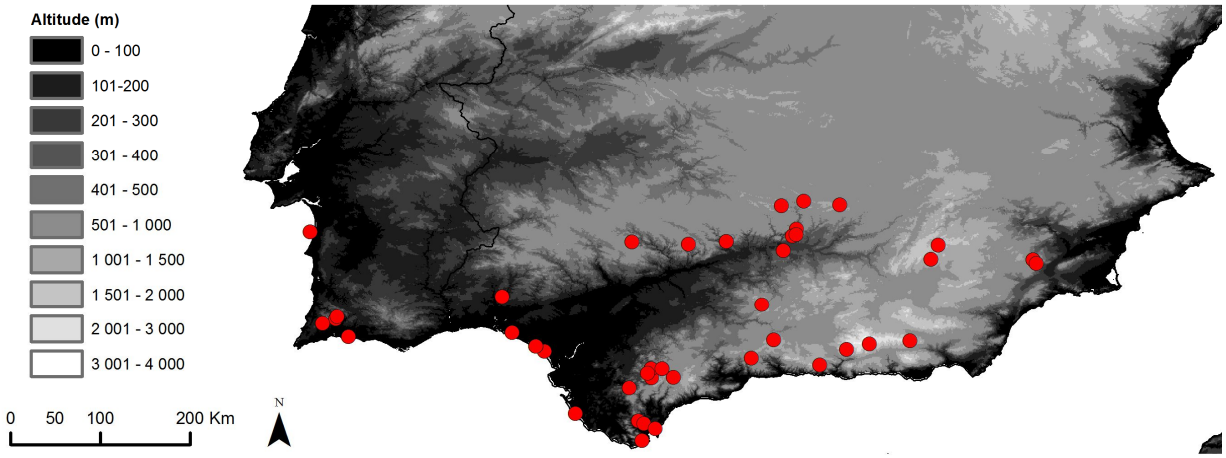

Figure 5 Distribution of *Vipera latastei* samples considered in the population genetic analyses conducted in Southern Iberia.

1.2. Laboratory procedures

1.2.1. DNA extraction

Genomic DNA was extracted from tissue samples (tail tip of 74 new specimens) using the QIAGEN's EasySpin Kit or the QIAGEN's QIAmp® DNA MicroKit, to assure DNA quality in case of limited amount of tissue. Buccal swabs DNA (4 samples) were extracted using QIAGEN's QIAmp® DNA Blood Mini Kit Handbook, following a modified protocol (Appendix 2). The museum samples DNA (16 samples) were extracted following an ancient DNA extraction technique optimized in Dabney *et al*. (2013). Since DNA from museum samples are often degraded and so extremely

vulnerable to DNA contamination, extraction and amplification procedures were performed under specific conditions optimized for the manipulation of low quality DNA in isolated and sterile rooms. The success of DNA extractions was evaluated by electrophoresis on agarose gels. When high amount or degraded DNA was found, samples were properly diluted with ultra-pure water to increase the yield of amplification. In addition, a total of 28 samples already extracted from previous projects (i.e. Instituto de Estudios Ceutíes Project, 2012) were used.

1.2.2. DNA amplification and sequencing of mitochondrial and nuclear DNA

Partial fragments of two mitochondrial genes, Cytochrome-b (Cytb) and Dehydrogenase subunit 4 (ND4) and two nuclear genes, *β*-fibrinogen (*β*-fib) and prolactin receptor (PRLP) were amplified by polymerase chain reaction (PCR) (for primers and conditions see appendix 3). These mitochondrial markers have been successfully used in previous studies within *Vipera latastei-monticola* (Velo-Antón *et al.,* 2012) and other *Vipera* species (e.g. Barbanera *et al.,* 2009; Martínez-Freiría *et al.,* 2015; Zinnenko *et al*., 2016). The two nuclear genes were also successfully used for assessing genetic variation in squamate lizards and were chosen because of their high variability shown in previous studies at lower phylogenetic levels (e.g. Godinho *et al*., 2006; Townsend *et al*., 2008), and which might be appropriate to conduct in *Vipera latastei-monticola*. Polymerase Chain Reactions were performed in a total of 10 µl reaction volumes containing 5µl of QIAGEN PCR MasterMix (for Cytb, ND4 and PRLP amplification) or MyTaq (MyTaqTM Mix, Bioline) (for *β*-fib amplification), 3.2 µl of ultrapure water, 0.4 µl of both reverse and forward primers at a concentration of 10 µM, and 1-3 µl of DNA (approximately 50 ng/ µl). PCR conditions were subjected to re-adjustments for samples with lower quantities of DNA (often by decreasing the annealing temperature and increasing the number of cycles). After the amplification, the PCR products were accessed by electrophoresis on agarose gels. PCR products were purified with ExoSap (USB® ExoSAP-IT® PCR Product Cleanup, Affymetrix) following manufactures instructions and then, sequenced following the BigDye®Terminator 3.1 Cycle protocol (Applied Biosystems) using only the forward primer (for the mitochondrial genes) or both primers (for nuclear genes). PCRs were conducted on BioRad C1000 Thermocycler and sequenced on the automatic sequencer ABI 3130*xl* genetic analyzer (Applied Biosystems).

The obtained sequences were manually aligned and edited using Geneious v4.8.5 and heterozygous sites in nuclear sequences were identified in the chromatograms and coded with IUPAC nucleotide ambiguity codes. A total of 63 ND4 and 66 Cytb sequences published in Velo-Antón *et al*. (2012) and 19 ND4 sequences already available from previous projects were also included.

1.2.3. Microsatellites amplification and genotyping

Ten microsatellite loci originally developed for *V. berus* (Carlsson *et al.,* 2003; Ursenbacher *et al.,* 2009) were amplified in this study. These markers were already successfully used in a previous study conducted in a contact zone among the three Iberian vipers (*V. aspis, V. latastei and V. seoanei*) in northern Spain (Tarroso *et al.,* 2014). Microsatellite loci were amplified in two multiplex reactions (following the protocol used in Tarroso *et al.*, 2014); forward primers were labelled with fluorescent dye markers (FAM, NED, VIC and PET; Oetting *et al.,* 1995). The multiplex reactions (optimized in the previous study) were tested and several re-adjustments were performed in order to maximize amplification success (for primers and conditions see appendix 4). PCR amplifications were performed on 10 µl final volume containing 1 µl of DNA (approximately 50 ng), 5 µl of QIAGEN PCR MasterMix and 1 µl of primer mix. PCRs were amplified on BioRad C1000 Thermocycler and then visualized under UV light after electrophoresis on agarose gels.

PCR products were genotyped on an ABI 3130*xl* genetic analyzer (Applied Biosystems). GeneScan™-500 Liz was used as fragment size standard to score allele sizes on GeneMapper 4.0 (Applied Biosystems).

1.3. Data analyses

1.3.1. Phylogenetic analyses

Phylogenetic relationships were assessed using a Bayesian Inference (BI) method performed in BEAST v 1.7.5 (Drummond *et al.,* 2012), using only the concatenated mitochondrial dataset (Cytb and ND4). The nuclear genes were not included in this phylogenetic analysis as they resulted to be uninformative in this study system (see results). The best-fit partitioning schemes and the substitution models for each mtDNA gene were selected using the Bayesian Information Criterion (BIC) in PartitionFinder v1.1 (Lanfear *et al*., 2012). For both genes, the best inferred model was TrN+G for

each gene, without partition into codon positions. Three independent runs of 100 million generations were performed, sampling trees and parameter estimates every 10000 generations with 10% of the trees discarded as burn-in. A coalescence constant population size model, most suitable for intra-specific phylogenies, was implemented; and a log-normal relaxed-clock model was used as tree prior to allow rate variation among branches (Drummond *et al*., 2006). The convergence of the parameters was verified by looking at the effective sample sizes of all parameters using Tracer v1.7 (ESS > 300). Trees obtained from multiple independent runs were then combined using LogCombiner v 1.7.5. and summary trees were generated with TreeAnnotator v1.7.1 and the final tree visualized in FigTree v1.4.2.

Haplotype phases of both nuclear genes were reconstructed using a coalescent-based Bayesian method implemented in PHASE (Stephens *et al*., 2001; Stephens & Donnelly, 2003) and run in DNAsp (Librado & Rozas, 2009). Then, haplotype networks for the two nuclear genes and for the concatenated mitochondrial dataset (Cytb and ND4) were constructed to visualize haplotypes relationships within *V. latastei-monticola* using statistical parsimony implemented in TCS v1.21 (Clement *et al.,* 2000) and the graphical output was visualized in TCSBU (dos Santos *et al.*, 2015).

1.3.2. Population genetic analyses

For the population genetic analyses, populations were defined based on the mitochondrial groups identified in the phylogenetic analyses. MICROCHECKER v.2.2.3 (Van Oosterhout *et al.,* 2004) was used to assess the existence of null alleles, large allele dropout and stuttering bands for each locus and population. Tests of pairwise Linkage Disequilibrium (LD) and deviations from the Hardy-Weinberg Equilibrium (HWE) for each locus and each population were computed in GENEPOP v4.2 (Rousset, 2008) using a Markov chain method with 1000 batches, 10000 iterations and 10000 dememorization steps. Both tests were performed using a Bonferroni correction for multiple tests. The Bayesian clustering approach implemented in STRUCTURE v2.3.3 (Pritchard *et al.,* 2000; Falush *et al.,* 2003) was used to examine the genetic structure of the studied individuals. The analyses were run for a number of clusters (K) from 1 to 10 with a burn-in period of 100000 generations followed by 1 million iterations, using an admixture model with allele frequencies correlated. STRUCTURE HARVESTER v.0.6.94 was used to determine the probability of each (K) and select the most likely number of clusters, based on Evanno's Delta K method (Evanno *et al.,*

2005). For the best K, runs were merged and graphically displayed using Pophelper (Francis *et al*., 2016). Defining populations based on the mitochondrial lineages and sublineages may lead to artificial deviations related with violation of HWE assumptions, such as random mating and lack of genetic migration. Thus, in order to test the influence of the most problematic loci (loci with signs of HWE disequilibrium), STRUCTURE analyses were conducted with and without these loci.

1.4. Spatial analysis

Before conducting further comparative analyses on ecological and morphological variation, records and georeferenced specimens not represented in the genetic sampling were assigned to the mitochondrial groups identified within the species distribution. Sampling limitations in North Africa precluded further divisions in this region and thus genetic assignments were done at three distinct levels, mainly focusing on Iberian populations: (1) North Africa vs. Iberia; (2) three Iberian lineages (WEST, EAST and SOUTH); and (3) four Iberian sublineages (IBW-CNSW, IBW-CWS, IBE-NSC, IBE-SOU).

The first level of assignment was performed on ArcMap 10.1, differentiating records and georeferenced specimens from North Africa and Iberia. To achieve the second and third levels of assignment, PHYLIN (Tarroso *et al*., 2015), an R package designed to spatially interpolate genetic information from the phylogenetic tree, was used. PHYLIN implements a modified method of kriging interpolation by linking a matrix of genetic distances with a matrix of geographical euclidean distances between sample locations to derive a statistical model of autocorrelation, and to generate interpolated surfaces, providing a probability of occurrence for each pre-defined genetic cluster (lineage or sublineage). The final mitochondrial phylogenetic tree (148 concatenated sequences for Cytb and ND4), excluding *V. aspis* as outgroup, was used as input file. After several trials with distinct models and parameters, an exponential model (sill = 0.002; range = 0.75; nugget = 0) was fit to the semi-variogram (i.e. the function that indicates the spatial correlation in genetic distances at sample locations; see appendix 5). Then, maps of probability of occurrence were created for each Iberian lineage and sub-lineage. The “regular sampling” function was used to visualize global patterns of genetic distance among all samples. Maps of probability of lineage occurrence were imported to ArcMap 10.1, where the probabilities of lineages occurrence were extracted for each sample (from both morphological and ecological datasets). A

threshold of 0.5 of probability was used to assign each sample to a genetic group. This threshold is a compromise to maximize the relationship among general parapatric distribution of Iberian lineages and sublineages and the number of samples attributed to each genetic group.

2. Analyses of ecological variability

2.1. Species occurrence

A total of 962 records at 1 x 1 km grid (WGS 1984 datum) covering most of *Vipera latastei-monticola* distributional range were available to develop ecological niche-based models (Fig. 6). Records were obtained from field work expeditions conducted by BIODESERTS members and collaborators (see acknowledgements), georeferenced specimens from museum collections and other databases (e.g. Asociación Herpetológica Española database). Our dataset was obtained from uneven sampling schemes and was spatially biased with over-representation of more accessible and extensively surveyed areas, which could potentially impact the reliability of the models predictions (Merow *et al*., 2013; Yackulic *et al*., 2013). Thus, in order to decrease potential sampling bias, and to environmentally homogenize both datasets (Merow *et al*., 2013), the number of observations within clusters were subsequently reduced, using Nearest Neighbor Index function of ArcGIS (ESRI, 2006) to assess the degree of data clustering (e.g. Martínez-Freiría *et al*., 2015). The final dataset included 589 records showing a low clustered distribution.

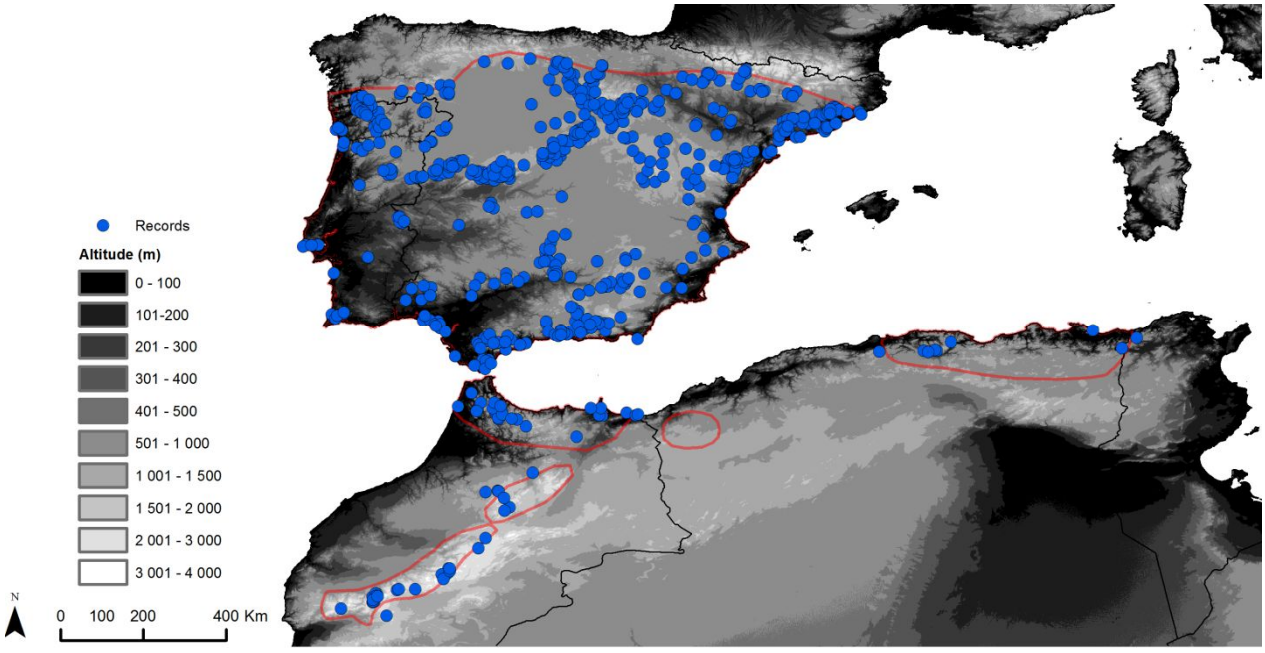

Figure 6 Distribution of *Vipera latastei-monticola* records available to conduct ecological variability analyses.

2.2. Environmental factors

A total of 21 eco-geographical variables (EGVs) were initially considered according to their importance for the species ecology and distribution (Santos *et al.*, 2006; Brito *et al.*, 2008, Martínez-Freiría *et al.*, 2008, Santos *et al.*, 2014). EGVs consisted of two topographic, altitude and slope, and 19 bioclimatic, temperature and precipitation related variables. Altitude and bioclimatic variables were downloaded from WorldClim, version 1.4 (Hijmans *et al.*, 2005) at ca. 1 km^2 of resolution. Slope was derived from altitude, using the Slope function of ArcGis 10 (ESRI, 2010). EGVs were tested for correlation and a set of seven uncorrelated variables ($R < 0.7$; including slope and six bioclimatic variables) were used in the ecological models (Table 1).

Both variables and occurrence records were projected in the WGS84 datum and had a spatial resolution of 1km^2.

Table 1 Eco-geographical factors (WorldClim codes) used for modelling the distribution of *Vipera latastei-monticola* and the different groups. Ranges (and units) for the whole species range are depicted.

EGVs	RANGE AND UNITS
Slope	From 0 to 32% of rise
Isothermality (BIO3)	From 2.6 to 5.2 ^oC
Temperature Annual Range (BIO7)	From 12.2 to 41.7 ^oC
Mean Temperature of the Wettest Quarter (BIO8)	From 4.0 to 19.8 ^oC
Mean Temperature of Warmest Quarter (BIO10)	From 4.4 to 34.1 ^oC
Annual Precipitation (BIO12)	From 148 to 1300 mm
Precipitation Seasonality (BIO15)	From 20 to 73 mm

2.3. Ecological Niche-based Models

Ecological Niche-based Models (ENMs) were developed for the whole species distribution (i.e. Iberia + North Africa) and the three levels of genetic structuration within *Vipera latastei-monticola*: (1) North African and Iberian clades; (2) the three Iberian lineages (WEST, EAST and SOUTH); and (3) the four Iberian sub-lineages (IBW-CNSW, IBW-CWS, IBE-NSC, IBE-SOU). Distributions were modelled in study areas defined by 100 km buffer polygons around records. The three levels of genetic structuration were then projected to a larger area: the whole species distributional area for the first level, and the Iberian Peninsula for the second and the third levels.

Ecological models were performed with a Maximum Entropy approach using MAXENT v.3.3 (Phillips *et al.*, 2008). This method compares presence areas with the background (given by the study area) which includes both presence and non presence areas (Phillips *et al.,* 2009). For each ecological model, 50 replicates were run with random seeds using 30% of the total records for testing the model predictions and 70% for training, both selected randomly by bootstrap. The models were run with the default parameters.

To investigate how environmental variability for each clade, lineage or sublineage could occur outside its range, individual ecological models were projected to larger areas (i.e. the whole species distributional area for the first level, and the Iberian Peninsula for the second and third levels of genetic structure). Projections were assessed by using clamping masks and the "Fade by clampling" function of Maxent in order to constrain the values found in the projected areas into the range of values existing in the study area (Elith *et al.,* 2011). Model performance was measured using the area under the curve (AUC) of the receiving operator characteristics (ROC) plot. In addition, replicates standard deviation was used to assess prediction uncertainty.

2.3.1. Eco-geographical variables importance

The weight of each EGV for describing the distribution of each genetic group was determined by its average percentage contribution to the models. The relation between groups´ distribution and the most important EGVs was assessed by the visual examination of response curves profiles from univariate models (Martínez-Freiría *et al.,* 2008).

2.3.2. Suitability and sympatry maps

In order to determine the suitable areas, projections from average probability models of each group occurrence were imported into ArcMap 10.1 where they were converted to binary models (absence/presence squares) using a five percentile training presence threshold (meaning that 5% of the squares with the lowest probabilities are assumed as absences). This more restrictive threshold was used as an alternative to other thresholds, to avoid the overestimation of the suitability areas without excluding extensive areas with records presence (Liu *et al.,* 2005*,* 2013). Then, binary models were summed to spatially represent and calculate areas where two groups could coexist (i.e. sympatry areas; see Martínez-Freiría *et al*., 2008).

2.4. Niche overlap tests

Ecological niches overlap between the two main lineages (Iberian and African) and between pairs of Iberian lineages and sublineages were measured following the 'PCA-env' approach proposed by Broennimann *et al.* (2012). This method performs a Principle Component Analyses (PCA) to summarize the environmental variability found in the study area and applies a kernel density function to determine occurrences density in the PCA space. The study areas were delimited using minimum convex polygons around the occurrences of each genetic group, using ArcMap 10.1.

Pairwise niche overlap was quantified using Schoener's D index (Warren *et al.,* 2008), which range from 0 (no overlap) to 1 (complete overlap). Niche equivalency and similarity randomization tests were performed, both based on 100 permutations. The former determines whether niches of two groups are equivalent by comparing the observed value of niche overlap (*D*) with the value obtained after randomly split the two datasets and reallocate the occurrences among the two study areas; the later addresses whether the niche of one group is more similar (or different) to the other than would be expected by chance by randomly shifting the occurrences of one group in its available environmental space (given by the study area) and measuring the overlap of the simulated niche with the observed niche of the other group (Broennimann *et al*., 2012). Niche overlap tests were performed in R 2.15.2 using scripts provided by Broennimann *et al.* (2012).

3. Analyses of morphological variability

3.1. Sampling

Morphological data of 953 adult specimens including 902 Iberian samples (393 males, 282 females and 227 specimens with unknown sex) and 51 North African samples (21 males, 18 females and 12 specimens with unknown sex), covering the whole species distribution, were available for the morphological analyses (Fig. 7). Data for most of the specimens (n = 805) were previously used in works aimed to analyse the morphological variability of the species along its distributional range (n = 678; Brito *et al.*, 2006, 2008) or in contact zones (n= 127; Martínez-Freiría *et al.*, 2009). These specimens were mostly from museum collections (e.g. Natural History Museum of Lisbon, Portugal; Natural History Museum of Porto, Portugal; Museo Nacional de Ciencias Naturales, Spain; Departamento de Biología Animal, Barcelona University, Spain; Muséum national d'Histoire naturelle, France; see Brito *et al.,* 2006, 2008),

fieldwork in northern Spain until 2008 year (Martínez-Freiría *et al*., 2009) or literature review (Dollfus & Beaurieux, 1928; Wettstein, 1933; Bons, 1958; Saint-Girons, 1977; Beerli *et al*., 1986). Additionally, morphological measurements for 133 specimens, collected from field work developed from 2009 to 2016 and further visits to museum collections during this period (e.g. Laboratoire de Biogéographie et Écologie des Vertébrés, Montpellier University, France; Natural History Museum of London, UK; Estación Biológica de Doñana, Spain) were added. The data was collected through direct examination of the specimens and by the examination of specimens photographs.

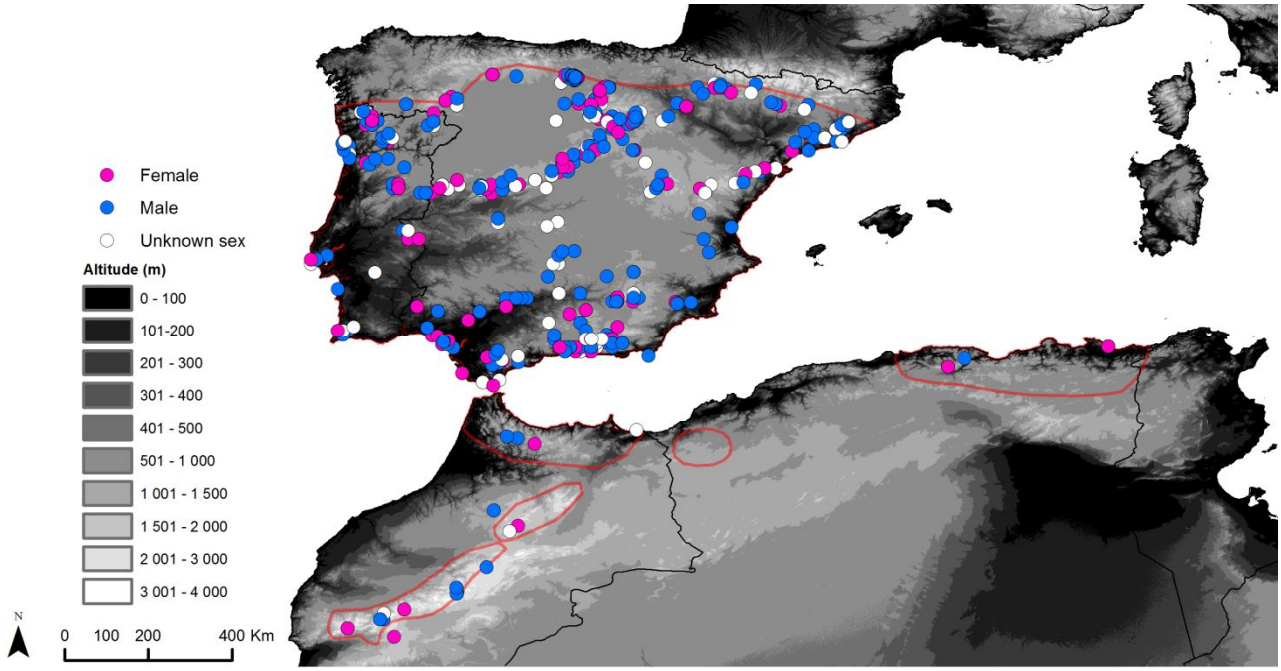

Figure 7 Distribution of *Vipera latastei-monticola* specimens considered in the morphological analyses.

3.2. Morphological characters

A set of 11 meristic traits were considered in the morphological analyses (Table 2). Most of these characters were reported to present geographic variation in previous studies on the morphological variability within the species (Brito *et al.*, 2006, 2008, Martínez-Freiría *et al*., 2009; Santos *et al.,* 2014) and some of them (e.g. number of dorsal rows at midbody, number of ventral scales) were recognized as diagnostic for the discrimination of *Vipera latastei* subspecies (Saint-Girons, 1977; Beerli *et al.*, 1986).

Table 2 Morphological characters used to assess the morphological variability within *Vipera latastei-monticola*.

MORPHOLOGICAL CHARACTERS	CODES
number of intercantal plus intersupraocular scales	INTER
number of supralabial scales	SUPRA
number of loreal scales	LOR
number of subcaudal scales	SUBC
number of infralabial scales	INFRA
number of periocular scales	PERI
number of total apical scales	N-APIC
number of small apical scales	S-APIC
number of ventral scales	VENT
number of marks from the dorsal design between the head and the vent	DMARK
number of dorsal rows at midbody	N-DORS

For the bilateral characters (SUPRA, LOR, INFRA, PERI) we only considered the right side of the head.

3.3. Statistical analyses

In order to examine the morphological variability within *Vipera latastei-monticola* and to assess whether morphological and genetic patterns are concordant, statistical analyses were conducted considering the three levels of genetic structure previously identified (the two main lineages, Iberia and North Africa, and the Iberian lineages and sublineages). Univariate analyses were performed for each morphological characters and multivariate analyses were conducted only for the characters that revealed differences between groups. All statistical analyses were conducted in SPSS v.24 (IBM SPSS Statistics 24).

3.3.1 Univariate analyses

For each character, preliminary tests were performed to evaluate morphological differences between sexes. When sexual dimorphism was found, the following analyses were conducted separately for males and females. Then, independent statistical tests were conducted for each level of genetic structure allowing us to compare the morphological patterns among (1) North Africa and Iberia, (2) the Iberian

lineages and (3) the Iberian sublineages separately. These tests were performed using student t-test/analyses of variance (ANOVA) or alternative non-parametric versions of these tests in case of assumptions violation (e.g. normality and homoscedasticity). Classic parametric statistical tests such as t-test and ANOVA are reliable tests to assess differences between groups. However if their assumptions are violated the results are typically invalid with increasing risk of committing Type I a II errors, especially in unbalanced designs (groups with different sample sizes) and small sample sizes (Wilcox, 1998, 2001; Zimmerman, 1998). As an alternative, rank-based procedures (non parametric tests) can be applied to assure results robustness (Zimmerman & Zumbo, 1993; Erceg-Hurn & Mirosevich, 2008).

Consequently, before conducting the statistical analyses we examined the distribution of the data by visual inspection of the histograms and by a normality test (Shapiro Wilk test) and we assessed the homogeneity of within-group variances (Levene´s test). In case of violation of the normality assumption, a Kruskal-Wallis test was performed (non parametric version of ANOVA) while in case of variances heterogeneity an ANOVA with Welch correction for heterocedastic data and unequal sample sizes were conducted. In case of non normal distribution and heterocedasticity, we performed a Welch ANOVA based on ranks (Zimmerman & Zumbo, 1993). Multiple comparison tests were performed for the characters that report significant p-values in the above analyses: Dunn´s test for Kruskall-Wallis test, Tukey-Krammer test for ANOVA (equivalent to a Tukey test but corrected for unequal sample sizes) and Games-Howell test for Welch´ANOVA (both based on ranks when the two assumptions are not met).

3.3.2 Multivariate analyses

Multivariate analyses were performed separately for males and females, considering only the characters that reported morphological differentiation among groups in the univariate tests. Principal Component Analyses (PCA), Hierarchical Cluster Analyses (HCA) and Discriminant Function Analyses (DFA) were conducted in order to examine the morphological patterns found within *Vipera latastei-monticola*. The multivariate analyses were performed without (for both PCA and HCA) and with (for DFA) *a priori* assignment of specimens into genetic groups. Thus, for PCA and HCA, we examined the patterns of morphological differentiation among North Africa and Iberia using the initial dataset and within Iberia using a reduced dataset only with Iberian specimens. For DFA, independent analyses were conducted for each level of genetic structure.

PCAs were conducted in order to reduce the morphological characters under study in principal components that still account for most of the data variance. The first components that explained most of the morphological variability were plotted to examine the morphological structure of the data. Following analyses were performed on the components extracted in the PCA instead of the raw data.

A DFA (analogue to a MANOVA) was used to classify specimens into the predetermined groups. DFA works by creating discriminant functions (linear combinations of the original variables) that maximize the differences between groups. The significant functions were plotted and classification tables of correct and incorrect estimates were obtained.

Finally, HCA were performed to identify homogeneous groups within the data and examine how specimens are grouped. This analysis uses an algorithm that starts with each case in a separate cluster and combines them until they are all merged. HCA based on squared euclidean distances was performed using UPGMA as a cluster method. A dendrogram was then produced showing graphically how the clusters are merged. Specimens that represented alone a distinct cluster (i.e outlier specimens) were removed from the dataset and the analyses were conducted again. A range of k (number of clusters) was examined and the best was represented in a map to allow the visualization of the morphological patterns.

V. Results

1. Genetic analyses

1.1 Laboratory overview

The amplification of the two mitochondrial regions (Cytb and ND4) and the PRLP nuclear fragment was successful, with more than 80% of the initial samples amplified (Cytb, 60 from 68; ND4 fragment amplified with F2 and Leu primers, 59 from 67; ND4 fragment amplified with F and R3 primers, 50 from 67; PRLP, 28 from 33). For the βfib fragment the amplification success was significantly lower (64%; 16 from 25 samples). Regarding the microsatellites, 42 out of the 65 samples analysed were successfully amplified for the two multiplexes.

1.2 Phylogenetic analyses

Bayesian analyses inferred with the combined mitochondrial dataset (517bp Cytb + 752bp ND4; 147 sequences) recovered two divergent clades highly structured: one Iberian clade and one African clade (Figs. 8, 9). However, the low posterior probabilities provided by the tree did not support the monophyly of these clades.

Within Iberia, three main lineages were identified, although with low support: the Western, Eastern and Southern lineages (Figs. 8, 9). The Western lineage is further subdivided in two well supported sublineages: I) Central Northwest-Southwest (CNSW), comprising samples from central northwest and southwest Iberia (light blue) and II) Central-West-South (CWS), which includes central western and southern specimens (dark blue). The Eastern lineage is also differentiated in two well supported sublineages: I) Northeast-Southeast Central North (NSC), including specimens from northeast, central north and southeast Iberia (light green) and II) Southeast (Sou), comprising samples from southeast Iberia (dark green). The Southern lineage (South) comprises specimens from the southern tip of the Peninsula (orange).
The African clade is subdivided in four main sublineages (Fig. 8; Fig. 9): I) a Central High-Atlas / Anti-Atlas sublineage (red); II) a Western High Atlas sublineage, a previously undescribed phylogenetic group (purple); III) an Algerian sublineage (yellow); and IV) a Moroccan sublineage, including *Vipera latastei* and *Vipera monticola* specimens from the Rif, Middle-Atlas and Eastern High-Atlas (brown). The

relationships among the African sublineages are not well resolved due to the low Bayesian posterior probabilities obtained for the Central High Atlas/ Anti-Atlas lineage and the Western High Atlas lineage; however, the tree provided good support for a sister group relationship among the Algerian and Moroccan sublineages and for the separation between these two groups and the Central High Atlas/Anti-Atlas and the Western High Atlas sublineage (Fig. 8).

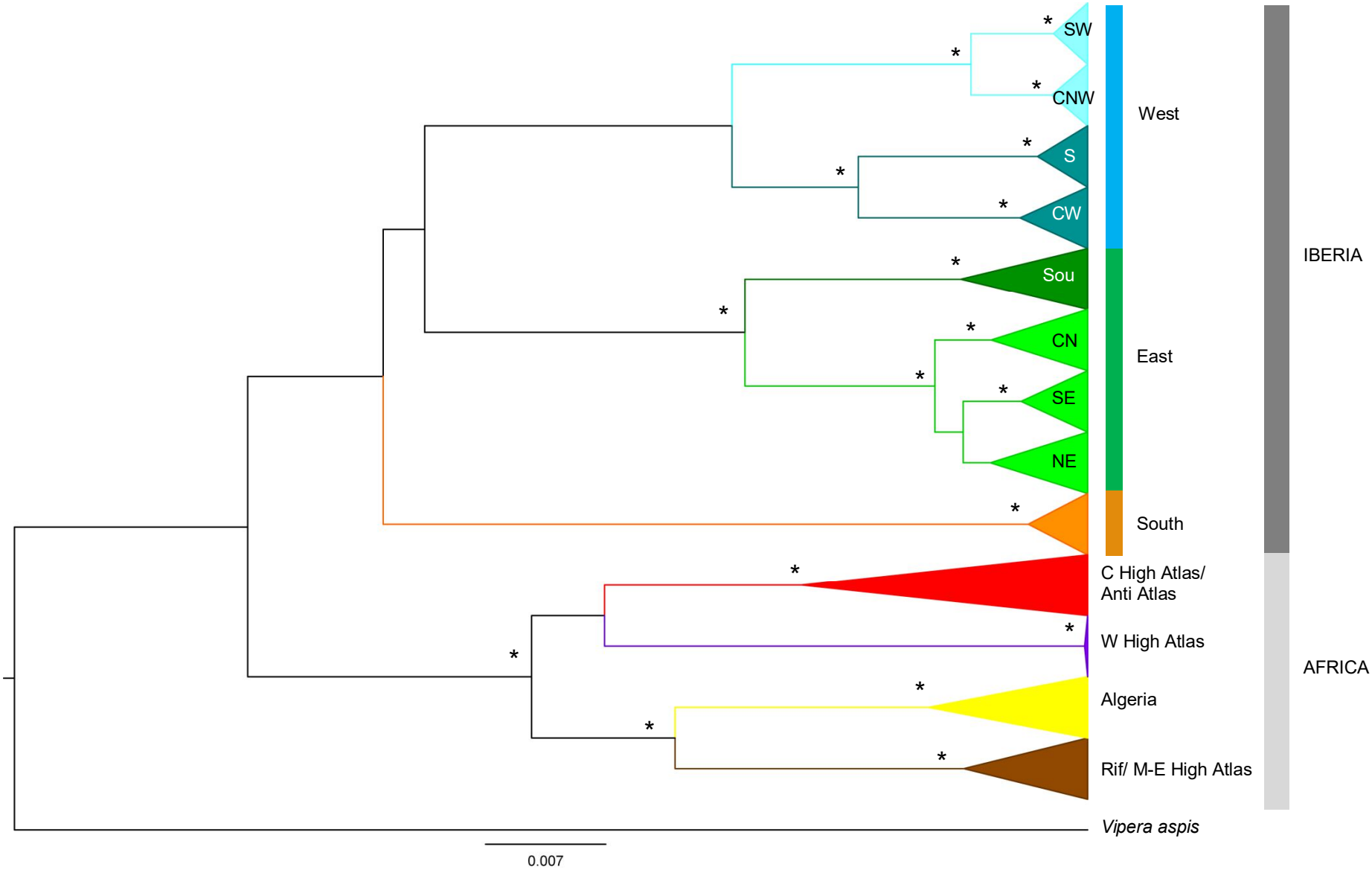

Figure 8 Bayesian phylogenetic tree based on the combined mtDNA dataset (Cytb and ND4) for the *Vipera latastei-monticola* complex, rooted with anoutgroup (*Vipera aspis*). The asterisks on the nodes show posterior probabilities higher than 0.9. Scale bar represents 0.7% of sequence divergence.

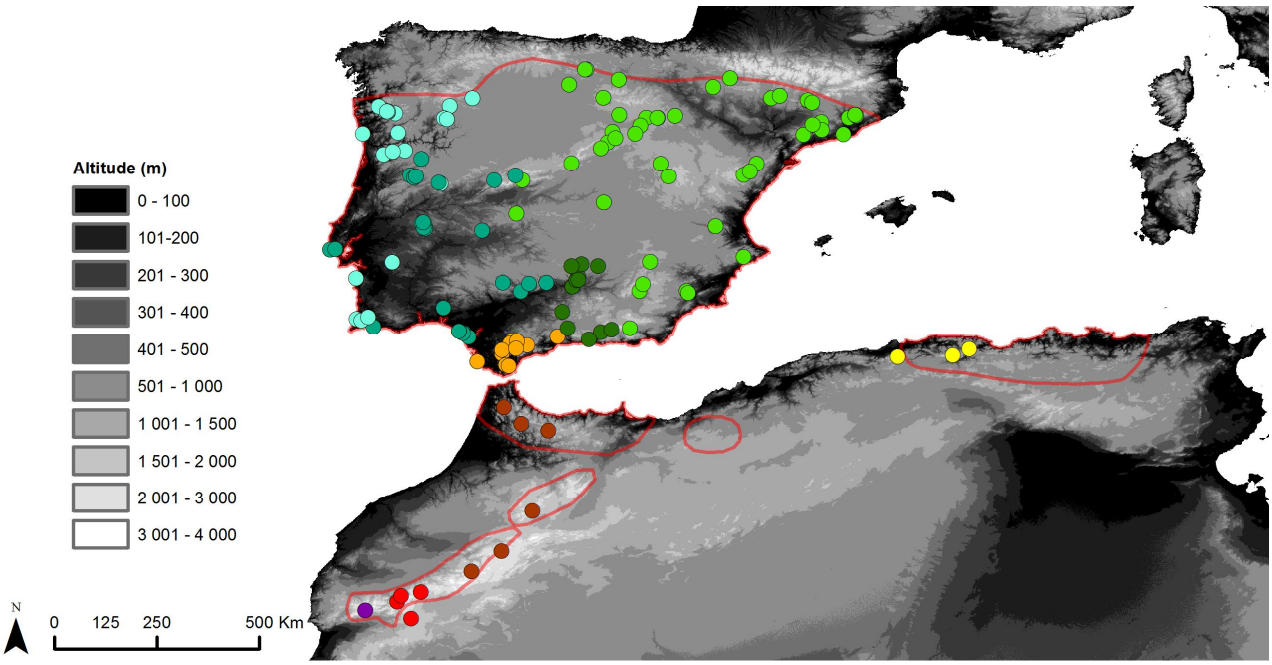

Figure 9 Distribution of *Vipera latastei-monticola* lineages and sublineages recovered in the phylogenetic analyses. Symbol colours are concordant with Figure 8. IUCN range polygons are represented.

1.3 Haplotype networks

Haplotype networks based on the mitochondrial concatenated dataset (reduced to 90 sequences with no missing data) found high genetic structure and haplotype diversity (Fig. 10). Among the 90 samples analysed, 63 haplotypes were identified, most of them comprising only one sample. From the 1269 sites, 285 were polymorphic; no insertions or deletions were found in the dataset.

Within Iberia, the haplotype networks recovered the same genetic structure found on the phylogenetic analyses (Fig. 8). Seven independent networks were obtained, one corresponding to the Southern lineage, other to the Western sublineage CNSW, two consistent with the central western (CW) and southern (S) groups of the Western sublineage CWS, one to the Eastern sublineage Sou and two corresponding to the northeast/southeast (NE-SE) and central north (CN) groups of the Eastern sublineage NSC.

Regarding the North African lineage, five independent networks consistent with the four sublineages identified in the phylogenetic tree and an additional network comprising the Anti-Atlas samples were recovered (Fig. 10).

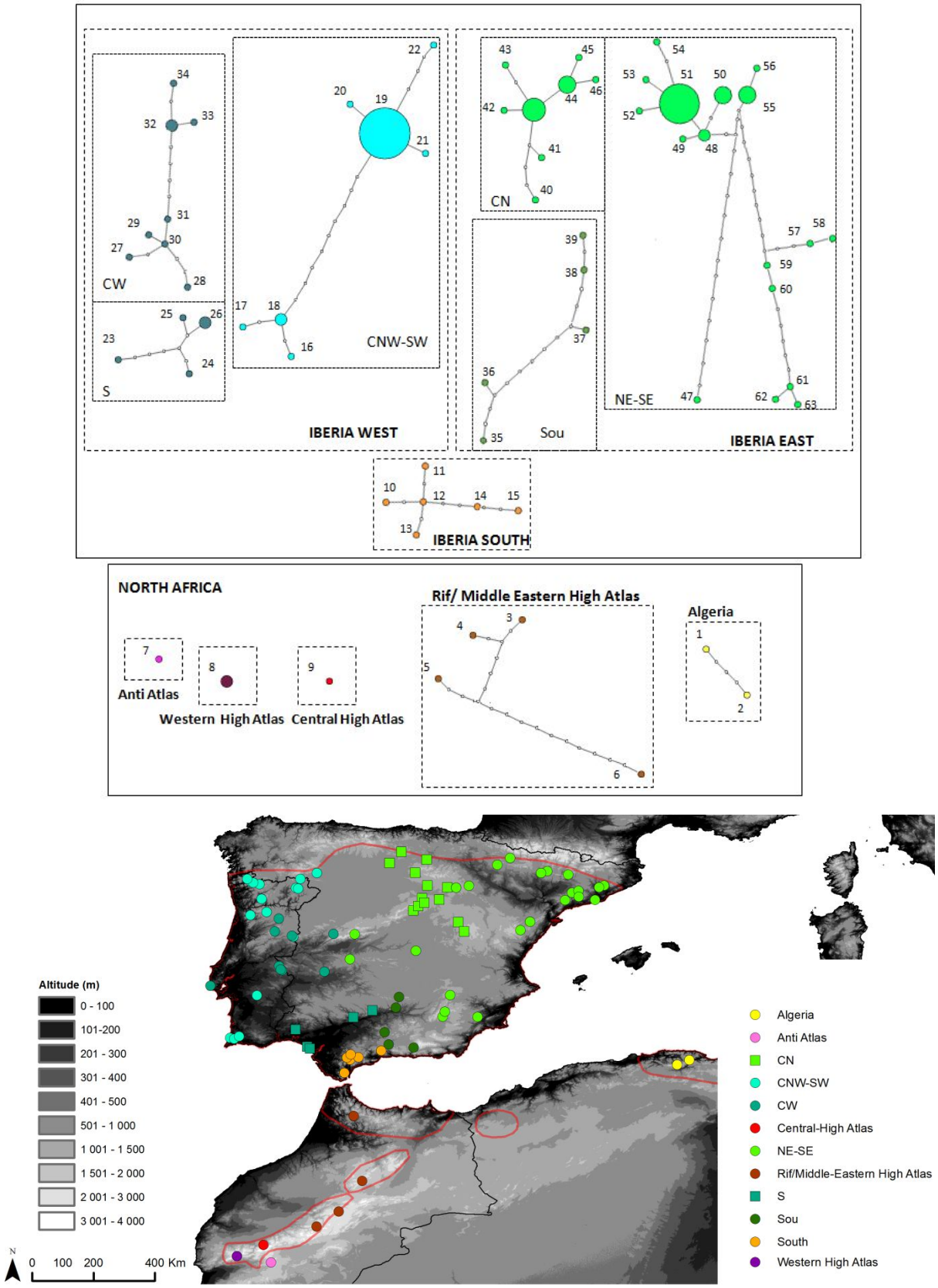

Figure 10 TCS haplotype network for the *V. latastei-monticola* complex based on the concatenated mitochondrial dataset. Each circle represents a different haplotype identified with the corresponding number. The size of each circle is proportional to the number of sequences sharing the same haplotype (up). Spatial distribution of the haplogroups recovered in the haplotype network (down).

In contrast, haplotype networks constructed for the two nuclear datasets, βfib and PRLP (with 46 and 48 sequences, respectively), showed no genetic structure and geographic coherence. The βfib dataset yielded 10 haplotypes with four heterozygous individuals. From the 596 sites analysed, 13 polymorphic sites; a insertion of 5 bp and a deletion of 4 bp were detected. Regarding the PRLP nuclear region, among the 48 sequences analyzed, 14 haplotypes were identified with nine heterozygous individuals. Twelve out of the 524 sites were polymorphic. No insertions or deletions were found within the fragment. In both haplotype networks, the most common haplotype (HAP1 for both PRLP and βfib) included sequences of specimens from all North African and Iberian lineages (Fig. 11).

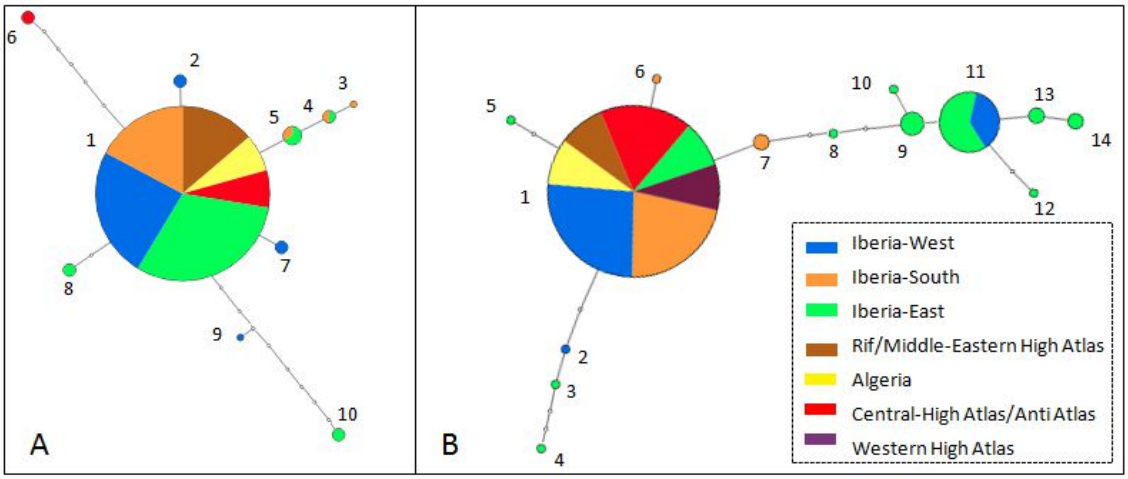

Figure 11 TCS haplotype network for the *V. latastei-monticola* complex based on the nuclear datasets, βfib (A) and PRLP (B). Each circle represents a different haplotype identified with the corresponding number. The size of each haplotype is proportional to its frequency.

1.4 Population genetic analyses

The final dataset of 42 samples included nine samples from the Southern lineage, 16 from the Eastern lineage (11 from the Eastern sublineage Sou and five from the Eastern sublineage NSC) and 17 from the Western lineage (11 from the Western sublineage CWS and six from the Western sublineage CNSW).

From the total ten loci, two (VBA8 and VBD17) reported significant deviations of the Hardy-Weinberg equilibrium (due to heterozygotes excess) and null alleles for three out of the five groups. In addition, four loci (VB37, VBB18, VB11 and VB64) presented the same problems but for one group only (for the Eastern sublineage Sou or for the Western sublineage CWS). No linkage disequilibrium was detected in the analyses.

Overall, the ten microsatellite loci were highly variable with a number of allelles ranging from 10 (VB71) to 37 (VBB10) (see appendix 4).

The optimal number of clusters indicated by Delta K were K=3 and K=6 (Fig. 12 A). As shown in the bar plot (Fig. 12 B), the STRUCTURE analyses failed to detect a clear genetic structure in the data. Nonetheless in K=6, all specimens from Doñana National Park were clustered together altough this pattern may reflect some degree of relatedness among them.

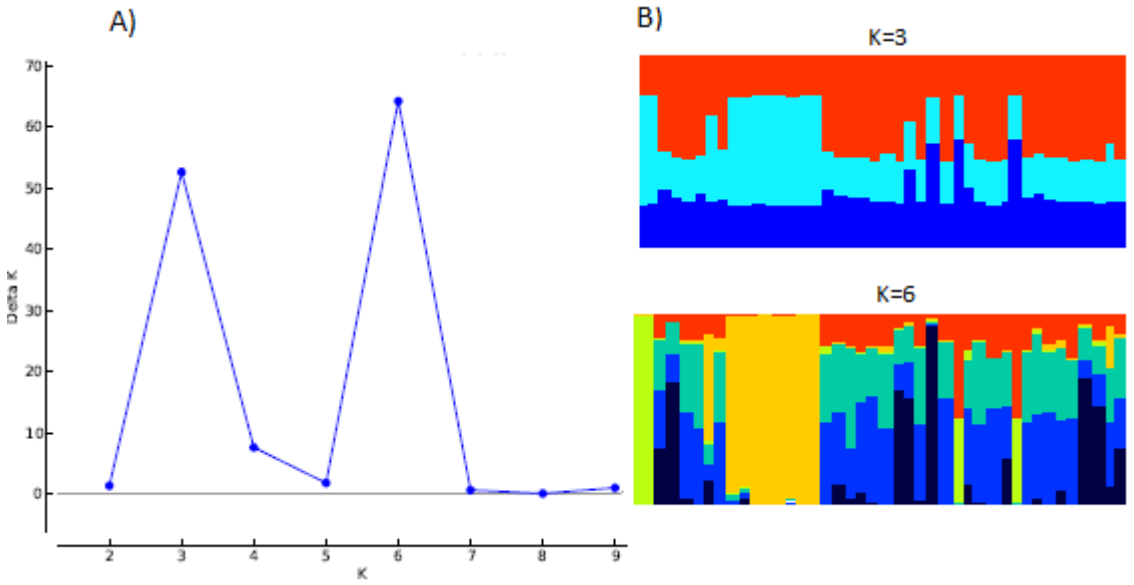

Figure 12 A) Evanno's Delta K showing the most probable K (K=3 and K=6) and B) Population structure of *Vipera latastei* in Southern Iberia Peninsula for K=3 and K=6.

1.5 Spatial interpolations of mitochondrial data

The map of potential contact zones within *Vipera latastei-monticola* represented by the average probability of multiple lineages presence was derived by using multiple thresholds along the mitochondrial phylogenetic tree. This sample scheme clearly shows the patterns of genetic divergence found within the species, with more divergent lineages presenting higher probabilities. Accordingly, contact zones at the margins of the main Iberian lineages ranges (Western, Eastern and Southern lineages) are highlighted with higher probabilities in comparison with the contact zones within Iberian sublineages. Sampling limitations in North Africa precluded a robust assessment of the exact spatial distribution of the phylogenetic groups, and thus potential contact zones among them are not accurately represented (Fig. 13).

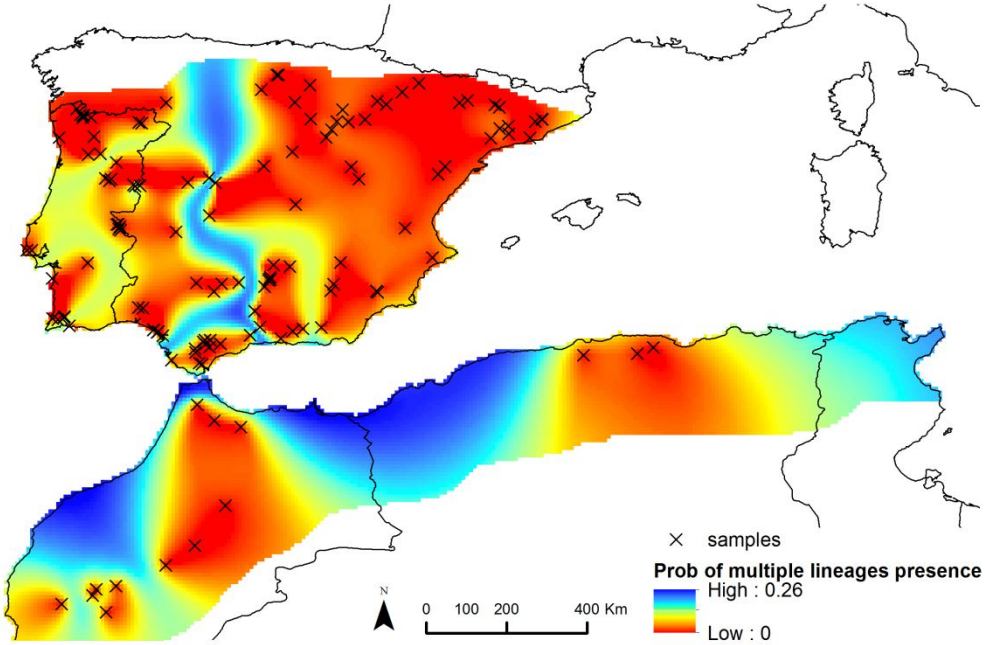

Figure 13 Map of the potential contact zones within *Vipera latastei-monticola* represented by the average probability of multiple lineages presence.

The maps for each Iberian lineage and sublineage predicted occurrence depicted well their spatial distribution, showing consistent classifications of Iberian specimens from both morphological and ecological datasets in phylogenetic groups (Fig. 14; appendix 6).

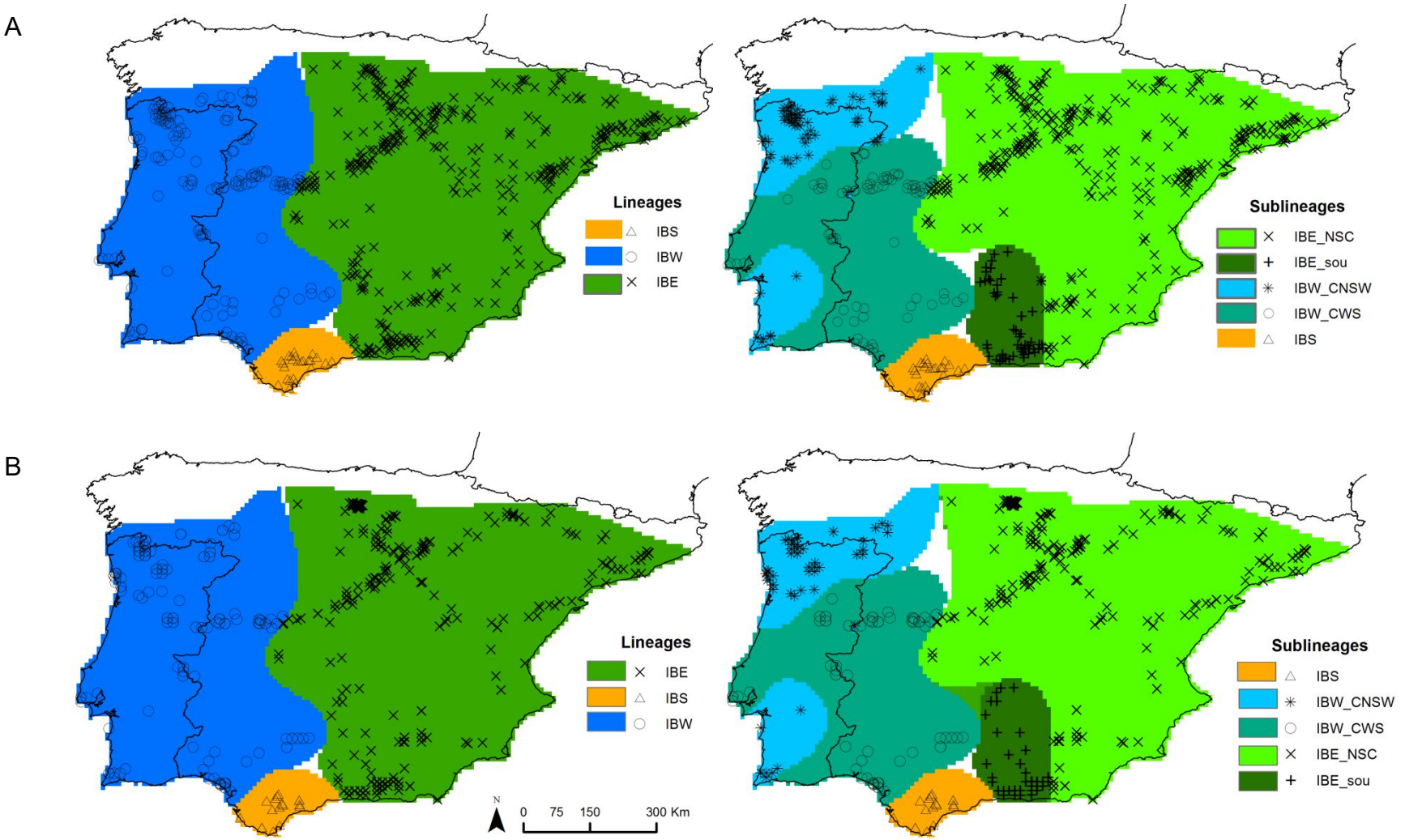

Figure 14 Maps of Iberian lineages and sublineages presence defined using a probability of 0.5 as threshold, used to assign Iberian samples from both ecological (A) and morphological (B) datasets in phylogenetic groups.

2. Ecological niche-based models

2.1 Models evaluation

Overall, ten ENMs were developed for the following mitochondrial groups: IBNA, Iberian and North African distributions; IB, Iberian distribution only; NA, North African distribution only; IBW, Western Iberian distribution; IBE, Eastern Iberian distribution; IBS, Southern Iberian distribution; IBW-CWS central western and southern Iberian distributions; IBW-CNSW, central northwestern and southwestern Iberian distributions; IBE-NSC, central northeastern and southeastern Iberian distributions; and IBE-sou, southeastern Iberian distributions only.

For each model developed the ROC curves averaged over 50 replicate runs showed high AUC for both training and test datasets and low standard deviations, suggesting that the models were highly accurate (Table 3). The lowest values of training and test AUC were obtained for the Iberian model (0.85 ± 0.01 and 0.82 ± 0.02 respectively) while the highest values were obtained for the North African model (0.95 ± 0.01 and 0.93 ± 0.04, respectively; Table 3).

2.2 Eco-geographical correlates

Two of the seven EGVs showed consistently high percentage of contribution in the different ENMs (Table 3): slope and annual precipitation. Annual precipitation presented the highest values of percentage contribution for the total distribution model (IBNA) and for the models of NA, IB, IBE, IBW-CNSW, and IBE-NSC. In addition, slope was identified as the main variable for the two southern Iberian groups (IBS and IBE-sou).

The mean temperature of the warmest quarter and the mean temperature of the wettest quarter are also highly related to the lineages and sublineages distribution: the first presents high percentage of contribution for the total distribution model (IBNA), and for IB, IBE, IBS, IBW-CNSW and IBE-NSC models; the latter affects the distribution of the remaining group models.

In addition, precipitation seasonality is also related to the species distribution, particularly for the Iberian populations (Table 3).

Average profiles of response curves were generated for the two most important EGVs, annual precipitation and slope, and for the mean temperature of the warmest

quarter and precipitation seasonality, representing only the responses of the groups affected by these variables.

The average profiles of response curves for the two most important EGVs suggested that the species occurs in areas with a wide range of annual precipitation (500 to 1750 mm) and slope (0 to 20%) (Fig.15).

Response curves for slope revealed differences within Iberia: lineages located in the south (IBS, IBE-sou, IBW-CWS) occur in areas with medium slope (10%), while lineages located in the north (IBE-NSC, IBW-CNSW) tend to occur in areas with high slope (20%). Similar profiles were obtained for Iberia and North Africa with high probability of occurrence in areas that range from 0 to 20% slope suggesting that these lineages include groups with very distinct responses to this variable (Fig. 15).

For annual precipitation, response curves revealed differences between the North African and Iberian populations; the first are more frequently distributed in areas with higher precipitation values (1000 to 1500 mm), while the later tends to occur in areas with lower precipitation levels (500 to 800 mm). Within Iberia, the response curves show distinct patterns among groups: (1) the Southern lineage occur more frequently in areas with 1000 mm of annual precipitation; (2) the western lineage tend to occur in areas with low (500 to 750 mm), although there is a secondary peak of occurrence at high annual precipitation (1500 mm). The IBW-CWS sublineage is restricted to the first range while the IBW-CNSW sublineage occurs in both; and (3) the Eastern sublineage IBE-NSC seems to be restricted to areas with low annual precipitation (500 to 750 mm) with complete absence in areas with higher precipitation levels (Fig. 15).

Response curves for the mean temperature of the warmest quarter revealed similar patterns across all models, suggesting that the species prefers temperatures lower than 20 ^{o}C (Fig. 15). On the other hand, responses curves for precipitation seasonality revealed differences within Iberia. Both western and southern lineages are more frequent in areas with higher values of precipitation variation along the year (variation coefficient of 60 to 70). Nonetheless, while IBE-sou is restricted to areas with low precipitation variation (variation coefficient of 50), IBW-CNSW is present in areas with a wide range of precipitation variation, from 50 to 80 (Fig. 15).

Table 3 On top, details and metrics of the 50 model replicates developed for *Vipera latastei-monticola* genetic groups, including number of records used in the training and tests datasets, average (standard deviation) training and tests AUC and five percentile minimum training presence threshold; on bottom, average (standard deviation) percentage contribution of each variable to the model replicates.

	IBNA	**NA**	**IB**	**IBW**	**IBE**	**IBS**	**IBW-CNSW**	**IBW-CWS**	**IBE-sou**	**IBE-NSC**
N training/test samples	411/175	49/20	362/155	101/43	243/103	18/7	47/19	56/23	38/16	206/87
Training AUC	0.88 (0.01)	0.95 (0.01)	0.85(0.01)	0.91 (0.01)	0.88 (0.01)	0.95 (0.01)	0.94 (0.01)	0.91(0.02)	0.92 (0.02)	0.89 (0.01)
Test AUC	0.85(0.01)	0.93 (0.04)	0.82 (0.02)	0.86 (0.03)	0.84 (0.02)	0.92 (0.04)	0.91 (0.04)	0.87 (0.04)	0.88 (0.05)	0.85 (0.02)
5 percentile threshold	0.17	0.06	0.18	0.18	0.17	0.18	0.08	0.20	0.17	0.16
Isothermality	9.9 (1.9)	1.9 (1.7)	12.3 (3.3)	7.3 (3)	7.7 (3.1)	5.6 (5.9)	5.9 (1.9)	21.9 (7.7)	0.8 (1.3)	5.1 (2.9)
Temp Annual Range	2.9 (1)	0.9 (0.9)	4.4 (1.8)	3.9 (2.7)	3.2 (1.2)	9.6 (6.2)	4.5 (1.7)	2.8 (2.2)	6.1 (4.7)	3.9 (1.6)
Temp wettest quarter	2.6 (1)	25.6 (11.9)	5.8 (2.3)	35.4 (8.3)	4.5 (1.9)	0.1 (0.3)	8.8 (6.4)	38.8 (12)	21.9 (10.7)	3.7 (1.9)
Temp Warmest quarter	28.1 (4.5)	9.2 (7.5)	15.9 (3.2)	6.9 (3.2)	18.1 (4.9)	22.4 (14)	17.4 (6.9)	3.8 (2.8)	6.8 (4.6)	18.2 (7.8)
Annual precipitation	37.7 (4.6)	30.4 (9.5)	28.9 (3.8)	21.2 (6)	43.8 (5.1)	19 (16.1)	30.2 (13)	14.5 (4.7)	2.4 (2.3)	46.9 (6.8)
Precipitation seasonality	8.2 (0.9)	8 (3.7)	12.4 (2.1)	12.2 (2.8)	6.5 (1.9)	13.3 (9.9)	19.2 (6.5)	3.8 (2.4)	22.9 (8.4)	10.8 (4.2)
Slope	10.6 (2.4)	23.9 (11.1)	20.2 (4.7)	13 (6.2)	16.1 (3.7)	30.2 (16.2)	14.1 (8.3)	14.4 (7.6)	39.1 (11.1)	11.4 (3.3)

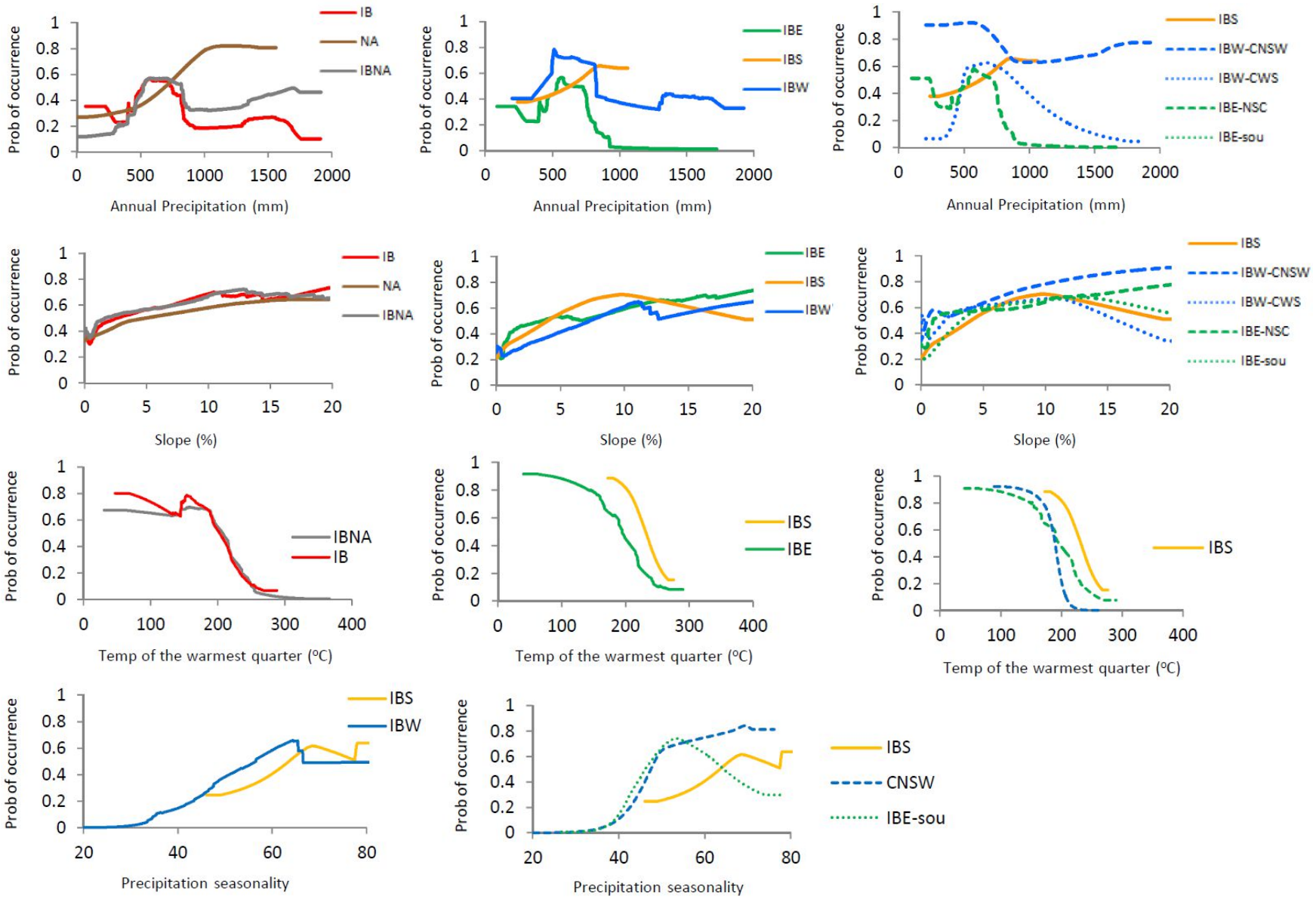

Figure 15 Response curves for the eco-geographical variables most related to the distribution of *V. latastei-monticola* groups within the three levels of genetic structure addressed in this study.

2.3 Predicted suitable areas

Overall, the model for the entire distribution of *Vipera latastei-monticola* predicted potential areas of occurrence consistent with the known distribution of the species (appendix 7). Low values of standard deviation among the 50 replicas were obtained across all study area suggesting that model predictions are highly accurate (appendix 7). The binary model reflected well the predictions from the probability model (Fig 16; appendix 7). In North Africa, the areas with potential occurrence were located in the Rif, in the Atlas Mountains and from Northeastern Algeria to Tunisia. In the Iberian Peninsula, extensive areas of potential occurrence were identified, including areas outside the range of the species in Northern Spain and Southern France where the other Iberian vipers (i.e. *Vipera aspis* and *Vipera seoanei*) are present.

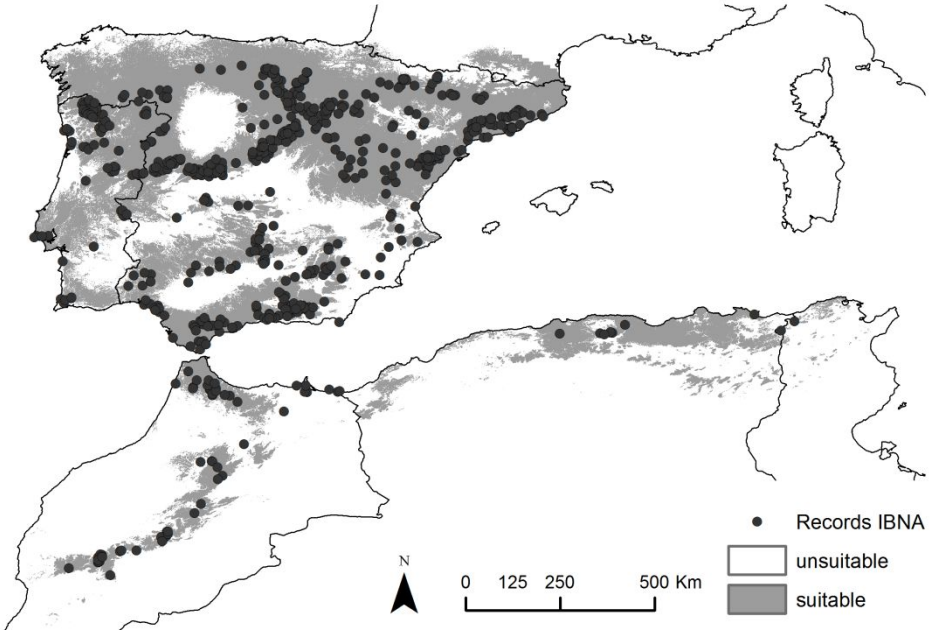

Figure 16 Predicted suitable areas for the occurrence of *Vipera latastei-monticola*.

The model developed for the Iberian lineage predicted extensive areas of potential occurrence in the Iberian Peninsula, consistent with the areas predicted by the model developed for the entire distribution of the species but showing a more restricted potential distribution in northern regions (appendix 7). In contrast, model projections to North Africa overestimated the potential occurrence of the species for this region, particularly in the Atlantic coast and south of Morocco. The lack of accuracy of these results is reflected in the high values of standard deviation found in this region (appendix 7). In agreement with the probability models, binary models for the Iberian lineage predicted as suitable extensive areas along the entire study area (Fig 17; appendix 7).

The model for the distribution of the North African lineage identified similar areas of probable occurrence as the model developed for the species entire range (appendix 7). Model projections to Iberia predicted potential areas of occurrence mostly restricted to southern and western regions of the Iberian Peninsula. Higher values of standard deviation were obtained in particular in northwest Iberia (appendix 7). The binary model overestimated the suitable areas for the lineage occurrence in both regions, predicting extensive areas as suitable in western and southern Iberia and in North Africa (Fig 17; appendix 7).

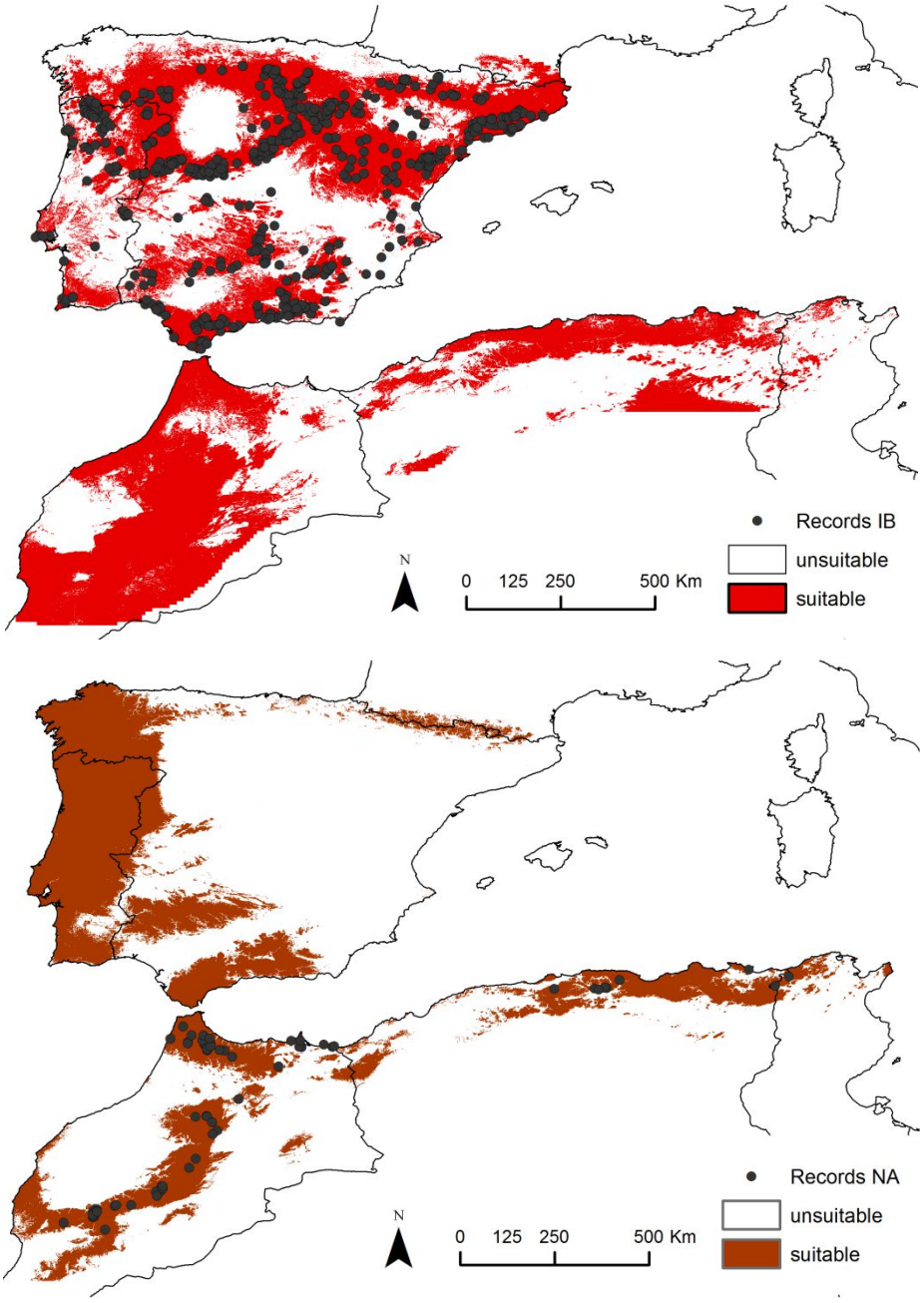

Figure 17 Predicted suitable areas for the occurrence of *Vipera latastei-monticola* Iberian (up) and North African (down) lineages, projected for the species entire distribution.

The Eastern, Western and Southern Iberian models predicted potential areas of occurrence that fit each group observational data (appendix 7). Model projections to Iberia predicted few areas for the presence of the Eastern lineage outside its distributional range. On the other hand, the Western and Southern lineages found extensive areas with high presence probability located outside their range: the model for the Western lineage predicts high probability of occurrence in the southern region of the peninsula while the model for the Southern lineage predicts extensive areas along the southwestern and northern coasts (from Southern Portugal to the Pyrenees) (Fig 18; appendix 7). In general, low values of standard deviation were obtain for the models, with the exception of the model for the Western lineage which presented high values of dissimilarity between replicas in the areas located outside de group range (appendix 7).

For each Iberian lineage, binary model predictions were highly consistent with the configuration of the probability models (Fig 18, appendix 7).

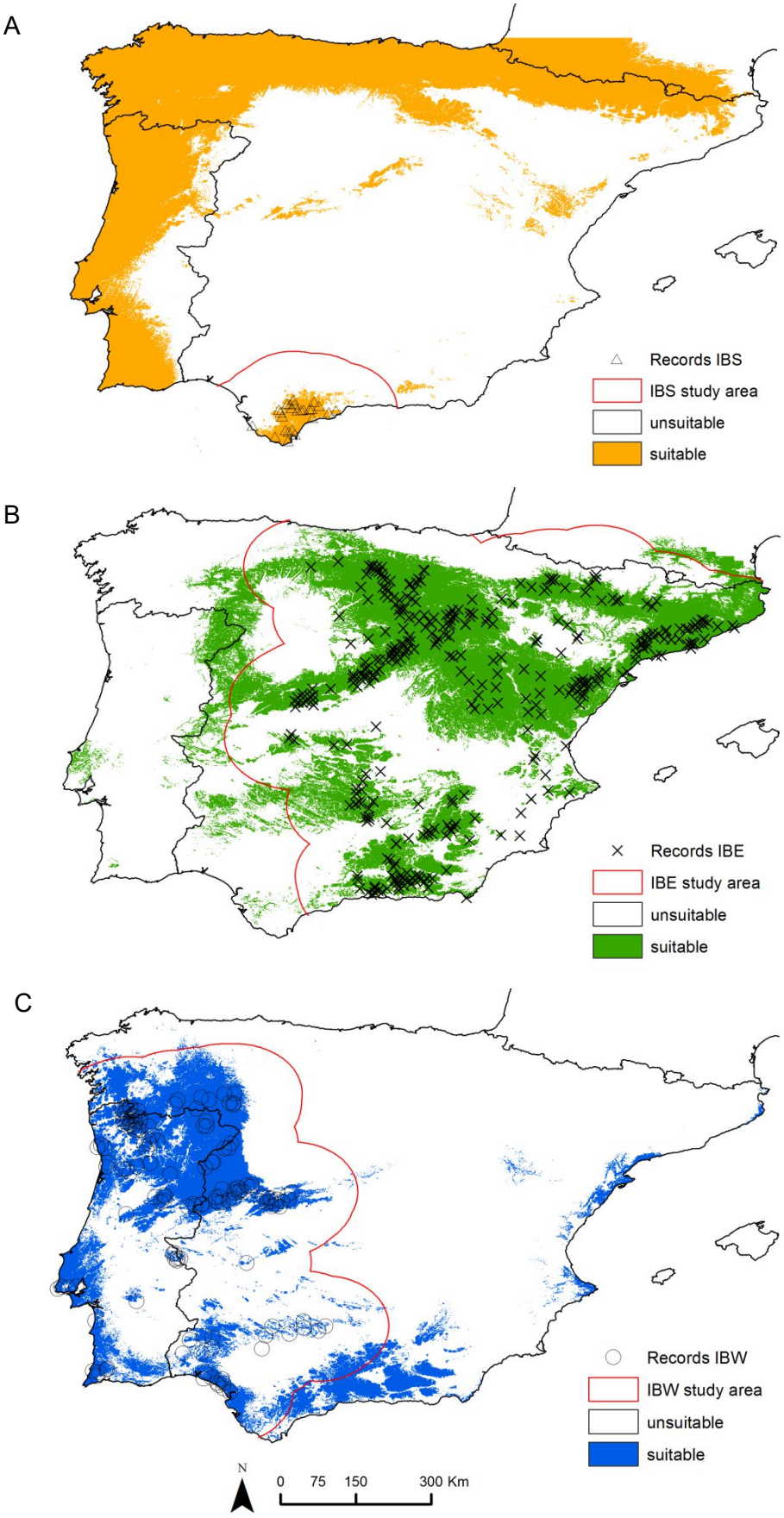

Figure 18 Predicted suitable areas for *Vipera latastei* Southern Iberian lineage (A), Eastern Iberian lineage (B) and Western Iberian lineage (C), projected to the whole Iberian Peninsula.

Models developed for the Iberian sublineages predicted potential areas of occurrence consistent with their distributional range (appendix 7). Models projections to Iberia predicted areas with high probability of occurrence located outside the sublineages range, with the exception of the model for the Eastern sublineage IBE-NSC that only predicted areas restricted to the known distribution of the group (Fig 19; appendix 7).
In particular, the model for the Eastern sublineage IBE-sou found potential areas of occurrence located in the Pyrenees and in northwestern Iberia, overlapping with the northern range of the Western sublineage IBW-CNSW. High values of standard deviation were obtained particularly in the northwestern region. Accordingly, the model developed for the Western sublineage IBW-CNSW predicted areas with high presence probability located in the southeastern region, consistent with the distribution of the Eastern sublineage IBE-sou; although with high values of standard deviation (Fig 19; appendix 7).

Regarding the Western sublineage, IBW-CWS, the model predicted extensive areas for the presence of the group across the entire region, in particular in the southeast (overlapping with the range of IBE-sou), northwest (overlapping with the range of IBW-CNSW) and in the northeastern coast of Spain. Nonetheless, high values of standard deviation were obtained in these areas, particularly in the northeastern coast of Iberia (Fig 19; appendix 7). Overall, predictions from the probability and the binary models were highly consistent (Fig 19; appendix 7).

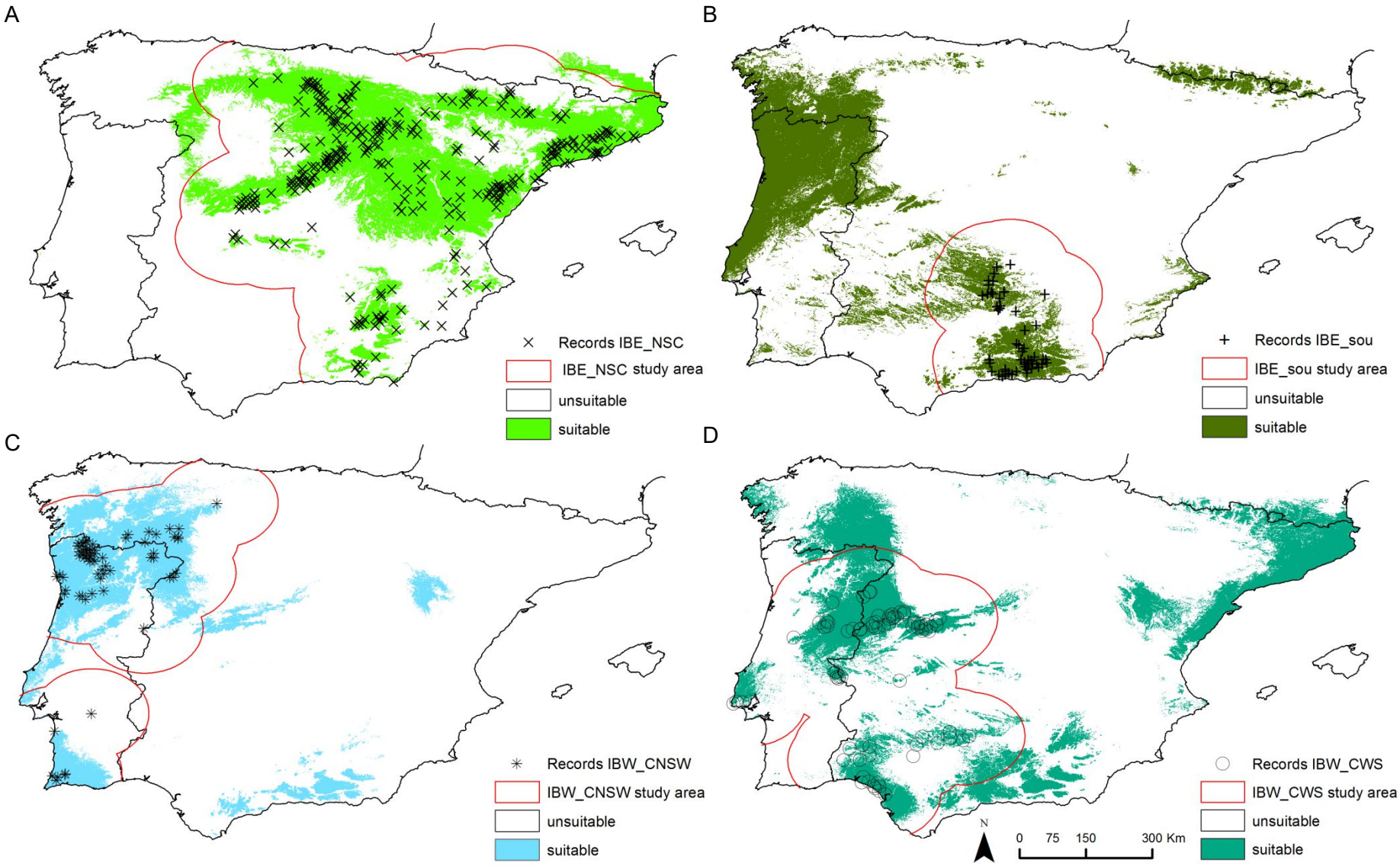

Figure 19 Predicted suitable areas for the occurrence of *Vipera latastei* Eastern Iberian sublineages IBE-NSC (A), IBE-sou (B) and the Western Iberian sublineages IBW-CNSW (C), IBW-CWS (D), projected to the whole Iberian Peninsula.

2.4. Sympatry areas for Iberian lineages and sublineages

Sympatry maps for Western and Eastern lineages reflect two potential areas of co-existence between these two lineages: one in southeastern Iberia and the other coinciding with the contact zone between the groups observed range in central Iberia (appendix 8). Sympatry maps for the Southern and Eastern lineages predicted suitable conditions for the two groups in Northern Iberia, outside the range of the species (appendix 8). Potential areas of sympatry between the Western and Southern lineages are mostly restricted to the western areas of the Peninsula and are more extensive than the remaining lineages (appendix 8). Indeed, Western and Southern lineages presented the highest extent of sympatry (Table 4).

Regarding the Iberian sublineages, the sympatry maps show extensive areas of potential sympatry among the western sublineages and the southern groups (IBS and IBE-sou; annex). In particular the Eastern sublineage IBE-sou has extensive areas of potential coexistence with the Western sublineages and the Southern lineage, mostly located in the northwestern region of Iberia. In contrast, potential areas of sympatry among this sublineage and the other Eastern sublineage, IBE-NSC, are extremely restricted, covering only 2.60% of the total area occupied by them (Table 4). Sympatry maps for the IBE-NSC predicted few areas where this group can coexist with the remaining (appendix 8).

Table 4 Extent of sympatry, in area (number of km^2) and as percentage regarding the total area of Iberia, among *V. latastei* Iberian lineages and sublineages given by the pairwise combination of the binary models.

Parwise Comparison	Sympatry (km^2)	Sympatry/total (%)
IBS-IBW	86,822	22.10%
IBW-IBE	62,456	14.20%
IBS-IBE	73,130	12.60%
sou-CNSW	81,118	37%
sou-CWS	89,737	30.50%
IBS-sou	106,520	27.50%
IBS-CNSW	87,447	25.20%
CNSW-CWS	63,243	24.20%
NSC-CWS	69,756	17.70%
IBS-CWS	75,921	17.20%
IBS-NSC	60,508	11.80%
NSC-CNSW	26,122	7.40%
NSC-sou	11,107	2.60%

2.5 Niche overlap tests

In general, the observed niche overlap (D) among groups is low, with a maximum D value of 0.334 obtained for the comparison between the eastern and western sublineage, IBE-NSC and IBW-CWS and a minimum D value of 0.02 obtained for the comparison of the two southern groups, IBS and IBE-sou (Table 5).

The niche equivalency hypothesis was rejected for all pairs ($p < 0.05$), signalling that ecological niches are significantly distinct (Table 5).

For most of the pairwise comparisons, the similarity tests failed to reject the null hypothesis of retained niche similarity (i.e. the D value falls within the null distribution [95% CI]) (Table 5). However for some comparisons, the null hypothesis is rejected with a D value falling on the right side of the distribution (results not shown), meaning that the niche occupied by one group is more similar to the niche of the other group than would be expected by chance. This pattern was observed for several pairwise comparisons including for the two main lineages: the North African lineage niche is more similar to the Iberian lineage niche than expected by chance suggesting that the later harbors a greater background environmental variability including also the environmental variability found in North Africa. The same conclusion is applied for the remaining significant pairwise comparisons (Table 5).

Table 5 Pairwise comparisons of the niche overlap showed between the three levels of genetic structure within *V. latastei-monticola*. Comparisons are based on calibrated Principal Component Analyses (PCA) of clades, lineages and sublineages occurrences and their respective climatic ranges. Values for percentage of variance explained by the first two components of each PCA (pc1 / pc2), Schoener's D Index of niche overlap, and probability for equivalency and similarity tests among different pairs of lineages are represented.

Comparison x-y	pc1	pc2	Niche overlap (D)	Equivalency	Similarity y->x	Similarity x->y
IB-NA	40.36	27.4	0.169	0.0198*	0.0198*	0.75248
IBS-IBE	50.08	23.71	0.063	0.0198*	0.91089	0.41584
IBS-IBW	46.76	31.55	0.072	0.0198*	0.71287	0.0198*
IBW-IBE	42.11	30.96	0.258	0.0198*	0.17822	0.0396*
IBS-NSC	51.06	23.22	0.068	0.0198*	0.91089	0.17822
IBS-sou	52.79	31.38	0.02	0.0198*	0.05941	0.45545
IBS-CNSW	52.46	27.58	0.111	0.0198*	0.75248	0.0198*
IBS-CWS	42.8	33.36	0.058	0.0198*	0.39604	0.0198*
NSC-sou	49.04	21.48	0.085	0.0198*	0.07921	0.277723
NSC-CNSW	42.08	34.7	0.109	0.0198*	0.41584	0.55446
NSC-CWS	43.32	27.76	0.334	0.0198*	0.0198*	0.29703
sou-CNSW	53.19	31.19	0.069	0.0198*	0.71287	0.0396*
sou-CWS	44.16	34.13	0.162	0.0198*	0.11881	0.05941
CNSW-CWS	51.65	31.19	0.207	0.0198*	0.0198*	0.51485

3. Morphological analyses

3.1 Univariate analyses

3.1.1 Sexual dimorphism

Before testing for sexual dimorphism in *V. latastei-monticola*, preliminary tests of normality and homogeneity of variances were conducted for each variable. The Levene's test for equality of variances revealed significant differences in N-DORS, S-APIC, N-APIC and SUBC. Through the visual examination of the histograms and the Shapiro Wilk test we confirmed that all variables deviated from a normal distribution.

Sexual dimorphism was found for INFRA, INTER, PERI, S-APIC, SUBC and N-APIC (appendix 8, 10). Therefore, further tests on these variables were performed separately for males and females.

3.1.2 Morphological differences between Iberian and North African lineages

All variables were tested again for normality and homogeneity of variances. Heterogeneity of variances was found for S-APIC (males), N-APIC (males), SUBC (males), LOR, VENT and N-DORS. All variables, with the exception of INTER (males and females), deviated from a normal distribution.

Significant morphological differences between North African and Iberian lineages were obtained for VENT, N-DORS, SUBC (females and males) and INTER for males only (appendix 8, 10). From all variables, only INTER revealed a distinct pattern between sexes on the morphological differentiation of North African and Iberian populations.

3.1.3 Morphological differences between Iberian lineages

Preliminary assumptions tests were performed for all variables. The Levene's test was significant for DMARKS, PERI (males), S-APIC (males), N-APIC (males), SUBC (males) and VENT. From all variables, only INTER (males and females) deviated from a normal distribution.

Morphological differences between the groups were detected for the following variables: DMARKS, LOR, VENT, INTER (for males and females), N-APIC (for males and females), PERI (for males and females) and S-APIC (for males and females). From all variables, only N-DORS, SUPRA, INFRA (males and females) and SUBC (males and females) reported non statistical significant differences (appendix 8, 10).

The traits INTER and PERI revealed a similar pattern: pairwise comparisons reported significant differences between the Eastern and Western lineages (appendix 9).

For both N-APIC and S-APIC, the *post hoc* tests revealed statistical significant differences between the Southern and Eastern lineages for both females and males. In addition, for the males morphological differences between the Southern and Western lineages were also detected (appendix 9).

For DMARKS, statistically significant differences between the Western lineage and the Southern and Eastern lineages were reported. On the other hand, for LOR morphological differences were detected between the Eastern lineage and the remaining (appendix 9).

Finally, *post hoc* tests on VENT reported significant differences for all pairwise comparisons: between the Western and Southern lineage, Eastern and Southern lineages, and the Eastern and Western lineages (appendix 9).

3.1.4 Morphological differences between Iberian sublineages

Preliminary tests of normality and homogeneity of variances were conducted. Heterocedasticity was reported for LOR, SUPRA, VENT, N-APIC (males), PERI (males), S-APIC (males) and SUBC (males). The normality assumption was rejected for all variables except for INTER in females.

Statistically significant differences between Iberian sublineages were obtained for DMARKS, LOR, VENT, INFRA (for females and males), INTER (for females and males), N-APIC (for females and males), S-APIC (for females and males) and PERI (males only) and SUBC (males only). For the remaining variables, N-DORS, SUPRA, PERI (females) and SUBC (females), there were no significant differences between groups (appendix 8, 10).

Post hoc tests on PERI (males), INTER (for both males and females), N-APIC (females) and S-APIC (females) only reported significant differences between the Western sublineage IBW-CWS and the Eastern sublineage IBE-NSC. However, pairwise comparisons revealed a different pattern for N-APIC and S-APIC in males with significant differences obtained between the southern groups IBS and IBE-sou and the remaining (appendix 9).

For INFRA (males) significant differences were found between the Eastern sublineage IBE-sou and the remaning Eastern and Western sublineages while the pairwise comparison of the two southern groups IBE-sou and IBS reported a non-significant p-value. For the females, non-significant differences were found between the groups after the Bonferroni correction (appendix 9).

Post-hoc tests on SUBC (males) detected significant differences between the southeastern sublineage IBE-sou and the northern groups IBE-NSC and IBW-CNSW and between the southwestern sublineage IBW-CWS and the northeastern sublineage IBE-NSC (appendix 9).

For LOR, significant differences were found between the Eastern sublineage IBE-NSC and the remaining groups (appendix 9).

Multiple comparisons on VENT identified three distinct morphological groups: one corresponding to the Eastern sublineages IBE-sou and IBE-NSC, other corresponding to the southern groups IBS and IBW-CWS and one including only the Western sublineage IBW-CNSW (appendix 9).

For DMARKS, differences between southern and northern groups were also detected: IBW-CNSW and IBE-NSC are statistically different from the southern groups IBW-CWS and IBE-sou. In addition, the southern lineage is also statistically different from IBW-CWS (appendix 9).

3.2 Multivariate analyses

3.2.1 Morphological differentiation between Iberian and North African clades

Principal Component Analyses

For both males and females, a PCA was performed on the traits that reported morphological differentiation among North African and Iberian specimens (Table 6). The two first components explained 60% and 68% of the total morphological variation for females and males respectively (Table 6). In females the first component was affected by VENT and INTER, and the second by SUBC and NDORS; in males, first component was affected by SUBC and VENT, and the second by NDORS and VENT (Table 6).

Table 6 Loading scores, eigenvalues and percentage of explained variance for the two principal components extracted in the PCA of the morphological characters that presented morphological differentiation among Iberian and North African specimens in both males and females. Loading values non-significant ($p<0.3$) were omitted. Variable INTRA was not analysed (NA) in males.

	Females		**Males**	
Variables	**PC1**	**PC2**	**PC1**	**PC2**
VENT	-0.77		0.587	0.36
SUBC		0.727	0.842	
NDORS		0.731		0.925
INTER	0.776		NA	NA
Eigenvalues	1.29	1.12	1.092	0.978
% variance	32	28	36	32

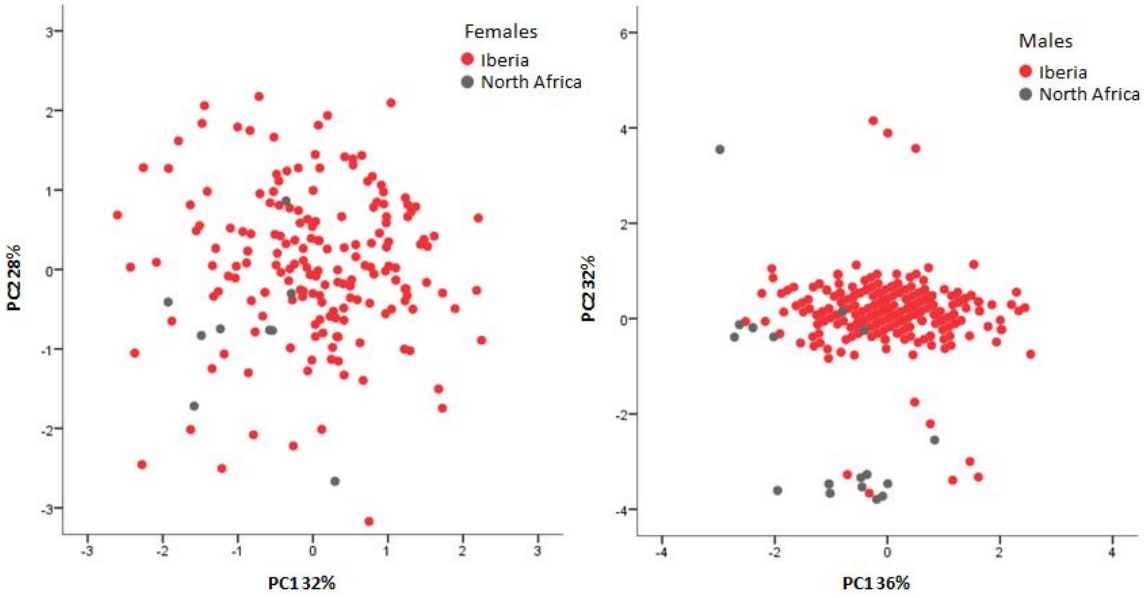

Figure 20 Graphic representation of the Iberian (red) and North Africa (grey) samples in the space of the two first principal components from a PCA over the traits that showed statistical differences, for females (left) and males (right).

The visualization of the morphological variability in the space of the two first components suggested high morphological variability in females but without clear geographic structure or differentiation among North African and Iberian groups. In males, the PC2 separates two morphological groups one including most of North African specimens and the other most of Iberian specimens (Fig. 20)

Discriminant function analyses

According with the Wilk's Lambda test, the discriminant functions derived by the DFA significantly differentiated the groups with $\Lambda = 0.907$ $\chi^2(4) = 17.684$ $p = 0.001$ for the females and $\Lambda = 0.662$ $\chi^2(3) = 122.933$ $p < 0.001$ for the males. For both males and females, the classification of the Iberian specimens was highly accurate with 98% of the specimens correctly classified (Table 7). However, the percentage of North African specimens correctly classified was substantially lower with 61.1% and 22.2%, for males and females respectively (Table 7).

Table 7 Percentage (and number in relation to the total) of correct classification for Iberian and North African individual males and females of *Vipera latastei-monticola* according to the discriminant function analyses.

		IB	NA
Females	**IB**	98.3 % (174/177)	1.7 % (3/177)
	NA	77.8 % (7/9)	22.2 %(2/9)
Males	**IB**	98.2 % (279/284)	1.8 % (5/284)
	NA	38.9 % (7/18)	61.1 % (11/18)

Hierarchical Cluster analyses

According with the DFA results, the examination of the dendrograms using a K=2 solution indicated the presence of two clusters in the males dataset consistent with the Iberian (K2 98.2%) and North African (K1 64.7%) groups (Table 8; Fig 21); in the females groups tend to cluster together suggesting no morphological differentiation (Table 8).

Table 8 Percentage (and number in relation to the total) for Iberian and North African individual males and females of *Vipera latastei-monticola* assigned to K1 and K2 according with hierarchical cluster analyses using a K=2 solution.

		K1	K2
Females	**IB**	96.5 % (166/172)	3.5% (6/172)
	NA	87.5 % (7/8)	12.5% (1/8)
Males	**IB**	1.8 % (5/280)	98.2% (275/280)
	NA	64.7% (11/17)	35.3% (6/17)

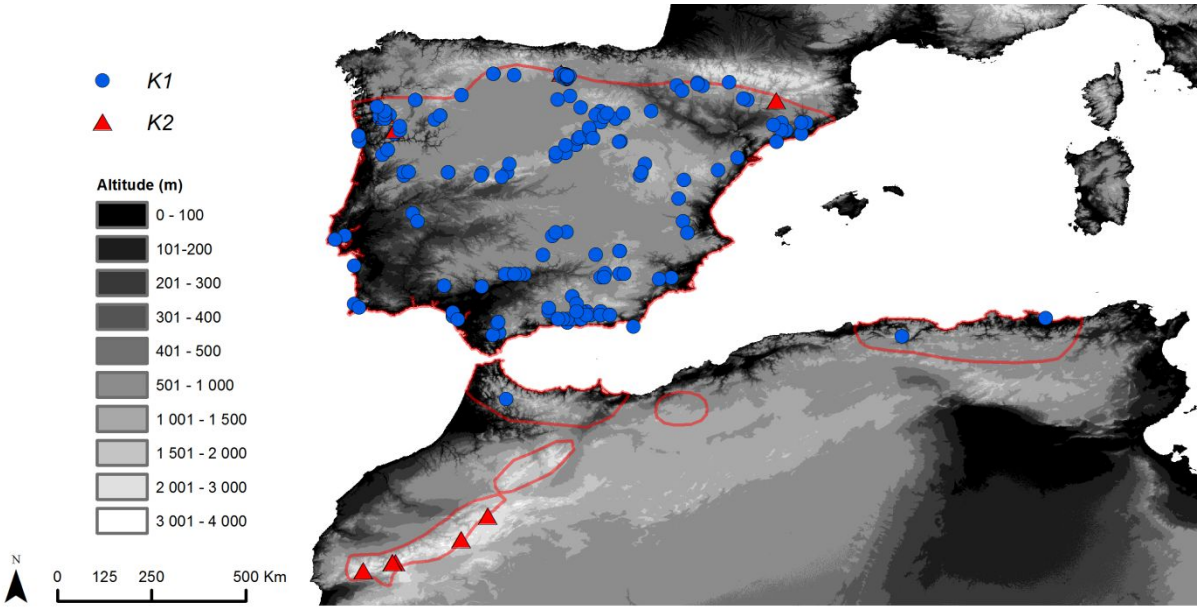

Figure 21 Spatial distribution of the two morphological clusters found within the males dataset using hierarchical cluster analyses.

3.2.2 Morphological differentiation within Iberia

Principal Component Analyses

Principal Component Analyses were conducted separately for males and females, considering only the characters that reported morphological differentiation within Iberian groups. For the females, the two first components explained 58.2% of the total morphological variability, while for the males the three first components explained most

of the variance, accounting for 56.8% of the variance present in the data (Table 9; Fig. 22). For both males and females, the first principal component explains most of the variability found in LOR, PERI and INTER. In the females, the second component is affected by all variables except PERI. In the males the second component accounts for the variability of N-APIC, INTER, SUBC, INFRA and the third by VENT and DMARKS (Table 9).

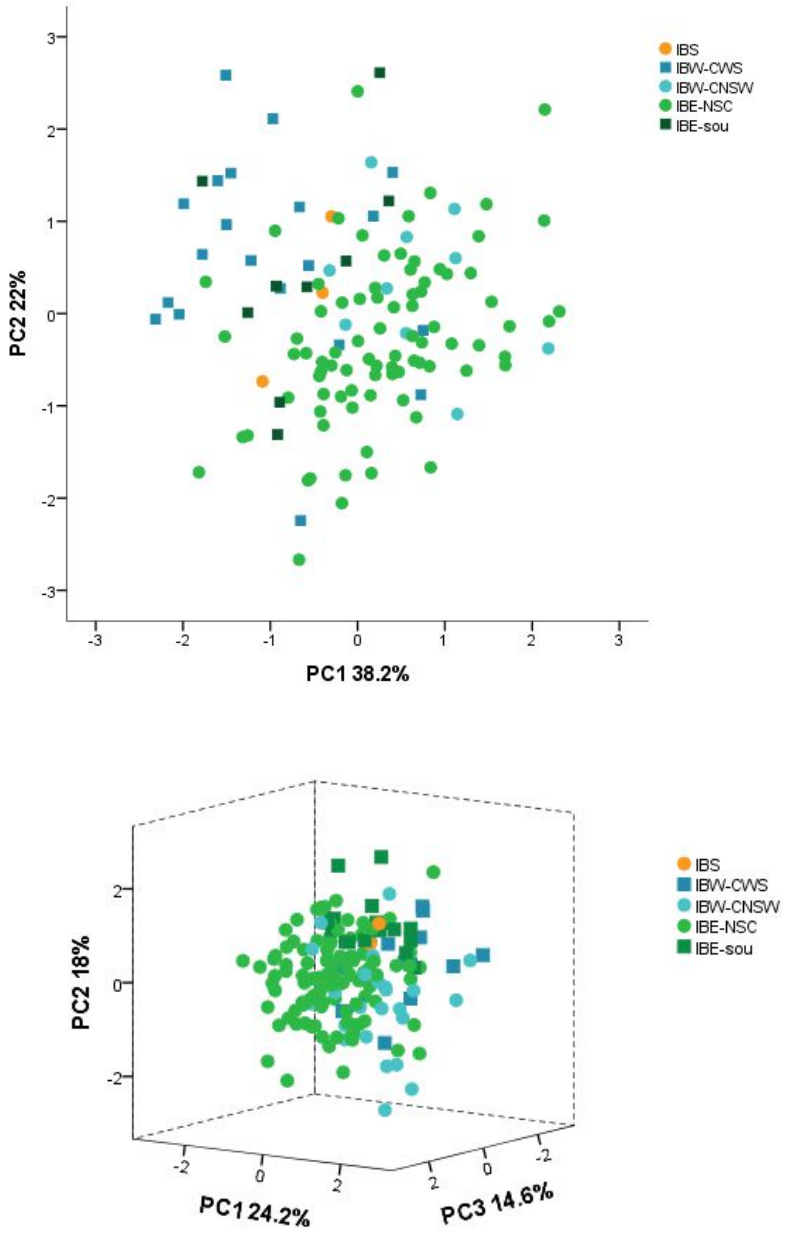

Figure 22 Graphic representation of individual females (up) and males (down) from Iberian Southern lineage (orange), Western sublineages (blue), and Eastern sublineages (green) in the space of the two and three first principal components from a PCA over the traits that showed statistical differences.

Table 9 Loading scores and eigenvalues and percentage of explained variance for the first principal components (PC1 and PC2 for females, PC1, PC2 and PC3 for males) extracted from a PCA for the morphological characters that presented morphological differentiation within Iberian groups in females and males. Loading values non-significant ($p < 0.3$) were omitted. Variables SUBC and INFRA were not analysed (NA) for females.

	Females		**Males**		
	PC1	**PC2**	**PC1**	**PC2**	**PC3**
NAPIC		-0.525		0.654	
VENT		0.772			0.778
LOR	0.717	-0.377	0.748		
DMARKS		0.731			0.762
PERI	0.829		0.757		
INTER	0.688	-0.323	0.619	0.427	
SUBC	NA	NA		0.635	
INFRA	NA	NA		0.673	
Eigenvalues	2.234	1.158	2.094	1.346	1.105
% Variance	38.2	20	24.2	18	14.6

The visualization of the morphological variability suggested high morphological variability in males and females; however, groups show high degree of overlap (Fig. 22).

Discriminant function analyses

Discriminant analyses on the Iberian lineages revealed two discriminant functions that accounted for 71.9% and 28.1% of the total variation (with eigenvalues of 0.235 and 0.092) in the females, and 86% and 14% of the variation (with eigenvalues of 0.431 and 0.070) in the males. In combination these functions significantly differentiate the groups, $\Lambda = 0.742$ $\chi^2(12) = 36.028$ $p < 0.001$ for the females and $\Lambda = 0.653$ $\chi^2(16) = 68.8$ $p < 0.001$ for the males.

However, the low percentages of corrected classifications obtained for the Western and Southern specimens suggested no morphological differentiation between the groups in both males and females (Table 10). In addition, the discriminant function plot of the specimens of each group and the groups centroids also indicate low morphological differentiation (Fig. 23).

Table 10 Percentage (and number in relation to the total) of correct classification of Western, Eastern and Southern Iberian individual males and females of *Vipera latastei-monticola* according to discriminant functions analyses.

		IBW	IBE	IBS
Females	**IBW**	43.3 % (13/30)	56.7 % (17/30)	0 %
	IBE	4.3 % (4/93)	94.6 % (88/93)	1.1 % (1/93)
	IBS	0 %	100 % (3/3)	0 %
Males	**IBW**	52.6 % (2/38)	47.4 % (18/38)	0 %
	IBE	5.5 % (7/127)	94.5 % (120/127)	0 %
	IBS	0 %	66.7 % (2/3)	33.3 % (1/3)

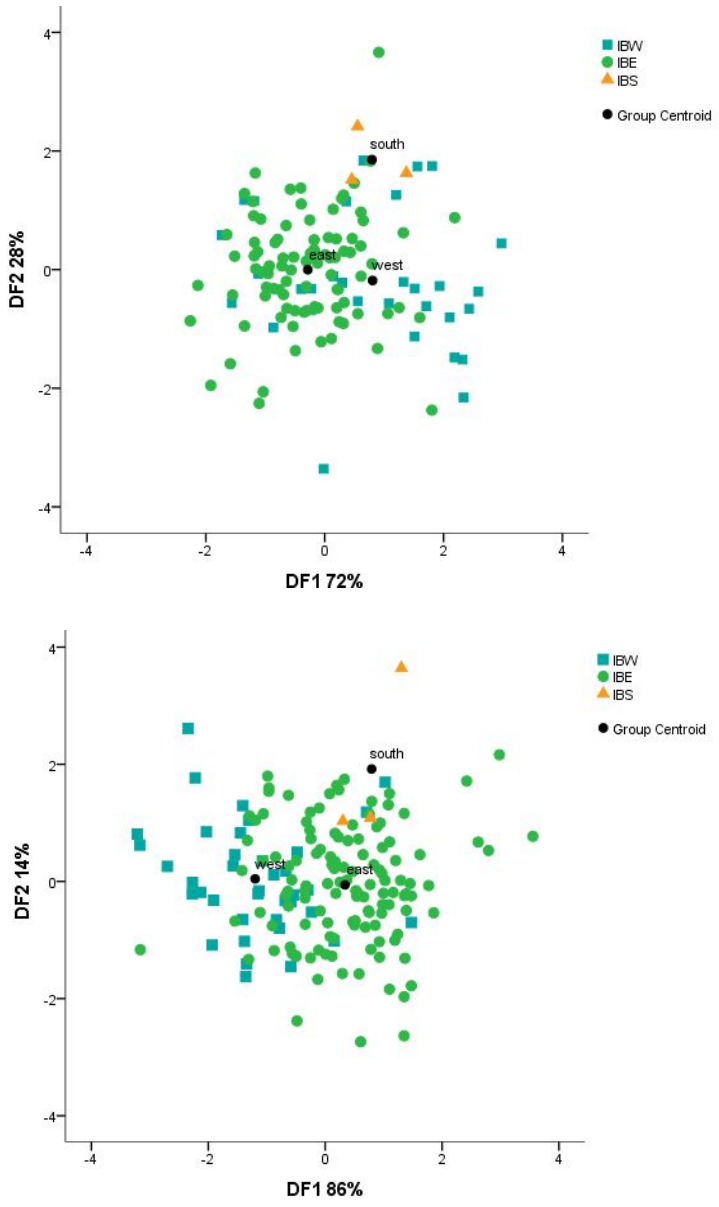

Figure 23 Graphic representation of females (up) and males (down) from the three main Iberian lineages, Southern (orange), Western (blue), and Eastern (green), in the space of their respective discriminant functions.

Discriminant analyses on the Iberian sublineages resulted in two significant discriminant functions for females ($\Lambda = 0.476$ $\chi^2(24) = 88.6$ $p < 0.001$ and $\Lambda = 0.808$ $\chi^2(15) = 25.4$ $p = 0.045$) and males ($\Lambda = 0.420$ $\chi^2(32) = 139.17$ $p < 0.001$ and $\Lambda = 0.642$ $\chi^2(21) = 71.24$ $p < 0.001$). The two functions accounted for 76% and 11.3% of the variance (eigenvalues of 0.697 and 0.104) and 51.1% and 36.6% (eigenvalues of 0.527 and 0.378) for females and males, respectively. For males and females, only the classifications of the sublineages IBE-NSC and IBW-CWS were accurate, although IBW-CWS reported a lower percentage of correct classification in males than in females (57.1 % for the males and 70% for the females). However, for the females 90% of the specimens from IBW-CNSW were assigned to IBE-NSC suggesting the existence of a homogeneous northern group (Table 11). These results are supported by the discriminant function plot that seems to differentiate northern and southern sublineages. On the other hand, the males plot showed lack of differentiation and morphological variability (Fig. 24).

Table 11 Percentage of correct classification (and number in relation to the total) for males and females of *Vipera latastei-monticola* in the corresponding Iberian sublineages according to discriminant functions analyses.

		IBS	IBW-CWS	IBW-CNSW	IBE-NSC	IBE-sou
Females	**IBS**	0%	33.3% (1/3)	0%	66.7 % (2/3)	0%
	IBW-CWS	5 % (1/20)	70 % (14/20)	0%	25 % (5/20)	0%
	IBW-CNSW	0%	0%	0%	90 % (9/10)	10 % (1/10)
	IBE-NSC	10 % (1/85)	2.4 % (2/85)	0%	96.4 % (81/85)	1.2 % (1/85)
	IBE-sou	11.1% (1/9)	22.2 % (2/9)	0%	55.6 % (5/9)	11.10 (1/9)%
Males	**IBS**	33.3 % (1/3)	0%	0%	66.7 % (2/3)	0%
	IBW-CWS	0%	57.1 %(8/14)	0%	42.9 % (6/14)	0%
	IBW-CNSW	0%	4.2 % (1/24)	37.5 % (9/24)	54.2 % (13/24)	4.2 % (1/24)
	IBE-NSC	0%	9 % (1/109)	3.7 % (4/109)	93.6 % (102/109)	1.8 % (2/109)
	IBE-sou	0%	0%	5.6 % (1/18)	44.4 % (8/18)	50 % (9/18)

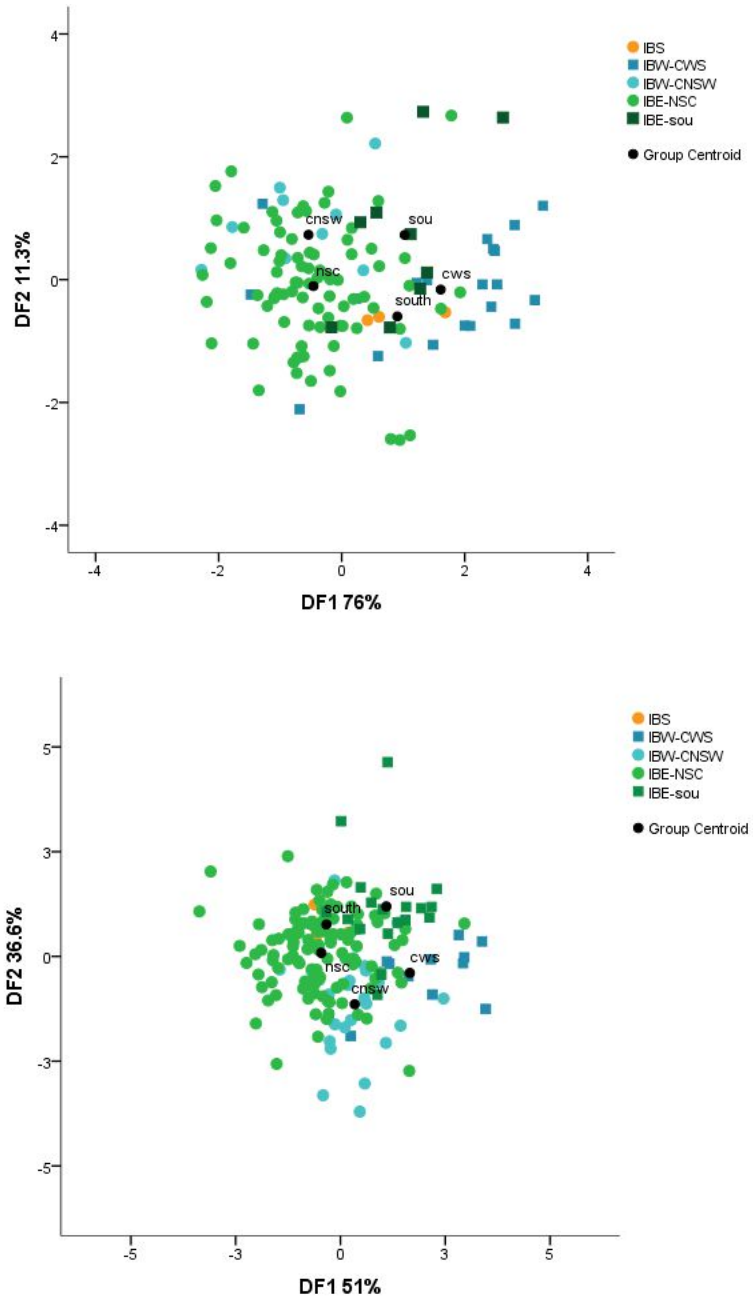

Figure 24 Graphic representation of females (up) and males (down) for the four Iberian sublineages (Western and Eastern sublineages, represented in blue and green respectively), and Southern lineage (orange) in the space depicted by their respective discriminant functions.

Hierarchical Cluster Analyses

In agreement with previous results, different morphological patterns were obtained for males and females. For the males, after examination of a wide range of K and removing outliers we conclude that the analyses were not able to cluster specimens into different morphological groups due to the morphological homogeneity found in the dataset.

In contrast, with a final solution of K=2, morphological differentiation between northern (IBE-NSC and IBW-CNSW) and southern sublineages (IBW-CWS and IBE-sou) was found for the females (Table 12; Fig 25), with the exception of the Southern lineage which clustered together with the northern sublineages.

Table 12 Percentage (and number in relation to the total) of Southern, Western and Eastern Iberian individual females of *Vipera latastei-monticola* assigned to K1 and K2 according with a hierarchical cluster analyses using K=2 solution.

	K1	K2
IBS	66.7 % (2/3)	33.3 % (1/3)
IBE-sou	45.5 % (5/11)	54.5 % (6/11)
IBE-NSC	93 % (84/90)	7.1 % (6/90)
IBW-CWS	25 % (5/20)	75 % (15/20)
IBW-CNSW	89% (8/9)	11.1 % (1/9)

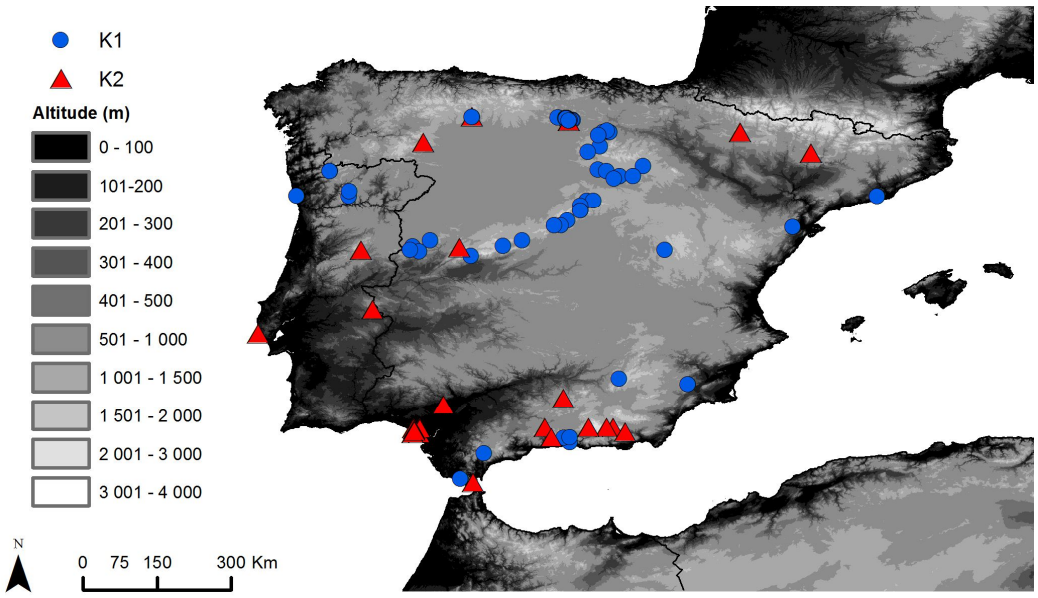

Figure 25 Spatial distribution of the two morphological clusters found within Iberia for females, using hierarchical cluster analyses.

V. Discussion

The Western Mediterranean Basin presents broad topographical and environmental variation, and has undergone a complex paleogeographic and climatic history, that shaped current biodiversity patterns and induced high levels of diversity and endemism (Weiss & Ferrand, 2007; Blondel *et al*., 2010). Several groups have been used to relate patterns of genetic and morphological variation to these factors (e.g. invertebrates: Ritchie *et al*., 2001; birds: Guillaumet *et al*., 2006; mammals: Gaubert *et al*., 2004; Cosson *et al*., 2015). Amphibians and reptiles, due to particular ecological constrains (i.e. ectothermic physiology) and life history traits (e.g. low dispersal), are highly suitable models to such purposes and thus, have been object of much research (e.g. Carranza *et al*., 2004, 2006; Velo-Antón *et al*., 2008, 2015; Miraldo *et al*., 2011; Kaliontzopoulou *et al*., 2011; Veríssimo *et al*., 2016). As a consequence of the increased knowledge on their biogeographical patterns, both groups have been subjected to important taxonomic revisions (e.g. in amphibians, *Hyla arborea-molleri*, Barth *et al*., 2011; *Bufo bufo-spinosus,* Recuero *et al*., 2012; in reptiles, *Timon lepidus-pater*, Paulo *et al*., 2008; *Natrix astreptophora-natrix*, Pokrant *et al*., 2016)

Previous studies on the Western Mediterranean viper *Vipera latastei-monticola* recovered strong patterns of genetic and morphological differentiation, suggesting that past geological and climatic events had a profound effect in its biogeographic history (Brito *et al*., 2006, 2008; Velo-Antón *et al*., 2012). However, no study has combined different biogeographical traits of the species, precluding the delimitation of coherent evolutionary and taxonomic units. In this work we addressed the intraspecific variability of *Vipera latastei-monticola* using an integrative approach that combines genetic, ecological and morphological variability analyses. We increased knowledge on genetic variability of the species and provided for the first time information regarding the intraspecific patterns of morphological and ecological variation of the species in an integrative framework.

1. Genetic variability analyses as tools to delimit evolutionary units

1.1. Inferences from mitochondrial DNA

In agreement with Velo-Antón *et al.* (2012), the inferred phylogenetic tree for the two mitochondrial genes recovered deep genetic lineages and high levels of genetic structure. The basal nodes of our phylogenetic tree were in general poorly supported preventing robust inferences regarding the relationships among the main lineages and sublineages of *Vipera latastei-monticola*; however the topology of the tree is similar to the referred work, supporting two highly structured vicariant clades, one Iberian and one African clade.

Both phylogenetic analyses and haplotype networks suggested that Southern Iberia harbors higher genetic diversity in comparison with the Central and Northern regions of the Iberian Peninsula, probably due to the presence of multiple refugia and important barriers to dispersal (Velo-Antón *et al*., 2012). Thus, the geographic distribution of the southern Iberian sublineages reflects the overall topographic complexity of the region with sublineages restricted to areas that acted as refugia during the glaciation periods (i.e. Betic mountains, Algarve), while northern Iberian lineages tend to be the result of posterior expansion processes (Velo-Antón *et al*., 2012). This pattern of genetic differentiation is highly concordant with the phylogeographic patterns observed in other species with low dispersal abilities (e.g. Martínez-Solano *et al.,* 2004; Santos *et al*., 2008; Gonçalves *et al.,* 2009).

The considerable improvement in sample size for North Africa allowed a better delimitation of already described sublineages and the identification of a new sublineage, the Western High Atlas. However, the lack of a fully resolved phylogenetic tree precludes an accurate assessment of the phylogenetic position of this sublineage.

1.2. Mitochondrial and nuclear discordances

In contrast with the high levels of genetic differentiation obtained with the mtDNA, the two nuclear genes, PRLP and β-fib*,* were not able to recover any genetic structure in *Vipera latastei-monticola*. These genes were initially chosen because of their high variability at lower phylogenetic levels shown in previous studies (e.g. Godinho *et al*., 2006; Townsend *et al,.* 2008). Other nuclear genes were already known to provide insufficient resolution to distinguish mitochondrial lineages within *V. latastei* (e.g. RAG 2; Velo-Antón *et al.,* 2012). This lack of population structure in nuclear genealogies in

contrast to deep patterns of genetic differentiation in mitochondrial lineages has been detected in multiple studies (e.g. in amphibians, Velo-Antón *et al*., 2008; in lizards: Pinho *et al*., 2007, 2008; in snakes, Rato *et al.,* 2009; Guo *et al.,* 2011). Incongruences between nuclear and mitochondrial markers may be explained by their highly different evolutionary rates and modes of inheritance (Godinho *et al*., 2008). The mitochondrial DNA accumulates nucleotide substitutions several times faster than a single-copy nuclear DNA (scnDNA), and so it provides better resolution for recent evolutionary events. Thus, while mtDNA can provide deep patterns of differentiation at lower taxonomic levels, nuclear markers may need more time to coalesce and match the patterns obtained with the mitochondrial data, resulting in common patterns of incomplete lineage sorting within species (Wan *et al*., 2004).

In addition, introgression and dispersal biased are among the most well documented causes of mito-nuclear discordances (e.g. Machado & Hey, 2003; Pinho *et al*., 2008; Barbanera *et al*., 2009; Boratyński *et al*., 2014). In particular, sex biased gene flow may lead to profound differences between nuclear and mitochondrial genealogies. As in other vipers, males of *Vipera latastei-monticola* frequently roam large distances during reproduction season to mate with females which practically display a sedentary behavior (see Martínez-Freiría *et al*., 2014), thus deeper patterns of genetic differentiation are expected in the mitochondrial DNA. However, the lack of differentiation between North African and Iberian specimens strongly suggests that the discordance between nuclear and mitochondrial DNA recovered in this study does not reflect the dispersal patterns of the species but rather with the insufficient resolution of the markers to differentiate the main mitochondrial groups.

1.3. Population genetic analyses

Microsatellite loci are mostly species specific, and as with many non-model species, such markers have not yet been developed for *Vipera latastei-monticola*. Thus, in the present study we have used ten microsatellite loci previously developed for *Vipera berus* (Carlsson *et al*., 2003; Ursenbacher *et al*., 2009). Cross-species amplification indicated that these markers could also be useful for studies with other species within the *Viperidae* family (Carlsson *et al*., 2003). Indeed, six microssatelite loci out of the 19 identified for *V. berus* performed well in a study conducted on the French populations of *V. ursinii* (Ferchaud *et al*., 2011). In addition, the same set of markers selected for this study was already successfully used in a previous study conducted in a contact zone among the three Iberian vipers (*V. aspis, V. seoanei and V. latastei*) (Tarroso *et*

al, 2014). Nonetheless, our population genetic analyses failed to identify genetic clusters within *Vipera latastei*. A possible scenario with extensive contemporary gene flow among Iberian mitochondrial lineages and sublineages in Southern Iberia does not fit the current distribution of the species with populations mostly isolated in undisturbed mountains (Santos *et al*., 2006), the existence of major barriers to gene flow in the region (e.g. Guadalquivir river) and their limited dispersal capabilities (see Martínez-Freiría *et al*., 2014). Therefore, this lack of genetic differentiation may indicate that this set of microsatellites is not appropriate to conduct intraspecific studies in the species, although we should not disregard the influence of the low sample size on the final result.

1.4. Spatial delimitation of mitochondrial groups

In this study we implemented a novel approach of spatial interpolation of genetic information from phylogenetic trees (Tarroso *et al*., 2015) to predict the spatial occurrence of the distinct lineages and sublineages identified within *Vipera latastei-monticola* and identify potential contact zones among them. The great improvement in sample size allowed a robust delimitation of Iberian lineages, in constrast sampling limitations in North Africa precluded the assessment of the exact spatial distribution of the phylogenetic groups.

This new method offers numerous advantages and considerable improves the delimitation of phygenetic groups in comparison with other alternatives commonly used such as 1) depicting the spatial distribution of lineages by representing samples in the map (e.g. Kidd & Liu, 2008) which usually involve a loss of genetic information when considering non alopatric groups (samples from contact zones are excluded, e.g. Anadón *et al.*, 2015); or 2) predicting the occurrence of distinct lineages using ecological niche modeling, which can led to overestimate potential areas of occurrence (e.g. Alvarado-Serrano & Knowles, 2014).

2. Analyses of ecological variability

2.1. Ecological niche based models

Previous studies using ecological models on *V. latastei-monticola* highlighted the association of climatic and topographic factors with the distribution of the species at regional (Santos *et al*., 2006; Brito *et al*., 2008; Brito *et al*., 2011) and local scales (Brito & Crespo, 2002; Martínez-Freiría *et al*., 2008; Tarroso *et al.*, 2014). Our ecological models developed at regional scale but using fine spatial resolution (1x1 km of pixel size) clearly followed these patterns as they found precipitation, temperature and slope as the most important factors affecting the distribution of the species, as well as, the distribution of each mitochondrial group. The average profiles of response curves indicated that the species occurs in areas with a wide range of annual precipitation (500 to 1750 mm) and slope (0 to 20%) and seems to be restricted to temperatures of the warmest quarter lower than 20°C. Interestingly, responses curves often revealed differences within the three levels of genetic structure addressed in this study. This pattern is particularly evident for annual precipitation: 1) Iberian populations tend to occur in areas with low precipitation (500 to 800 mm) while North African populations are more frequently distributed in areas with high precipitation (1000 to 1500 mm); 2) the three Iberian lineages frequently occur in areas with contrasted levels of precipitation, particularly the Southern lineage in relation to the other, and partially the Western lineage when compared to the Eastern; and 3) the Western sublineages IBW-CWS and IBW-CNSW tend to occur in areas with contrasting values of annual precipitation, the first is more frequent in areas with low (500 to 750 mm) and the later occurs in areas with high annual precipitation (1500 mm). Similar results were already reported by previous ecological models developed for North African populations (Brito *et al*., 2011), as well as for Eastern and Western morphological groups (Brito *et al.,* 2008; Santos *et al*., 2014), reinforcing that major phylogeographic groups within the species are restricted to habitats with contrasting precipitation conditions.

Regarding spatial predictions, the ecological model for the species was apparently robust identifying potential areas of occurrence mostly consistent with its distributional range (see Martínez-Freiría *et al*., 2014). Nonetheless, extensive areas of potential occurrence were identified outside the range of *V. latastei-monticola.* In North Africa, the model predicted potential suitability in the eastern Tellian Atlas in Algeria, where until now no vipers observations exist (Schleich *et al*., 1996, Brito *et al*., 2011). Lack of fieldwork in this politically unstable area might be related to this fact (Brito *et al*., 2011). In Northern and North-eastern Iberia the model predicted potential areas for the

species occurrence overlapping with the distribution of the other Iberian vipers, *Vipera aspis* and *Vipera seoanei.* The presence of other viper species has been referred as constrain in the distribution of *V. latastei* along northern suitable areas (Saint Girons, 1980; Santos *et al.*, 2006). This is further supported by studies developed at contact zones which suggested that interspecific competition might led to different habitat or microhabitat selection at local scale (e.g. Brito & Crespo, 2002; Martínez-Freiría *et al.*, 2006, 2008, 2009, 2010).

Iberian populations of *V. latastei* have been suggested to present high tolerance to contrasting environmental conditions as the species is present in humid, sub-humid and semiarid Mediterranean biotopes and from the sea level up to 3000 m (Santos *et al.*, 2006; Miras *et al.*, 2009; Martínez-Frería *et al.*, 2014). However, such variability seems reduced in North Africa, as the species is mostly link to humid Mediterranean areas located at moderate to high altitude (Brito *et al.*, 2011; Martínez-Frería *et al.*, 2014). Our models developed for each clade confirm these suggestions as the Iberian model projected to North Africa predicted extensive areas of potential occurrence across the entire region, while the North African lineage projected to the Iberian Peninsula identified more restrictive areas of potential occurrence, mostly located in southeastern and northwestern Iberia. However, the extensive areas of environmental suitability found across the entire North African region are clearly overestimated since they do not reflect the true ecological requirements of the species. Moreover, the low climatic stability at range limits may hamper the species persistence in these locations (e.g. Martínez-Freiría *et al.*, 2015).

In addition, ecological models for the Iberian lineages and sublineages projected to the entire region also retrieved interesting patterns. The Western and Southern lineages tend to extend their environmental suitability to the southern and western regions of the Iberian Peninsula, respectively, suggesting that these populations occur in similar habitat conditions. On the other hand the Eastern lineage comprise two well differentiated genetic groups with apparent distinct ecological preferences: the eastern sublineage IBE-NSC located in northeast-southeast-central north Iberia for which predicted suitable areas were restricted to the known distribution of the group; and the southeast sublineage (IBE-sou) confined to the Baetic mountains but with extensive areas of high presence probability located in Western Iberia. Accordingly, genetic groups located in the southern region (the southern lineage and the eastern sublineage IBE-sou) and the Western sublineages (IBW-CWS and IBW-CNSW) have extensive areas of potential coexistence. In contrast, sympatry maps for the eastern sublineage IBE-NSC and the remaining genetic groups are extremely restricted, particularly with

the other eastern sublineage IBE-sou. Notwithstanding, the low posterior probabilities obtained in general by the models (despite we had implemented a more restrictive threshold) probably overestimated the suitable areas for the lineages occurrence and thus the extent of sympatry among them.

2.2 Niche conservatism in *Vipera latastei-monticola*

The assessment of how the ecological niche of different evolutionary lineages or species evolved through time can provide valuable insights on their evolutionary history and help understand the role of ecological variability in species differentiation (Warren *et al.,* 2008). Although several studies suggest a general tendency of species to retain some aspects of their ancestral fundamental niche (e.g. Graham *et al.,* 2004; Wiens & Graham, 2005; Broennimann *et al.,* 2007), niche conservatism may also be associated to species differentiation, since it can limit geographic range expansion and also promote allopatric speciation (Wiens & Graham, 2005). Allopatric speciation is considered the most common mode of geographic speciation on which populations are geographically separated by extrinsic barriers that usually consist in unsuitable habitat for the species. Niche conservatism limits adaptation to these suboptimal conditions and allows the maintenance of independent units (Wiens & Graham, 2005).

Our tests of niche overlap revealed a general tendency to niche conservatism within *Vipera latastei-monticola*. Despite non equivalent, ecological niches were in most cases more similar than expected by chance. This implies that the differences observed between the niches are a result of habitat availability rather than a shift in habitat preferences (Warren *et al.,* 2008). These observations are largely consistent with previous studies that have shown that sister species tend to occupy similar niches but not equivalent (Broennimann *et al*., 2012). Nonetheless, for some pairwise comparisons the background similarity tests failed to reject the null hypothesis suggesting that the tests have insufficient power to predict niche differentiation or conservatism (Warren *et al.,* 2008).

In summary, ecological models predicted extensive areas of potential co-existence among *Vipera latastei-monticola* main lineages and sublineages, suggesting that these groups tend to occur in similar topoclimatic conditions. These results were further supported by the niche overlap tests that indicated a general tendency to niche conservatism in the species. In particular, the Eastern lineage presents an intriguing scenario: while the eastern sublineage IBE-sou found extensive areas of potential habitat suitability in the Western region of Iberia, IBE-NSC occurs in restricted habitat conditions. However, although ecological model predictions strongly suggest a clear difference in the niche of IBE-NSC in comparison with the remaining Iberian sublineages, similarity tests for this group were inconclusive and so we cannot exclude the possibility of niche conservatism.

3. Morphological variability within *Vipera latastei-monticola*

Previous studies on the geographical variability of morphological traits in *Vipera latastei-monticola* have shown a complex pattern of variation across the Strait of Gibraltar, with high variability of North African populations (Saint Girons, 1977; Brito *et al.,* 2006, 2008). The use of geostatistics over the morphological variability of the species allowed the identification of two distinct groups within Iberia (Western and Eastern Iberia) and three in North Africa (Rif/Middle Atlas, Algeria and High Atlas), which were highly concordant with the main mitochondrial lineages (Brito *et al*., 2008; Velo-Antón *et al.,* 2012). Concordant patterns of genetic and morphological differentiation may be a result of past historical events that favored the long persistence of populations in multiple refugia, leading to allopatric divergence with local adaptation to different environmental conditions involving phenotypic changes in morphological characters (see Santos *et al.,* 2014). However, clinal patterns of variability in some traits and correlation to environmental gradients might suggest local adaptation (e.g. number of ventral scales, number of subcaudal scales, dorsal-pattern variation; Shine 2000; Sanders *et al.,* 2004; Martínez-Freiría *et al.,* 2009). Species generally show geographic differences in morphological traits in response to changing selective pressures of the environments in order to enhance performance and fitness (Arnold, 1983; Kingsolver, 2003). Accordingly, a recent study on the geographic variability of the dorsal pattern shape within the Iberian populations of *V. latastei* identified two major groups, Western and Eastern, and highlighted the correlation between dorsal pattern variation and both genetic (historical phylogeography of the species) and environmental (adaptation to climate and lithogy) factors (Santos *et al.,* 2014). Our

morphological analyses recovered significant morphological patterns throughout the species entire range and provided support for some of the findings of previous studies.

Multivariate analyses on the morphological differentiation of Iberian and North African populations were largely consistent with patterns reported in previous studies (Saint Girons, 1977; Brito *et al.,* 2006, 2008). However, it was only recover for males, since females revealed lack of morphological differentiation among the populations of the two regions, probably due to the small sample size of the North African group. Populations from the Rif tended to cluster with the Iberian populations suggesting similar morphological variation patterns across the Strait of Gibraltar, which greatly contrasts with the patterns of genetic differentiation (Velo-Antón *et al.,* 2012). The most plausible explanation for this morphological pattern is that populations from both regions evolved under similar environmental conditions leading to morphological convergence of several traits (Brito *et al*., 2008). This is further supported by the ecological models that predicted suitable conditions for the North African populations occurrence in Iberia and vice-versa. In addition, our results indicated the clear differentiation of populations from the High Atlas (previously considered as part of *Vipera monticola*), mostly supported by differences in the number of dorsal rows. This morphological character has been used to separate populations from the High Atlas (N-DORS = 19) and Algeria (N-DORS = 23) from the remaining populations of the species (N-DORS = 21) (Saint Girons, 1977; Beerli *et al,* 1986). In fact, it has been suggested that the Algerian populations might represent an undescribed taxon (Saint Girons, 1977; Brito *et al.,* 2006, 2008). However, our study does not provide any evidences of that.

Within Iberian populations, two different scenarios of morphological differentiation were recovered in previous studies. Saint Girons (1977) and Brito *et al*. (2006) highlighted the low morphological differentiation found in this region in comparison with North Africa. In fact, the later study identified a homogeneous central area and peripheral isolated populations with morphological differentiation (Brito *et al*., 2006). Later, the use of geostatistics allowed the identification of complex biogeographic patterns of morphological variability within Iberia (Brito *el at.,* 2008). These authors identified a multivariate cline of morphological variation (including the number of dorsal marks and of ventral, loreal and infralabial scales) that divided the Iberian Peninsula along a north-east/south-west cline and lead to the differentiation of two morphological distinct groups, Western and Eastern Iberia, although these groups were considerable less supported than the remaining groups identified within North Africa. In concordance with this study, univariate analyses on the main Iberian lineages recovered a pattern of morphological differentiation among the Western and Eastern lineages while the

Southern lineage tended to be clustered with one of them (depending on the morphological trait). This pattern was obtained for all morphological characters that reported significant variation among Iberian lineages, with the exception of the number of apical scales that exhibited differences among the Southern lineage and the remaining lineages and the number of ventral scales which reported significant differences among all genetic groups. Thus, the taxonomic relevance of the number of ventral scales (Saint Girons, 1977, 1978; Beerli *et al.,* 1986; Brito *et al*., 2006) was further confirmed in our study. Nonetheless, despite the general concordance among different morphological characters, multivariate analyses were not able to recover any pattern of differentiation among Iberian lineages in both males and females.

Conversely, univariate analyses on the Iberian sublineages often recover distinct morphological patterns on different characters and clear differences between males and females. Multivariate analyses on males recover no morphological structure, supporting previous studies on the species that highlighted the low morphological differentiation of this region (Saint Girons, 1977; Brito *et al.,* 2006). On the other hand, multivariate analyses on the females reported significant differences between the western sublineage IBW-CWS and the eastern sublineage IBE-sou and the remaining groups, showing a clear pattern of morphological differentiation between southern and northern sublineages. The only exception was the Southern lineage that was consistently clustered with the northern sublineages (IBE-NSC and IBW-CNSW). However, this result may be an artefact of the low sample size of this group (less than five specimens analysed).

Overall, our results supported a complex scenario of morphological variability in *Vipera latastei-monticola* likely driven by a combination of genetic (i.e. historical events) and environmental factors (adaptation to environmental gradients). Nonetheless, consistent patterns of morphological and genetic differentiation were only obtained for the main Iberian lineages (Western and Eastern lineages), indicating that at lower levels of genetic structure local adaptation may play a major role in shaping morphological patterns. This is supported by two distinct evidences: 1) clear morphological differences between males and females at both univariate and multivariate analyses and 2) different patterns of morphological differentiation recovered by distinct traits. The role of local adaptation in shaping intraspecific morphological differentiation has been reported in multiple studies on reptile species (e.g. Brown & Thorpe, 1991; Thorpe & Baez, 1993; Malhotra & Thorpe, 1997). In particular, fitness related traits frequently present variation across different environmental and ecological conditions in order to meet the species specific needs and enhance performance (Arnold, 1983;

Kingsolver, 2003). This suggests that different characters tend to respond to different selective factors. For instance, in *Vipera latastei-monticola* dorsal pattern variation might improve their cryptic and thermoregulations capabilities across the study area (Santos *et al.,* 2014). Moreover, males and females are subjected to different ecological constrains, females for instance have specific requirements related with the reproduction effort, and so different patterns of morphological variation are expected (Shine, 1993, 2000).

4. Taxonomic implications

After more than 250 years of prevalence of comparative morphology in species discovery, the integration of molecular tools and modern morphological and ecological methods have been increasingly used to identify taxonomic units and study intraspecific variability, leading to important taxonomic revisions in several taxa (e.g. Ruiz-Sanchez & Sosa, 2010; Barata *et al*., 2012; Ahmadzadeh *et al*., 2013). However, the application of these methods often leads to taxonomic conflicts, particularly when delimiting recently evolved species since it is very unlikely that they have had sufficient time to reach evident and congruent separation of several taxonomic characters (Padial *et al*., 2010). Accordingly, in the last years extensive molecular and morphological studies in the European vipers (genus *Vipera*) have lead to conflicting results regarding the number of species within this group as well as the relationships among them (see Martínez-Freiría *et al*., 2014 and references herein). Surprisingly, no study has used an holistic approach that combines multiple lines of evidences to resolve such taxonomic uncertainties.

In the present study we combined genetic tools, morphological variation analyses and ecological niche modelling techniques to study intraspecific patterns within *Vipera latastei-monticola* and clarify its taxonomy. Overall, our results supported previous findings on the species and shed new light into several aspects of the intraspecific patterns of *V. latastei*. Within Iberia, consistent patterns of genetic and morphological differentiation were obtained between the Western and Eastern lineages. In addition, ecological analyses provided evidence for clear habitat differences between the wider Eastern sublineage IBE-NSC and the remaining mitochondrial groups, suggesting some degree of ecological differentiation between the Western and Eastern regions of the Iberian Peninsula. On the other hand, ecological analyses suggested that the Western and Southern lineages occur in similar habitat conditions. Furthermore, in agreement with a previous studies (Brito *et al*., 2008) morphological analyses failed to

detect variation in the Southern lineage, although this result may be related with low sample size.

Regarding the two main clades, Iberia and North Africa, morphological and ecological analyses did not provide evidence of clear morphological and ecological differentiation among the populations of the two regions. However, in light of the recently proposed "unified species concept" (de Queiroz, 2005, 2007) and the perspective of integrative taxonomy "by accumulation" (Padial *et al*., 2010), species are now considered separately evolving lineages of populations that may or may not acquire differentiating properties (e.g. morphological distinctiveness, occupation of different ecological niches) along the speciation continuum. Thus, these two lineages may fit well with this criterion for multiple reasons. First, they are highly divergent vicariant lineages that differentiated in the Miocene and evolved in allopatry since then. In fact, there are several cases of vicariant lineages separated by the Strait of Gibraltar that underwent similar evolutionary processes and display similar patterns of *Vipera latastei-monticola* that were described as independent species, most of them based on the mtDNA and their allopatric distribution (e.g. *Salamandra*: Vences *et al.,* 2014; *Alytes*: Martínez-Solano *et al*., 2004; *Pelobates*: García-París *et al*., 2003). Second, morphological distinctiveness and niche differentiation are no longer considered as primary species criteria (de Queiroz, 2005, 2007; Padial *et al*., 2010). In fact, despite commonly used in taxonomy, morphological characters are prone to convergence since they might reflect microevolutionary processes correlated with environmental and ecological conditions rather than historical population connectivity and differentiation. This has been extensively documented in reptiles (e.g. Brown & Thorpe, 1991; Thorpe & Baez, 1993; Malhotra & Thorpe, 1997) and more recently in mammals (e.g. Koepfli *et al*., 2015). Furthermore, recent studies highlighted that sister species tend to retain some aspects of their ancestor fundamental niche (e.g. Graham *et al*., 2004; Wiens & Graham, 2005; Broennimann *et al*., 2007).

Even though, the use of different molecular markers is fundamental to accurately delimit species/lineages (Godinho *et al*., 2008). Therefore, to a deep understanding of the systematics of *Vipera latastei-monticola,* further investigation may require the development of species-specific nuclear markers such as multiple independent nuclear loci to reconstruct accurately a species tree (e.g. Barlow *et al*., 2012, 2013) and infer contemporary genetic structure and patterns of gene flow among Iberian lineages (e.g. Ferchaud *et al*., 2011). In addition, future work on the species would benefit from a comprehensive sampling throughout the species entire range, particularly in North

Africa where sampling limitations prevented a deep investigation on its ecological and morphological variability.

VI. References

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VII. Appendices

Appendix 1: Summary table of samples used in the genetic analyses. Lineages and sublineages recover from the phylogenetic analyses based on the concatenated mitochondrial dataset are represented.

ID	Species	Locality, Region,Country	LAT	LONG	Lineage	Sublineage	Cytb	ND4	B-fibrinog	PRLP	MICROS
1	*V. latastei*	Argel,ALG	36.5	3.1	Africa-Algeria	Africa-Algeria	1	1			
2	*V. latastei*	Tizi-Ouzou,Kabylia,ALG	36.6	4.6	Africa-Algeria	Africa-Algeria	1	1	1	1	
3	*V. latastei*	P.N. Djurdjura,Tizi-Ouzou,Kabylia,ALG	36.5	4.3	Africa-Algeria	Africa-Algeria	1	1			
4	*V. latastei*	P.N. Djurdjura,Tizi-Ouzou,Kabylia,ALG	36.5	4.3			1				
5	*V. latastei*	P.N. Djurdjura,Tizi-Ouzou,Kabylia,ALG	36.5	4.3			1				
6	*V. monticola*	Azilal,Azilal,MOR	31.8	-6.3	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1			
7	*V. monticola*	Toubkal,Marrakech,Marrakech,MOR	31.1	-7.9	Central-High Atlas/Anti Atlas	Central-High Atlas/Anti Atlas	1	1	1	1	
8	*V. latastei*	Chefchaouen,Chaouen,MOR	35.2	-5.2			1				
9	*V. latastei*	Tetouan,MOR	35.4	-5.6	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1			
10	*V. latastei*	Tierta,Ketama,Al Hucemas,MOR	34.8	-4.6	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1			
11	*V. latastei*	Khénifra,MOR	33.1	-5.0	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1			
12	*V. monticola*	Toubkal,Marrakech,Marrakech,MOR	31.1	-7.9				1			
13	*V. monticola*	Oukaimeden,Marrakech,Marrakech,MOR	31.2	-7.8	Central-High Atlas/Anti Atlas	Central-High Atlas/Anti Atlas	1	1			
14	*V. latastei*	Bragança,Trás-os-Montes,PT	41.9	-6.8	Iberia-West	CNSW	1				
15	*V. latastei*	Portalegre,Alentejo,PT	39.3	-7.3	Iberia-West	CWS	1	1			
16	*V. latastei*	Serra da Malcata,Castelo Branco,Beira Baixa,PT	40.2	-7.0	Iberia-West	CNSW	1	1			
17	*V. latastei*	Serra da Malcata,Castelo Branco,Beira Baixa,PT	40.2	-7.1			1				
18	*V. latastei*	Mata de Albergaria,Braga,Minho,PT	41.8	-8.1			1				
19	*V. latastei*	Janes,Lisboa,Estremadura,PT	38.8	-9.4	Iberia-West	CWS	1	1			
20	*V. latastei*	Serra da Estrela,Viseu,Beira Alta,PT	40.4	-7.7			1				
21	*V. latastei*	Lindoso,Viana do Castelo,Minho,PT	41.8	-8.2	Iberia-West	CNSW	1	1			
22	*V. latastei*	Tarouca,Viseu,Beira Alta,PT	41.0	-7.7	Iberia-West	CNSW	1	1			
23	*V. latastei*	Paradela,Chaves,Trás-os-Montes,PT	41.8	-8.0	Iberia-West	CNSW	1	1			

24	*V. latastei*	Vairão,Porto,North,PT	41.3	-8.7	Iberia-West	CNSW	1	1				
25	*V. latastei*	Serra da Estrela,Viseu,Beira Alta,PT	40.4	-7.6	Iberia-West	CWS	1	1				
26	*V. latastei*	Vairão,Porto,North,PT	41.3	-8.7			1					
27	*V. latastei*	Ermelo,Vila Real,Trás-os-Montes,PT	41.4	-7.9	Iberia-West	CNSW	1	1				
28	*V. latastei*	Mata de Albergaria,Braga,Minho,PT	41.8	-8.1			1	1	1	1		
29	*V. latastei*	Samoucal,Faro,Algarve,PT	37.3	-8.8			1	1				
30	*V. latastei*	Samoucal,Faro,Algarve,PT	37.3	-8.8	Iberia-West	CNSW	1	1				
31	*V. latastei*	Portagem,Portalegre,Alentejo,PT	39.4	-7.4	Iberia-West	CWS	1	1				
32	*V. latastei*	Currais,Aveiro,Beira Litoral,PT	40.9	-8.2	Iberia-West	CNSW	1					
33	*V. latastei*	Melides,Setúbal,Alentejo,PT	38.2	-8.8	Iberia-West	CNSW	1	1			1	
34	*V. latastei*	Guarda,Beira Alta,PT	40.8	-7.4	Iberia-West	CWS	1	1				
35	*V. latastei*	Guarda,Beira Alta,PT	40.4	-7.5	Iberia-West	CWS	1	1				
36	*V. latastei*	Monte do Meio,Portalegre,Alentejo,PT	39.3	-7.3	Iberia-West	CWS	1	1				
37	*V. latastei*	Serra da Malcata,Guarda,Beira Alta,PT	40.3	-7.0	Iberia-West	CWS	1	1				
38	*V. latastei*	Serra de Monchique,Portimão,Algarve,PT	37.3	-8.6	Iberia-West	CNSW	1	1			1	
39	*V. latastei*	Belas,Lisboa,Estremadura,PT	38.8	-9.3	Iberia-West	CWS	1	1				
40	*V. latastei*	Lagos,Algarve,PT	37.1	-8.4	Iberia-West	CWS	1	1			1	
41	*V. latastei*	S. Estrela,Guarda,Beira Alta,PT	40.4	-7.5	Iberia-West	CWS	1	1				
42	*V. latastei*	Portalegre,Alentejo,PT	39.3	-7.3	Iberia-West	CWS	1	1				
43	*V. latastei*	Portalegre,Alentejo,PT	39.4	-7.3	Iberia-West	CWS	1	1				
44	*V. latastei*	Vila Chã,Porto,North,PT	41.3	-8.7			1					
45	*V. latastei*	Vila Chã,Porto,North,PT	41.3	-8.7			1					
46	*V. latastei*	S. Soajo,Viana do Castelo,Minho,PT	41.9	-8.3	Iberia-West	CNSW	1					
47	*V. latastei*	Porto,North,PT	41.3	-8.6			1					
48	*V. latastei*	Portimão,Algarve,PT	37.3	-8.7	Iberia-West	CNSW	1	1	1	1	1	
49	*V. latastei*	Aveiro,Beira Litoral,PT	40.9	-8.6				1				

50	*V. latastei*	S. Nogueira,Bragança,Trás-os-Montes,PT	41.7	-6.9	Iberia-West	CNSW	1	1		
51	*V. latastei*	S. Nogueira,Bragança,Trás-os-Montes,PT	41.7	-6.8	Iberia-West	CNSW	1	1		
52	*V. latastei*	Alferce,Portimão,Algarve,PT	37.3	-8.6	Iberia-West	CNSW	1	1		1
53	*V. latastei*	Valverde,Évora,Alentejo,PT	38.5	-8.0	Iberia-West	CNSW	1	1		
54	*V. latastei*	Barcelona,Catalunya,ES	41.4	1.5			1			
55	*V. latastei*	Tarragona,Catalunya,ES	41.5	1.3			1			
56	*V. latastei*	Tarragona,Catalunya,ES	41.6	1.4	Iberia-East	NSC	1	1		
57	*V. latastei*	Tarragona,Catalunya,ES	41.4	1.4	Iberia-East	NSC	1	1		
58	*V. latastei*	Huesca,Aragón,ES	42.1	0.5			1			
59	*V. latastei*	Jaén,Andalucía,ES	37.9	-2.8			1			
60	*V. latastei*	Córdoba,Andalucía,ES	37.4	-4.3	Iberia-East	SOU	1	1		
61	*V. latastei*	Castellón,Comunidad Valenciá,ES	40.7	0.0	Iberia-East	NSC	1	1		
62	*V. latastei*	Castellón,Comunidad Valenciá,ES	40.7	0.0			1			
63	*V. latastei*	Castellón,Comunidad Valenciá,ES	40.4	-0.3	Iberia-East	NSC	1			
64	*V. latastei*	Castellón,Comunidad Valenciá,ES	40.5	-0.2	Iberia-East	NSC	1	1		
65	*V. latastei*	Córdoba,Andalucía,ES	37.4	-4.3			1	1		1
66	*V. latastei*	Jaén,Andalucía,ES	38.0	-4.1	Iberia-East	SOU	1	1		
67	*V. latastei*	Jaén,Andalucía,ES	38.2	-4.0	Iberia-East	SOU	1	1		
68	*V. latastei*	Huelva,Andalucía,ES	37.2	-6.8			1			1
69	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.4	Iberia-South	SOUTH	1	1		
70	*V. latastei*	Jaén,Andalucía,ES	38.0	-4.1	Iberia-East	SOU	1	1		1
71	*V. latastei*	Córdoba,Andalucía,ES	37.9	-5.2	Iberia-West	CWS	1	1		
72	*V. latastei*	Málaga,Andalucía,ES	36.9	-4.4	Iberia-South	SOUTH	1	1		1
73	*V. latastei*	Ávila,Castilla y León,ES	40.3	-5.2	Iberia-East	NSC	1	1		
74	*V. latastei*	Málaga,Andalucía,ES	36.9	-4.4	Iberia-South	SOUTH	1	1		
75	*V. latastei*	Castellón,Comunidad Valenciá,ES	40.7	0.2			1			
76	*V. latastei*	Albacete,Castilla La Mancha,ES	38.5	-2.4	Iberia-East	NSC	1	1		

77	*V. latastei*	Cádiz,Andalucía,ES	36.7	-5.4	Iberia-South	SOUTH	1	1				1
78	*V. latastei*	Granada,Andalucía,ES	37.1	-4.2	Iberia-East	SOU	1	1	1	1	1
79	*V. latastei*	Huelva,Andalucía,ES	37.5	-6.9	Iberia-East	CWS	1	1				1
80	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.4	Iberia-South	SOUTH	1	1				1
81	*V. latastei*	Huelva,Andalucía,ES	37.2	-6.7			1					
82	*V. latastei*	Ciudad Real,Castilla La Mancha,ES	38.4	-3.5	Iberia-East	SOU	1	1	1	1	1
83	*V. latastei*	Ávila,Castilla y León,ES	40.3	-5.2	Iberia-West	CNSW	1	1				
84	*V. latastei*	Burgos,Castilla y León,ES	42.4	-4.1	Iberia-East	NSC	1	1				
85	*V. latastei*	Huelva,Andalucía,ES	37.3	-6.8				1				
86	*V. latastei*	Cádiz,Andalucía,ES	36.5	-5.6	Iberia-South	SOUTH	1	1				
87	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.3	Iberia-South	SOUTH	1	1				1
88	*V. latastei*	Málaga,Andalucía,ES	36.7	-5.1	Iberia-South	SOUTH	1	1				
89	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.4	Iberia-South	SOUTH	1	1				1
90	*V. latastei*	Málaga,Andalucía,ES	36.7	-5.2			1					1
91	*V. latastei*	Soria,Castilla y León,ES	41.7	-2.5	Iberia-East	NSC	1	1				
92	*V. latastei*	Soria,Castilla y León,ES	41.5	-2.6	Iberia-East	NSC	1	1				
93	*V. latastei*	Soria,Castilla y León,ES	41.3	-2.7	Iberia-East	NSC	1	1				
94	*V. latastei*	Segovia,Castilla y León,ES	40.9	-4.0	Iberia-East	NSC	1	1				
95	*V. latastei*	Sierra Filabres,Almeria,Andalucía,ES	37.9	-2.6	Iberia-East	NSC	1	1				1
96	*V. latastei*	Córdoba,Andalucía,ES	38.1	-4.6	Iberia-West	CWS	1	1				1
97	*V. latastei*	Viveiro Florestal (PNBLSX),Ourense,Galicia,ES	41.8	-8.1	Iberia-West	CNSW	1	1				
98	*V. latastei*	Viveiro Florestal (PNBLSX),Ourense,Galicia,ES	41.8	-8.1	Iberia-West	CNSW	1	1				
99	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.5	Iberia-East	CWS	1	1				
100	*V. latastei*	S. Nevada,Granada,Andalucía,ES	37.0	-3.4	Iberia-East	SOU	1	1				1
101	*V. latastei*	Zamora,Castilla y León,ES	42.1	-6.3	Iberia-West	CNSW	1	1				
102	*V. latastei*	Cáceres,Extremadura,ES	40.3	-6.9			1	1				

103	*V. latastei*	Granada,Andalucía,ES	36.8	-3.7	Iberia-East	SOU	1	1				1
104	*V. latastei*	Córdoba,Andalucía,ES	38.1	-5.0	Iberia-East	CWS	1	1	1	1		1
105	*V. latastei*	Granada,Andalucía,ES	37.0	-3.2	Iberia-East	SOU	1	1				1
106	*V. latastei*	Jaén,Andalucía,ES	38.2	-3.9	Iberia-East	SOU	1	1				1
107	*V. latastei*	Jaén,Andalucía,ES	38.1	-4.0	Iberia-East	SOU	1	1				1
108	*V. latastei*	Ciudad Real,Castilla La Mancha,ES	38.5	-3.9	Iberia-East	SOU	1	1				1
109	*V. latastei*	Jaén,Andalucía,ES	38.1	-3.9	Iberia-East	SOU	1	1				1
110	*V. latastei*	Granada,Andalucía,ES	38.0	-2.5	Iberia-East	NSC	1	1				1
111	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.4	Iberia-South	SOUTH	1	1				1
112	*V. latastei*	Andujar,Jaén,Andalucía,ES	38.1	-5.6	Iberia-East	CWS	1	1				1
113	*V. latastei*	S. Morena,Cordoba,Andalucía,ES	38.1	-4.6	Iberia-East	CWS	1	1				1
114	*V. latastei*	Murcia,Murcia,ES	37.9	-1.6	Iberia-East	NSC	1	1				1
115	*V. latastei*	Bagues,Barcelona,Catalunya,ES	41.3	1.9	Iberia-East	NSC	1	1				
116	*V. latastei*	Huesca,Aragón,ES	42.1	0.3	Iberia-East	NSC	1	1				
117	*V. latastei*	P.N. Saint Llorenç,Barcelona,Catalunya,ES	41.7	2.0	Iberia-East	NSC	1	1				
118	*V. latastei*	Lleida,Catalunya,ES	42.1	1.1	Iberia-East	NSC	1	1				
119	*V. latastei*	Cádiz,Andalucía,ES	36.8	-5.3	Iberia-South	SOUTH	1	1				1
120	*V. latastei*	Murcia,Andalucía,ES	37.9	-1.6	Iberia-East	NSC	1	1				1
121	*V. latastei*	Zaragoza,Aragón,ES	42.3	-1.0	Iberia-East	NSC	1	1				
122	*V. latastei*	Huesca,Aragón,ES	42.5	-0.6	Iberia-East	NSC	1	1				
123	*V. latastei*	Ávila,Castilla y León,ES	40.4	-5.3	Iberia-West	CWS	1	1				
124	*V. latastei*	Madrid,Madrid,ES	40.7	-4.1	Iberia-East	NSC	1	1				
125	*V. latastei*	Cáceres,Extremadura,ES	39.6	-5.3	Iberia-East	NSC	1	1				
126	*V. latastei*	Bab-Taza,MOR	35.0	-5.2	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1	1	1		
127	*V. latastei*	Alicante,Comunidad Valenciá,ES	38.6	-0.3	Iberia-East	NSC	1	1				
128	*V. latastei*	Guadalajara,Castilla La Mancha,ES	40.7	-2.1	Iberia-East	NSC	1	1				

129	*V. latastei*	Viseu,Beira Alta,PT	40.9	-8.0	Iberia-West	CNSW	1	1	1	1	
130	*V. latastei*	Cuenca,Castilla La Mancha,ES	40.4	-2.0	Iberia-East	NSC	1	1	1	1	
131	*V. latastei*	Cáceres,Extremadura,ES	40.3	-5.8	Iberia-West	CWS	1	1	1	1	
132	*V. monticola*	Imilchil,MOR	32.2	-5.6	Rif/Middle-Eastern High Atlas	Rif/Middle-Eastern High Atlas	1	1	1		
133	*V. monticola*	High Atlas, central,MOR	31.3	-7.4	Central-High Atlas/Anti Atlas	Central-High Atlas/Anti Atlas	1	1	1	1	
134	*V. latastei*	Viana do Castelo,Minho,PT	42.0	-8.3			1	1			
135	*V. latastei*	Burgos,Castilla y León,ES	42.7	-3.8	Iberia-East	NSC	1	1			
136	*V. latastei*	Burgos,Castilla y León,ES	42.7	-3.8	Iberia-East	NSC	1	1			
137	*V. latastei*	Cádiz,Andalucía,ES	36.4	-6.2	Iberia-South	SOUTH	1	1	1	1	1
138	*V. latastei*	P.N. Alcornocales,Cádiz,Andalucía,ES	36.6	-5.6	Iberia-South	SOUTH	1	1	1		1
139	*V. latastei*	P.N. Alcornocales,Cádiz,Andalucía,ES	36.3	-5.5	Iberia-South	SOUTH	1	1	1	1	1
140	*V. latastei*	Zarza de Montánchez,Cáceres,Extremadura,ES	39.2	-6.1	Iberia-West	CWS	1	1	1	1	
141	*V. latastei*	La Guardia - Villatobas,Toledo,Castilla La Mancha,ES	39.8	-3.4	Iberia-East	NSC	1	1		1	
142	*V. latastei*	Almeria,Andalucía,ES	37.1	-2.8	Iberia-East	NSC	1	1			1
143	*V. latastei*	Ciudad Real,Castilla La Mancha,ES	38.4	-4.1	Iberia-East	SOU	1				1
144	*V. latastei*	Almenar,Soria,Castilla y León,ES	41.7	-2.2	Iberia-East	NSC	1	1			
145	*V. latastei*	Almenar,Soria,Castilla y León,ES	41.7	-2.2	Iberia-East	NSC	1	1			
146	*V. latastei*	Moncayo (Cueva de Agreda),Soria,Castilla y León,ES	41.7	-1.8	Iberia-East	NSC	1	1			
147	*V. latastei*	sierra de la Espuña,Murcia,Murcia,ES	37.9	-1.5	Iberia-East	NSC	1	1			1
148	*V. latastei*	Laguarres,Huesca,Aragón,ES	42.2	0.5	Iberia-East	NSC	1	1	1	1	
149	*V. latastei*	Málaga,Andalucía,ES	36.6	-5.3	Iberia-South	SOUTH	1	1			
150	*V. latastei*	Rupelo,Burgos,Castilla y León,ES	42.1	-3.4	Iberia-East	NSC	1	1	1	1	
151	*V. latastei*	Muro de Aguas,La Rioja,La Rioja,ES	42.1	-2.1			1				
152	*V. latastei*	Navarra,Navarra,ES	42.1	-1.5	Iberia-East		1	1	1	1	
153	*V. latastei*	Navarra,Navarra,ES	42.1	-1.4	Iberia-East		1	1			
154	*V. monticola*	plateau du Tichka,MOR	30.9	-8.6	Western High Atlas	Western High Atlas	1	1			

155	*V. monticola*	plateau du Tichka,MOR	30.9	-8.6	Western High Atlas	Western High Atlas	1		1	1	
156	*V. latastei*	Cádiz,Cádiz,Andalucía,ES	36.3	-5.5	Iberia-South	SOUTH	1	1			1
157	*V. latastei*	Valencia,Comunidad Valenciá,ES	39.3	-0.9	Iberia-East	NSC	1	1			
158	*V. latastei*	Cañón Río Lobos,Soria,Castilla y León,ES	41.7	-3.0	Iberia-East	NSC	1	1			
159	*V. latastei*	Barcelona,Catalunya,ES	41.7	2.1	Iberia-East	NSC	1	1			
160	*V. latastei*	Barcelona,Catalunya,ES	41.7	2.1	Iberia-East	NSC	1	1			
161	*V. latastei*	Lleida,Catalunya,ES	42.0	1.2	Iberia-East	NSC	1	1			
162	*V. latastei*	Barcelona,Catalunya,ES	41.7	2.1	Iberia-East	NSC	1	1			
163	*V. latastei*	Tarragona,Catalunya,ES	41.3	1.0	Iberia-East	NSC	1	1			
164	*V. latastei*	Passanant,Tarragona,Catalunya,ES	41.5	1.2	Iberia-East	NSC	1	1			
165	*V. latastei*	Leiva,La Rioja,La Rioja,ES	42.5	-3.1	Iberia-East	NSC	1	1			
166	*V. latastei*	Soria,Castilla y León,ES	42.0	-2.3			1	1			
167	*V. latastei*	Huelva,Andalucía,ES	36.9	-6.3	Iberia-West	CWS	1	1			
168	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.5	Iberia-West	CWS	1	1			1
169	*V. latastei*	Huelva,Andalucía,ES	37.1	-6.5			1				
170	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6			1				
171	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1			1
172	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6			1				
173	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1			1
174	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1			1
175	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1			1
176	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1	1	1	1
177	*V. latastei*	Huelva,Andalucía,ES	37.0	-6.6	Iberia-West	CWS	1	1			1
178	*V. latastei*	Valencia,Comunidad Valenciá,ES	39.0	-0.9			1				
179	*V. latastei*	Belorado,Burgos,Castilla y León,ES	42.4	-3.2			1				
180	*V. latastei*	Cádiz,Andalucía,ES	36.2	-5.4			1		1	1	1
181	*V. latastei*	Cádiz,Andalucía,ES	36.1	-5.5						1	1

182	*V. latastei*	Soria,Castilla y León,ES	41.4	-3.2	Iberia-East	NSC	1	
183	*V. latastei*	Majaelrayo,Guadalajara,Castilla La Mancha,ES	41.1	-3.3	Iberia-East	NSC	1	
184	*V. latastei*	Condemios de Arriba,Guadalajara,Castilla La Mancha,ES	41.2	-3.1	Iberia-East	NSC	1	
185	*V. latastei*	Puebla de la Sierra,Madrid,Madrid,ES	41.0	-3.4	Iberia-East	NSC	1	1
186	*V. latastei*	Huelva,Andalucía,ES	37.5	-6.8			1	1
187	*V. monticola*	Jebel Sirwa,Anti-Atlas,MOR	30.7	-7.6	Central-High Atlas/Anti Atlas	Central-High Atlas/Anti Atlas	1	1

Appendix 2: Buccal swab DNA extraction protocol using spin columns (modified from the QIAamp DNA Blood Mini Kit Handbook)

1. Turn on the heat block to 56 °C
2. Pipet 600µl of PBS into a 2 ml microcentrifuge tube
3. Add 20µl of Protease stock solution (stored at 4 °C after re-suspension) to the 2 ml microcentrifuge tube.
4. Place the buccal swab into the 2 ml microcentrifuge tube.
5. Cut off the swab so that it will fit tinto the microcentrifuge tube.
6. Add 600 µl of Buffer AL. Do not add protease directly to Buffer AL.
7. Mix immediately by vortexing for 15 seconds.
8. Incubate for 10 min at 56 °C.
9. Briefly centrifuge to remove drops from inside the lid.
10. Add 600 µl of ethanol (96-100%) and mix thoroughly by vortexing. It is very important that the ethanol and sample be completely mix.
11. Briefly centrifuge to remove drops from inside the lid.
12. Pipet 600 µl of the mixture from step 11 into the QIAamp spin column placed in a 2 ml collection tube.
13. Centrifuge at 8000 rpm for 1 min. Discard flow-through.
14. Repeat steps 12-13 until all of the swab solution has been put through the column
15. Add 500 µl of Buffer AW1 to the spin column.
16. Centrifuge at 8000 rpm for 1 min. Discard the flow-through and collection tube.
17. Place AE at 56 °C
18. Add 500 µl of Buffer AW2 to the spin column.
19. Centrifuge at 14000 rpm for 3 min. Discard flow-through and collection tube.
20. Place spin column into a new 2 ml collection tube.
21. Spin the column again at 14000 rpm for 1 min.
22. Place the QIAamp spin column in a clean labeled 1.5 ml microcentrifuge tube.
23. Pipet 150 µl of AE (heated) directly onto the QIAamp membrane.
24. Incubate at room temperature for 1 min
25. Centrifuge at 8000 rpm for 1 min to elute the DNA.
26. Repeat the elution by repeating steps 22-25
27. Store at 4°C.

Appendix 3: PCR conditions for the sequenced genes of mtDNA and nuDNA

Cytb

Primers CB1 and CB2 (Palumbi, 1996)

Amplification step	Temperature (°C)	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	53 (Touchdown: -0.5)	30 seconds	5
Extension	72	1 minute	
Denaturation	95	30 seconds	
Annealing	51	30 seconds	35
Extension	72	1 minute	
Final extension	60	10 minutes	1

ND4

Primers ND4 F2 and Leu (Areválo *et al.*, 1994)

Amplification step	Temperature (°C)	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	60 (Touchdown: -1)	30 seconds	11
Extension	72	1 minute	
Denaturation	95	30 seconds	
Annealing	50	30 seconds	29
Extension	72	1 minute	
Final extension	60	10 minutes	1

Primers ND4 F and R3 (Areválo *et al.*, 1994)

Amplification step	Temperature (°C)	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	56	30 seconds	40
Extension	72	1 minute	
Final extension	60	10 minutes	1

PRLP

Primers PRLR_F1 and PRLR_R3 (Townsend *et al.*, 2008)

Amplification step	Temperature (°C)	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	56	30 seconds	40
Extension	72	45 seconds	
Final extension	60	10 minutes	1

β-fib (nested PCR)

1º Primers Bfx-7 and Bfx-8 (Prychitko & Moore, 1997)

Amplification step	Temperature	Duration	Number of cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	
Annealing	50	45 seconds	40
Extension	72	1 minute	
Final extension	72	7 minutes	1

2º Primers Bfx-F and Bfx-R (Prychitko & Moore, 1997)

Amplification step	Temperature	Duration	Number of cycles
Initial denaturation	95	10 minutes	1
Denaturation	95	30 seconds	
Annealing	55	45 seconds	40
Extension	72	1 minute	
Final extension	72	7 minutes	1

Appendix 4: Multiplexes information and PCR conditions

Multiplex	Name	Primers (5'-3')	Repeat motif	Repeat pattern	Number of alleles	Allele size range (bp)	Vol (100 µl mix)	Tail
M1	VB21	CCAGTGGCACATAAGTAG GTTCCATCATCAAAACAT	TG	Perfect	15	168-204	3	NED
	VB37	CTAAAGATGTCTTAGGGTCACT ATCCAGCCAGAACTGAT	(CT)TT(CT)	Perfect	35	309-415	5	FAM
	VBB18	TGCTGGTGGGAATCACAATG CACAAAAGCTGCCTGCAAAG	GA	Imperfect	15	96-158	1.4	FAM
	VB71	TTGGCAAGAATCGAGGAGCTG TGTGCCGACTTTTTGTGCTGA	(AC)TC(AC)	Perfect	10	139-159	2	VIC
	VB3	CAAGAAATGGAGATGAGC GAAACCTATGAGCCAGTA	AC	Perfect	16	167-205	2	VIC
M2	VBA8	ATTTCACCATGCCTCCAGAA GGTACACTCATTGTGATGAAC	CA	Perfect	25	197-255	4.1	PET
	VBB10	CGTGAGGTGTGTAAAATGAAG- CTATTTGAATCCCACCAGTG	GA	Imperfect	37	191-293	2.5	NED
	VBD17	TTTCTGCCCCATTTTACGAC TGTAAGATGTTCCGAGTAGC	AAG	Imperfect	22	184-241	2	FAM
	VB64	AGGCTCTGCTAAATGACC GATCCCCTGAATTGATTA	(TG)TT(TG)	Imperfect	21	249-295	3	VIC
	VB11	GCAGCAGTCAGGACCGTTA CCCCTTTCCTCTCCTTCTT	TC	Imperfect	24	133-185	1.5	VIC

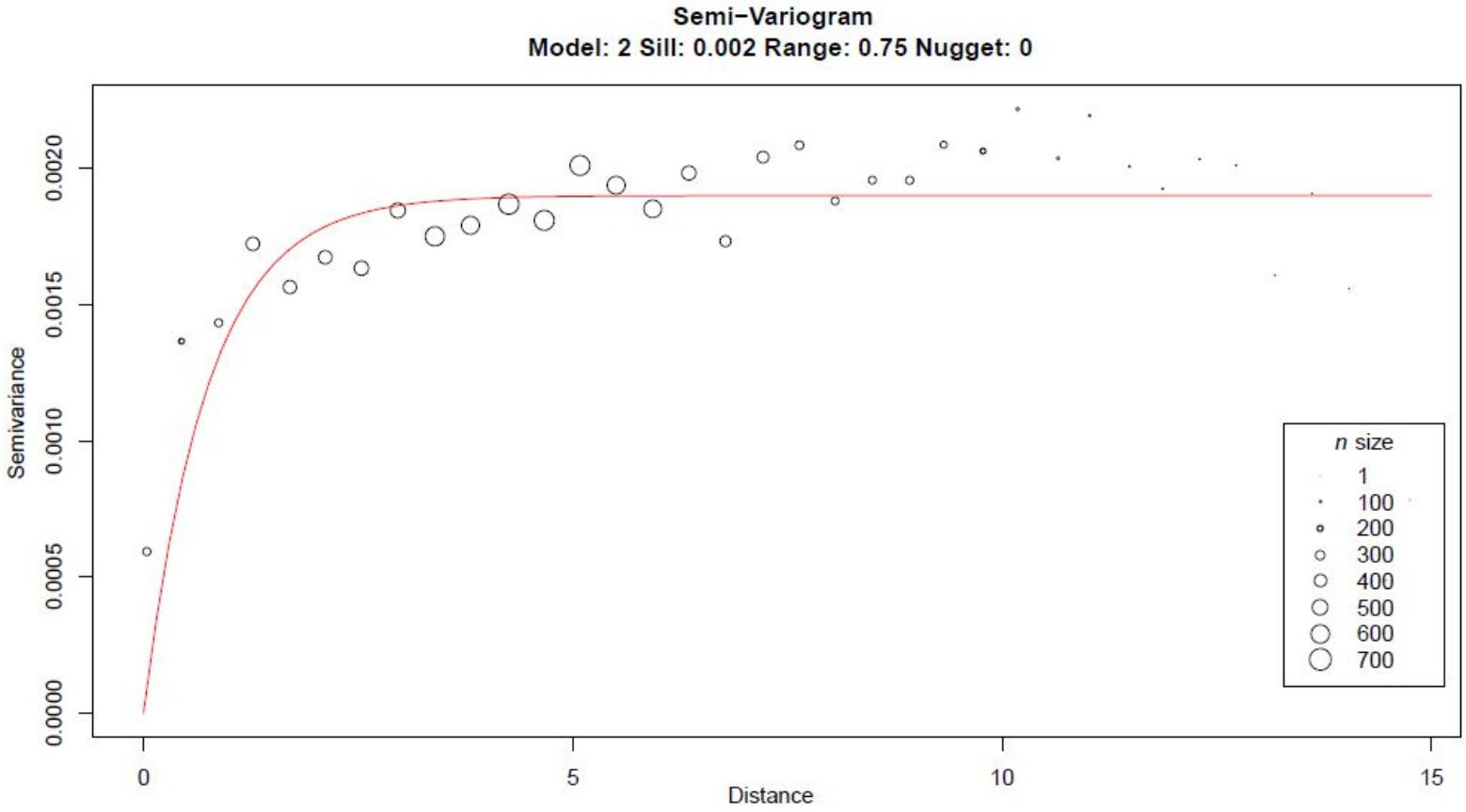
Multiplex 1:

Amplification step	Temperature	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	54	30 seconds	32
Extension	72	45 seconds	
Denaturation	95	30 seconds	
Annealing	53	30 seconds	8
Extension	72	45 seconds	
Final extension	60	30 minutes	1

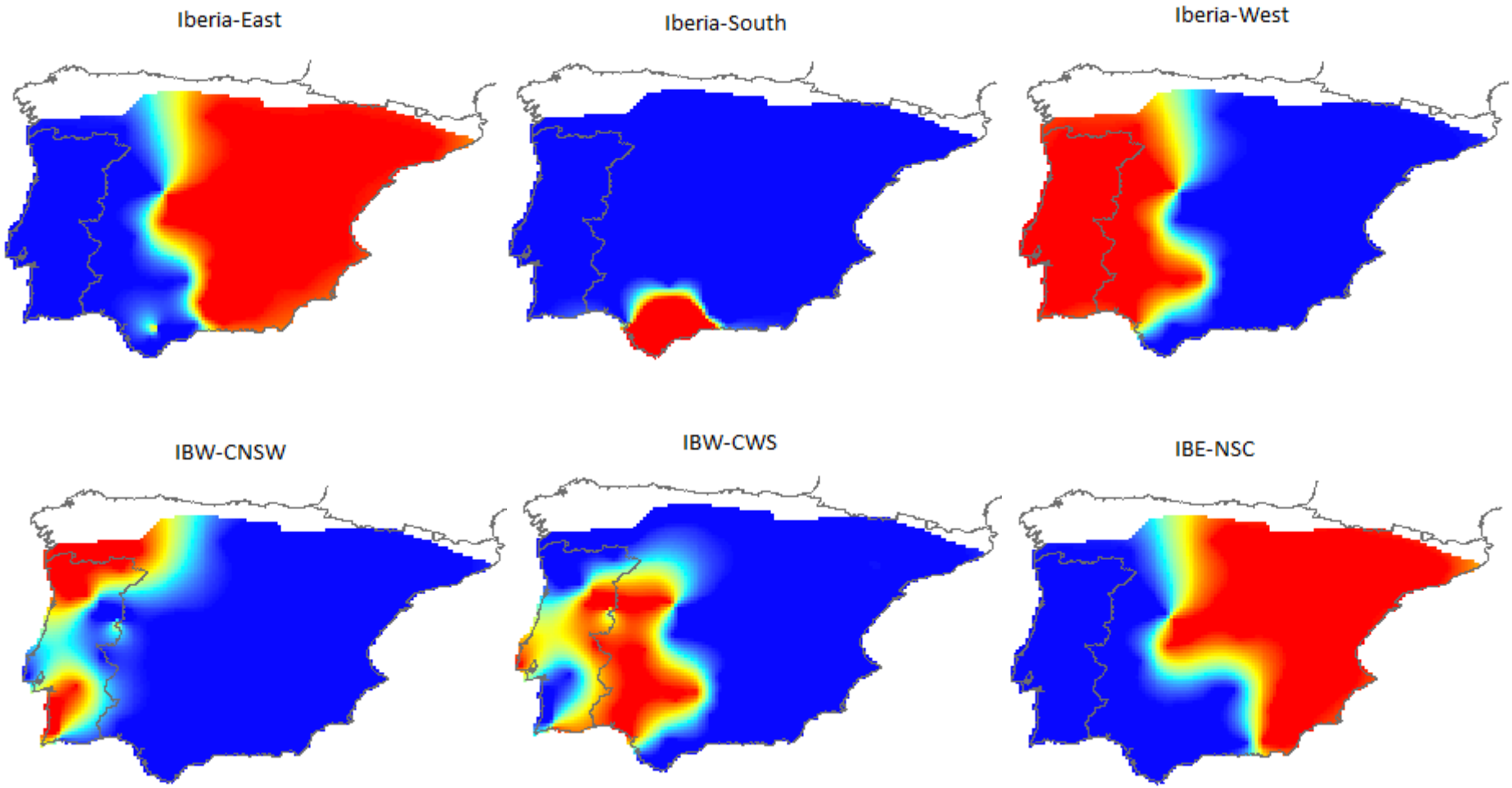
Multiplex 2:

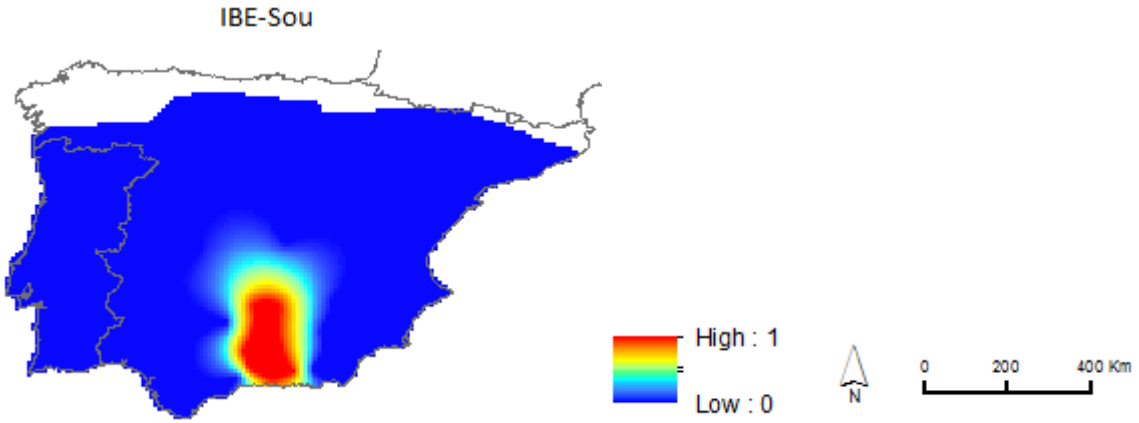
Amplification step	Temperature	Duration	Number of cycles
Initial denaturation	95	15 minutes	1
Denaturation	95	30 seconds	
Annealing	56	30 seconds	32
Extension	72	1 minute	
Denaturation	95	30 seconds	
Annealing	53	30 seconds	8
Extension	72	1 minute	
Final extension	60	30 minutes	1

Appendix 5: Semi-variogram with fitted model showing the spatial dependence of the ecological data. The number of pairwise samples within each distance class is represented with different circle size.

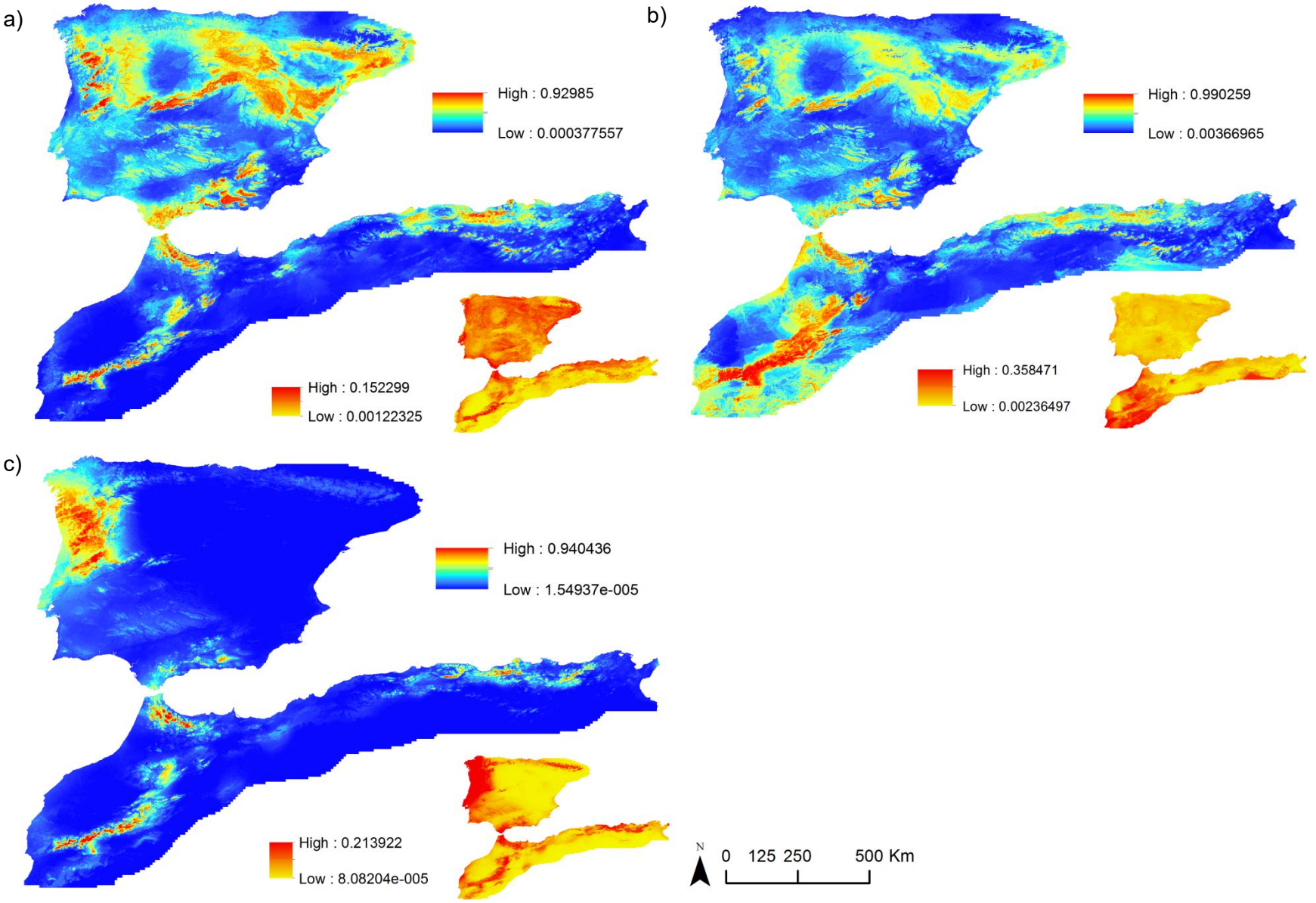


Appendix 6: Spatial interpolations of Iberian lineages and sublineages occurrence.

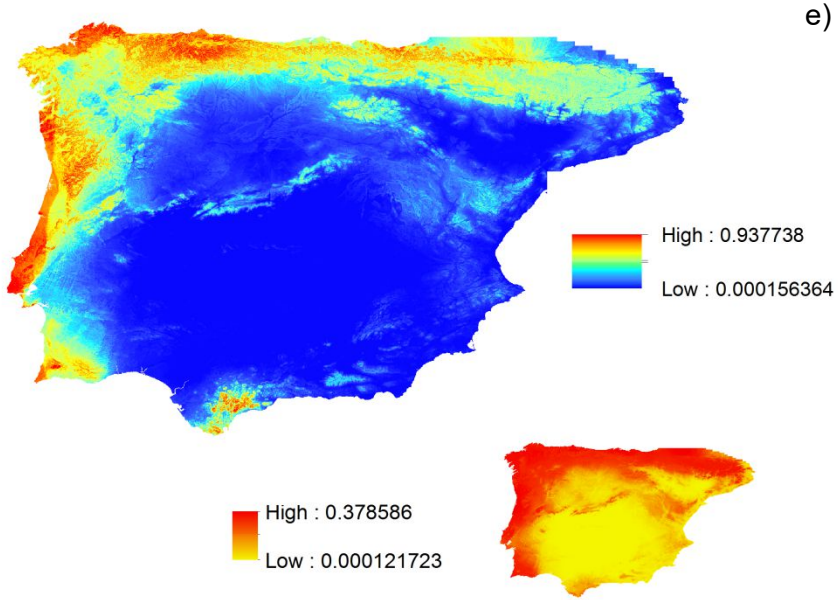



IBE-Sou
High : 1
Low : 0
N
0
200
400 Km

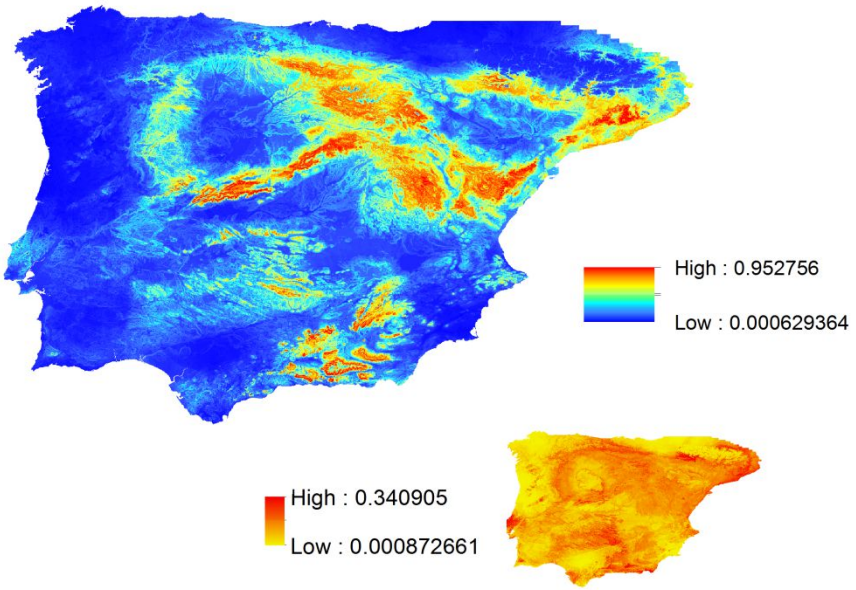
Appendix 7: Average probability of occurrence and standard deviations (small insets) for *Vipera latastei-monticola* (a), for the Iberian (b) and North African (c) lineages projected for the species entire range, for the Southern (d), Eastern (e) and Western (f) lineages projected to Iberia, for the Western sublineages, CNSW (g) and CWS (h), and Eastern sublineages, NSC (i) and Sou (j), projected to Iberia.



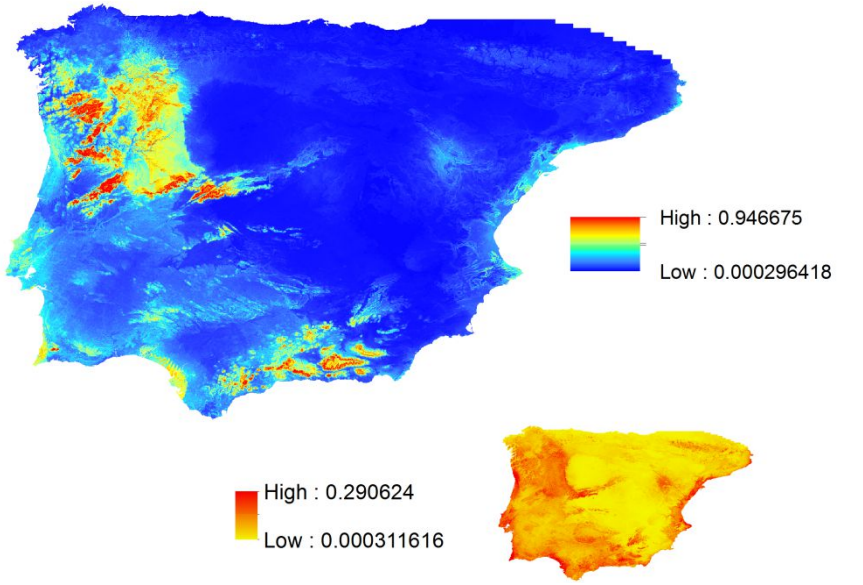
d)

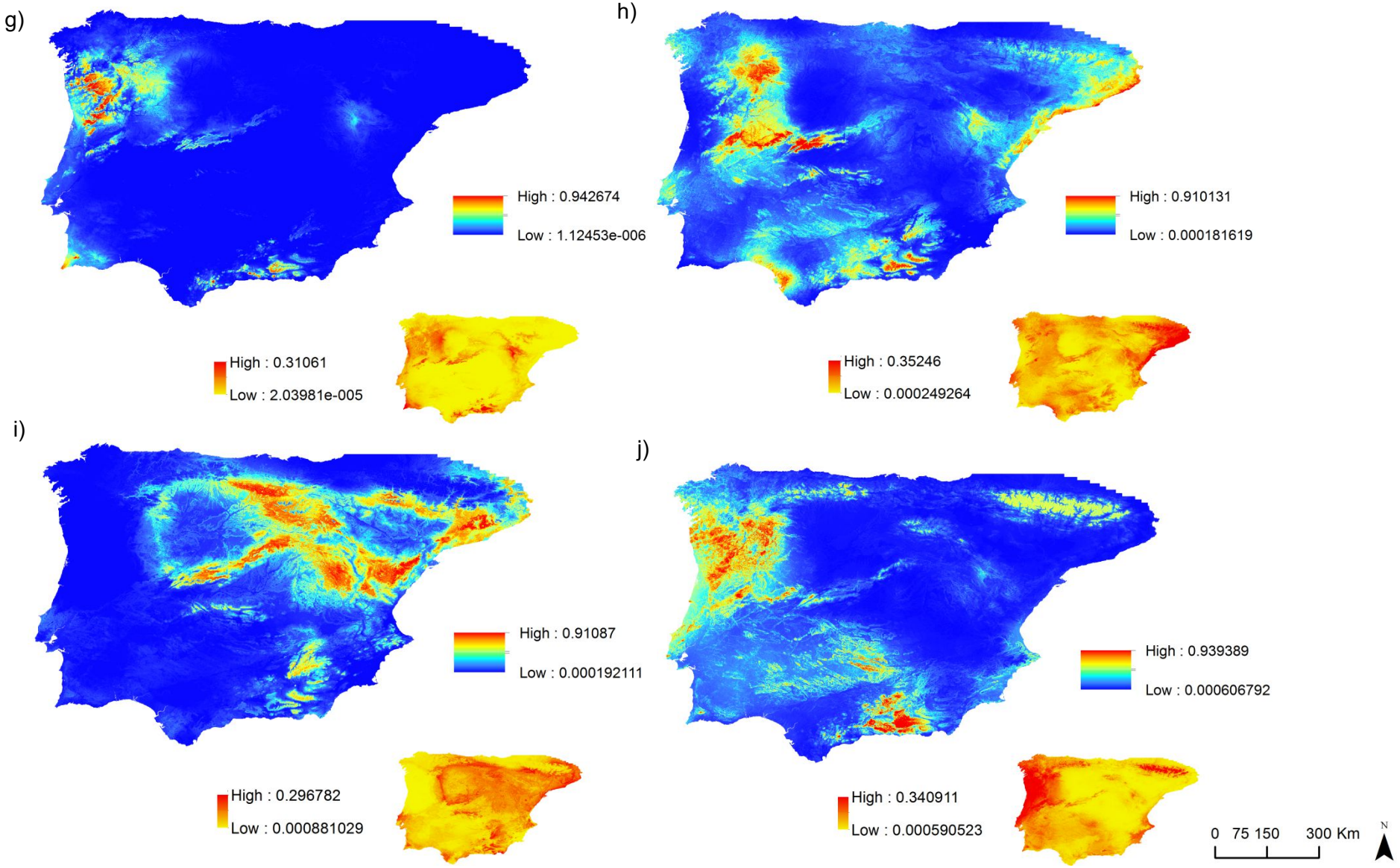

High : 0.937738
Low : 0.000156364
High : 0.378586
Low : 0.000121723

e)

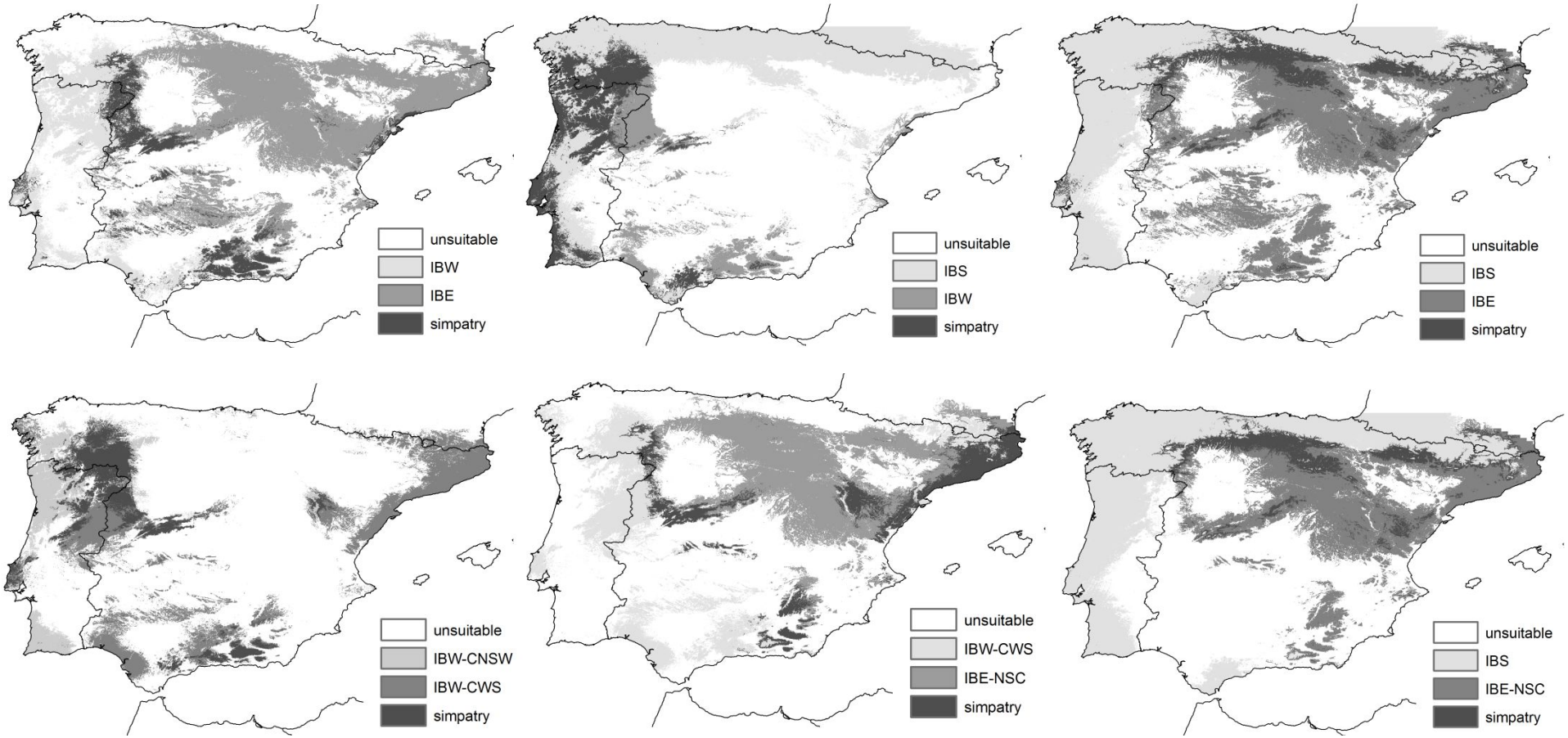

High : 0.952756
Low : 0.000629364
High : 0.340905
Low : 0.000872661

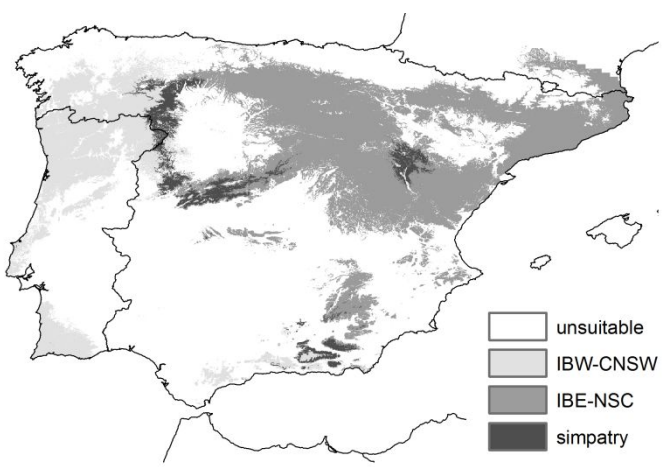
f)

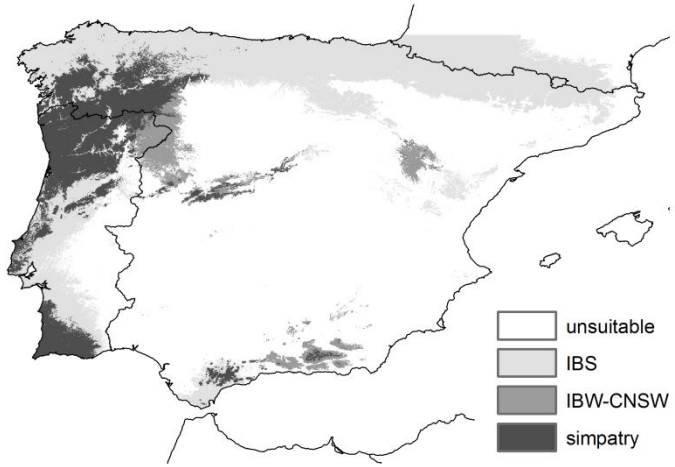

High : 0.946675
Low : 0.000296418
High : 0.290624
Low : 0.000311616

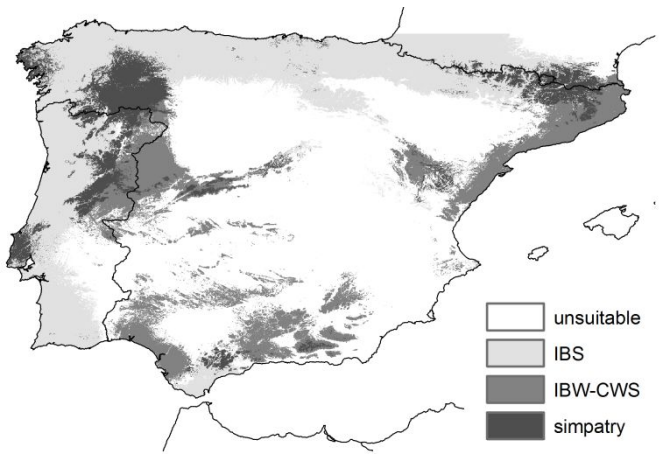

g)
High : 0.942674
Low : 1.12453e-006
High : 0.31061
Low : 2.03981e-005
h)
High : 0.910131
Low : 0.000181619
High : 0.35246
Low : 0.000249264
i)
High : 0.91087
Low : 0.000192111
High : 0.296782
Low : 0.000881029
j)
High : 0.939389
Low : 0.000606792
High : 0.340911
Low : 0.000590523
0 75 150 300 Km
N

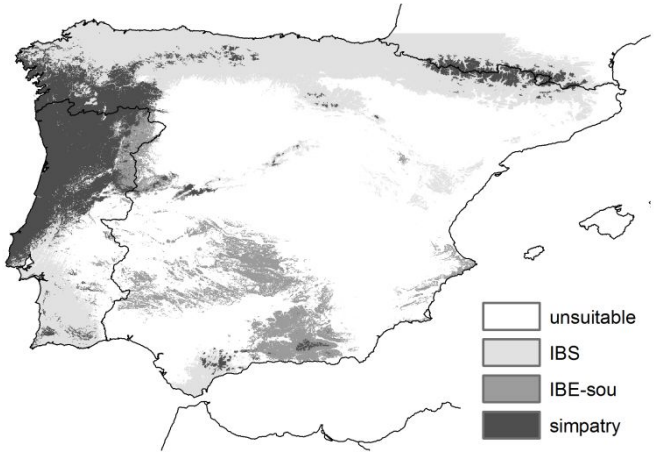
Appendix 8: Sympatry maps among Iberian phylogeographic groups given by the pairwise combination of the suitability maps defined using the 5 percentile minimum training presence threshold.

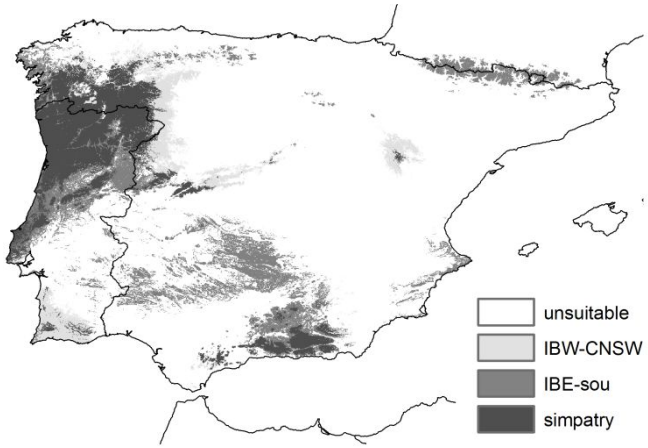


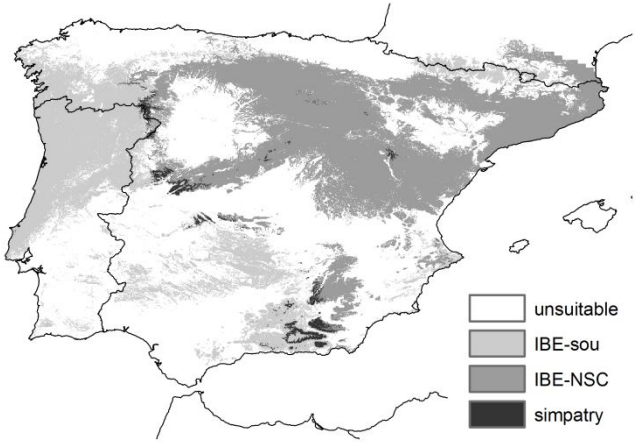

unsuitable
IBW-CNSW
IBE-NSC
simpatry

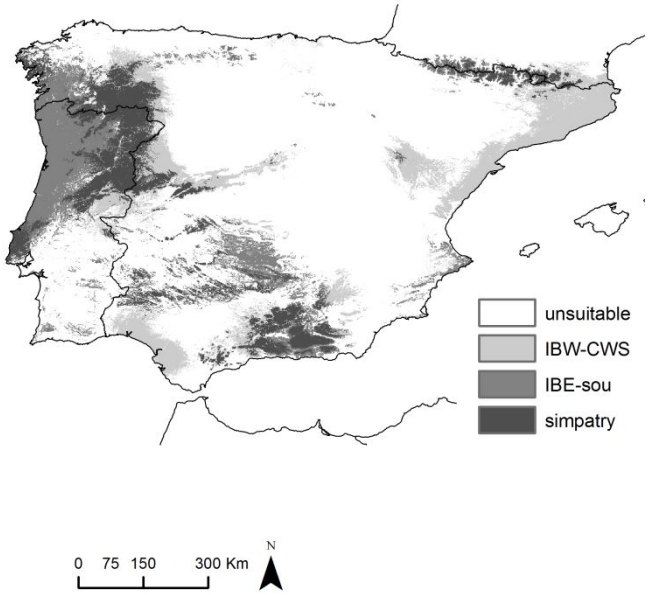

unsuitable
IBS
IBW-CNSW
simpatry


unsuitable
IBS
IBW-CWS
simpatry


unsuitable
IBS
IBE-sou
simpatry


unsuitable
IBW-CNSW
IBE-sou
simpatry


unsuitable
IBE-sou
IBE-NSC
simpatry


unsuitable
IBW-CWS
IBE-sou
simpatry
0 75 150 300 Km
N

Appendix 9: Statistical analyses performed for each morphological character to investigate differences between sexes and within the three levels of genetic structure.

Differences between males and females

Variable	Test	Statistic	DF1	DF2	p
DMARKS	Kruskall-Wallis test	0.329	1	-	0.566
LOR	Kruskall-Wallis test	0.186	1	-	0.667
SUPRA	Kruskall-Wallis test	2.178	1	-	0.140
VENT	Kruskall-Wallis test	0.939	1	-	0.133
N-DORS	Welch ANOVA	0.041	1	479.978	0.840
INFRA	Kruskall-Wallis test	5.802	1	-	0.016
INTER	Kruskall-Wallis test	7.340	1	-	0.007
PERI	Kruskall-Wallis test	7.483	1	-	0.006
S-APIC	Welch ANOVA	27.251	1	480.094	<0.001
SUBC	Welch ANOVA	698.753	1	601.313	<0.001
N-APIC	Welch ANOVA	25.525	1	585.463	<0.001

Differences between Iberia and North Africa

Variable		Test	Statistic	DF1	DF2	p
DMARKS		Kruskall-Wallis test	0.961	1	-	0.327
		Welch ANOVA	0.263	1	39.559	0.611
LOR		Kruskall-Wallis test	0.054	1	-	0.816
SUPRA		Welch ANOVA	81.171	1	49.381	<0.001
VENT		Welch ANOVA	31.136	1	43.794	<0.001
N-DORS						
INFRA	**Males**	Kruskall-Wallis test	0.122	1	-	0.122
	Females	Kruskall-Wallis test	0.227	1	-	0.634
INTER	**Males**	ANOVA	5.880	1	321	0.016
	Females	ANOVA	2.427	1	237	0.121
PERI	**Males**	Kruskall-Wallis test	2.483	1	-	0.115
	Females	Kruskall-Wallis test	0.100	1	-	0.752
S-APIC	**Males**	Welch ANOVA	0.154	1	11.388	0.702
	Females	Kruskall-Wallis test	2.878	1	-	0.090
SUBC	**Males**	Welch ANOVA	28.104	1	21.146	<0.001
	Females	Kruskall-Wallis test	9.820	1	-	0.002
N-APIC	**Males**	Welch ANOVA	0.240	1	11.364	0.633
	Females	Kruskall-Wallis test	3.722	1	-	0.054

Differences between Iberian lineages

Variable		Test	Statistic	DF1	DF2	p
DMARKS		Welch ANOVA	18.847	2	27.155	<0.001
LOR		Kruskall-Wallis test	53.231	2	-	<0.001
SUPRA		Kruskall-Wallis test	3.029	2	-	0.220
VENT		Welch ANOVA	17.705	2	39.783	<0.001
N-DORS		Kruskall-Wallis test	0.651	2	-	0.722
INFRA	Males	Kruskall-Wallis test	4.891	2	-	0.087
	Females	Kruskall-Wallis test	3.788	2	-	0.151
INTER	Males	ANOVA	5.469	2	305	0.003
	Females	ANOVA	4.980	2	220	0.008
PERI	Males	Welch ANOVA	7.991	2	17.811	0.003
	Females	Kruskall-Wallis test	6.858	2	-	0.032
S-APIC	Males	Welch ANOVA	8.384	2	17.099	0.003
	Females	Kruskall-Wallis test	8.175	2	-	0.017
SUBC	Males	Welch ANOVA	0.527	2	15.562	0.601
	Females	Kruskall-Wallis test	0.504	2	-	0.777
N-APIC	Males	Welch ANOVA	8.292	2	17.113	0.002
	Females	Kruskall-Wallis test	9.522	2	-	0.009

Differences between Iberian sublineages

Variable		Test	Statistics	DF1	DF2	p
DMARKS		Kruskall-Wallis test	111.689	4	-	<0.001
LOR		Welch ANOVA	19.739	4	84.659	<0.001
SUPRA		Welch ANOVA	1.734	4	79.248	0.151
VENT		Welch ANOVA	43.498	4	87.042	<0.001
N-DORS		Kruskall-Wallis test	5.226	4	-	0.265
INFRA	Males	Kruskall-Wallis test	25.528	4	-	<0.001
	Females	Kruskall-Wallis test	11.490	4	-	0.022
INTER	Males	Kruskall-Wallis test	20.022	4	-	<0.001
	Females	ANOVA	3.497	4	218	0.009
PERI	Males	Welch ANOVA	4.385	4	38.045	0.005
	Females	Kruskall-Wallis test	9.084	4	-	0.059
S-APIC	Males	Welch ANOVA	12.435	4	39.926	<0,001
	Females	Kruskall-Wallis test	16.118	4	-	0.003
SUBC	Males	Welch ANOVA	6.975	4	35.843	<0.001
	Females	Kruskall-Wallis test	8.347	4	-	0.080
N-APIC	Males	Welch ANOVA	12.534	4	39.973	<0.001
	Females	Kruskall-Wallis test	18.231	4	-	0.001

Appendix 10: Multicomparison tests for the morphological characters that reported significant p-values in the univariate analyses on the morphological differences between Iberian lineages and sublineages.

VENT

Games-Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	-53.640	18.462	0.01
	South	146.968	38.167	0.00
South	East	-200.608	35.914	0.00

Games-Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
	South	0.373	38.229	1.000
CWS	CNSW	-276.230	25.750	0.000
	NSC	-209.124	18.984	0.000
	South	276.603	40.090	0.000
CNSW	NSC	67.106	22.499	0.027
	Sou	133.253	30.948	0.000
NSC	South	209.497	36.119	0.000
	South	143.350	41.907	0.015
Sou	CWS	142.977	28.495	0.000
	NSC	-66.147	25.595	0.083

LOR

Dunn's test

(i)	(j)	Statistic	Std error	p
West	East	109.096	15.992	0.000
	South	-42.655	47.637	1.000
South	East	-151.751	46.516	0.003

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
	CWS	35.726	56.465	0.968
South	CNSW	58.283	55.056	0.825
	NSC	177.606	53.395	0.030
	Sou	60.017	57.405	0.831
	CNSW	22.557	26.295	0.912
CWS	NSC	141.88	22.611	0.000
	Sou	24.291	30.911	0.934

CNSW	NSC	119.323	18.820	0.000
	Sou	1.734	28.257	1.000
NSC	Sou	-117.589	24.865	0.000

DMARKS

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	-110.054	17.784	0.000
	South	-110.343	41.412	0.046
South	East	0.289	39.041	1.000

Dunn's test

(i)	(j)	Statistic	Std error	p
	CWS	162.332	53.891	0.026
South	CNSW	-9.933	58.823	1.000
	NSC	-17.558	51.778	1.000
	Sou	134.810	56.094	0.162
	CNSW	-172.265	30.359	0.000
CWS	NSC	-179.890	19.334	0.000
	Sou	-27.522	28.972	1.000
CNSW	NSC	-7.625	26.426	1.000
	Sou	144.742	34.116	0.000
NSC	Sou	152.368	24.820	0.000

INTER (females)

Tukey krammer test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	2.689	0.870	0.006
	South	0.042	2.619	1.000
South	East	2.647	2.580	0.561

INTER (males)

Tukey krammer test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	2.663	0.805	0.003
	South	1.797	2.818	0.800
South	East	0.866	2.786	0.948

Dunn's test

(i)	(j)	Statistic	Std error	p
	CWS	-41.213	36.638	1.000
South	CNSW	-4.371	37.805	1.000
	NSC	18.933	36.848	1.000
	Sou	-29.907	40.015	1.000
	CNSW	36.842	17.375	0.340
CWS	NSC	60.147	15.180	0.001
	Sou	11.306	21.77	1.000
CNSW	NSC	23.305	12.913	0.711
	Sou	-25.536	20.254	1.000
NSC	Sou	-48.841	18.406	0.080

N-APIC (females)

Dunn's test

(i)	(j)	Statistic	Std error	p
West	East	14.900	8.068	0.194
	South	-51.626	25.276	0.123
South	East	66.526	24.912	0.023

Dunn´s test

(i)	(j)	Statistic	Std error	p
	CWS	36.689	26.016	1
South	CNSW	68.02	26.165	0.093
	NSC	69.309	24.967	0.055
	Sou	42.309	28.656	1
	CNSW	31.331	12.924	0.153
CWS	NSC	32.62	10.285	0.015
	Sou	6.009	17.424	1
CNSW	NSC	1.289	10.656	1
	Sou	-25.322	17.645	1

NSC	Sou	-26.611	15.815	0.924

N-APIC (males)

Games-Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	-159.858	10.689	0.296
	South	99.286	23.961	0.008
South	East	83.328	23.086	0.021

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
South	CWS	-88.090	25.576	0.038
	CNSW	107.399	25.385	0.012
	NSC	93.227	23.263	0.029
	Sou	27.625	24.161	0.781
CWS	CNSW	19.309	17.482	0.804
	NSC	5.137	14.227	0.996
	Sou	-60.465	15.652	0.002
CNSW	NSC	-14.172	13.880	0.845
	Sou	-79.774	15.337	0.000
NSC	Sou	-65.602	11.490	0.000

PERI (females)

Dunn's test

(i)	(j)	Statistic	Std error	p
West	East	21.914	8.379	0.027
	South	11.039	24.558	1.000
South	East	10.875	24.119	1.000

PERI (males)

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	34.464	10.222	0.003
	South	-20.162	19.645	0.580

South	East	54.627	18.876	0.050

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
	CWS	7.613	21.875	0.996
South	CNSW	29.690	20.800	0.624
	NSC	55.815	19.030	0.105
	Sou	47.088	24.610	0.345
	CNSW	22.077	16.535	0.67
CWS	NSC	48.201	14.244	0.010
	Sou	39.475	21.123	0.347
CNSW	NSC	26.124	12.531	0.235
	Sou	17.397	20.010	0.907
NSC	Sou	-8.727	18.161	0.989

S-APIC (females)

Dunn's test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	12.590	8.126	0.364
	South	-51.824	25.458	0.125
South	East	64.413	25.092	0.031

S-APIC (males)

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
West	East	-16.977	10.757	0.257
	South	-100.218	24.080	0.008
South	East	83.241	23.167	0.022

Games Howell test

(i)	(j)	Mean differences (i-j)	Std error	p
	CWS	88.933	25.725	0.037
South	CNSW	108.396	25.525	0.011
	NSC	93.089	23.340	0.030
	Sou	27.830	24.274	0.779
	CNSW	19.464	17.676	0.806

CWS	NSC	4.156	14.342	0.998
	Sou	-61.103	15.816	0.002
CNSW	NSC	-15.308	13.98	0.809
	Sou	-80.566	15.489	0.000
NSC	Sou	65.258	11.540	0.000

SUBC (males)

Games Howell test

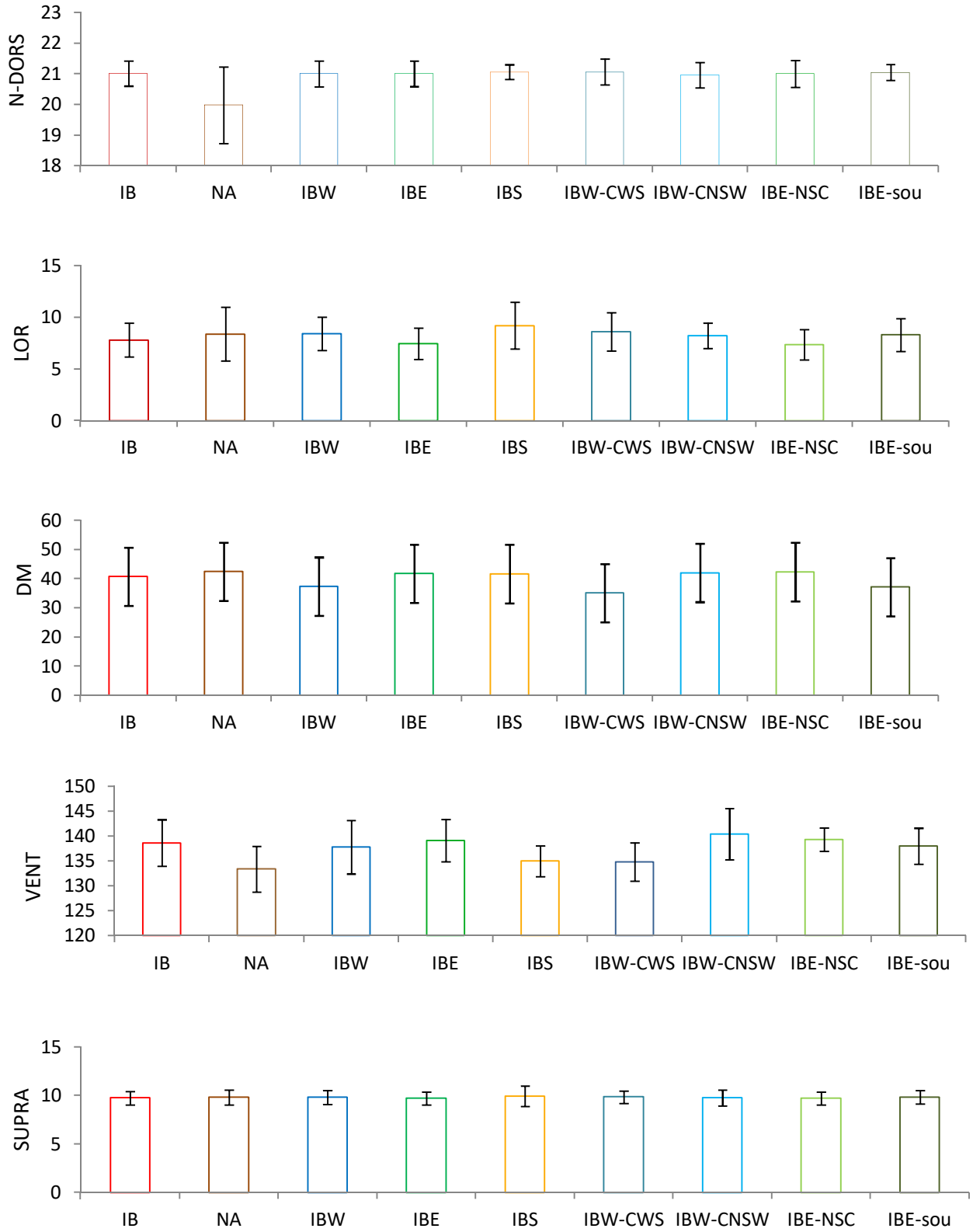
(i)	(j)	Mean differences (i-j)	Std error	p
South	CWS	-22.899	22.980	0.852
	CNSW	10.406	22.451	0.989
	NSC	21.551	19.734	0.806
	Sou	-57.750	22.828	0.150
CWS	CNSW	33.305	19.095	0.412
	NSC	44.451	15.813	0.049
	Sou	-34.851	19.538	0.391
CNSW	NSC	11.146	15.033	0.946
	Sou	-68.156	18.912	0.005
NSC	Sou	-79.302	15.591	0.000

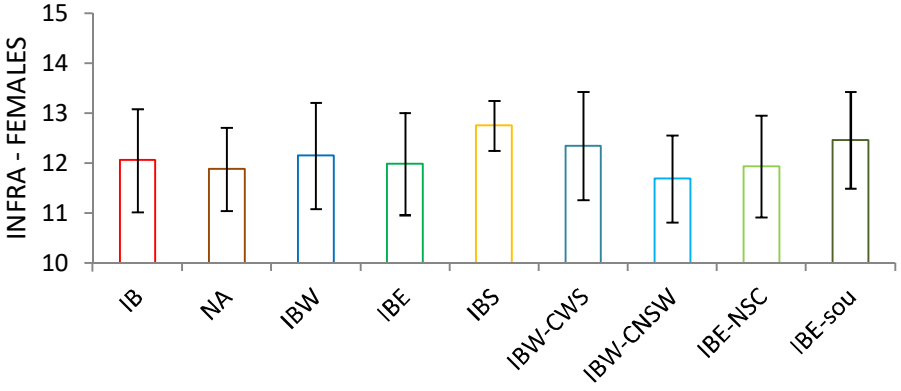
INFRA (males)

Dunn's test

(i)	(j)	Statistic	Std error	p
South	CWS	35.715	31.097	1.000
	CNSW	60.044	31.097	0.535
	NSC	39.560	29.471	1.000
	Sou	-25.013	32.060	1.000
CWS	CNSW	24.329	16.240	1.000
	NSC	3.845	12.856	1.000
	Sou	-60.728	18.016	0.007
CNSW	NSC	-20.484	12.856	1.000
	Sou	-85.057	18.016	0.000
NSC	Sou	-64.573	15.038	0.000

Appendix 11: Bar graphs representing the mean and standard deviation of each morphological character for all phylogeographic groups.

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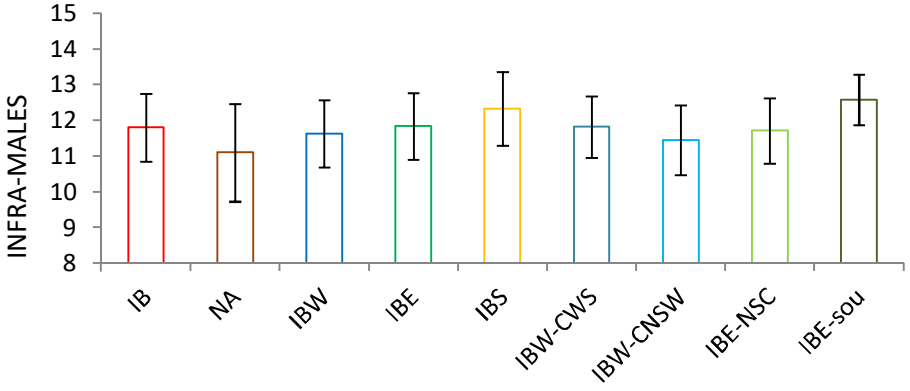
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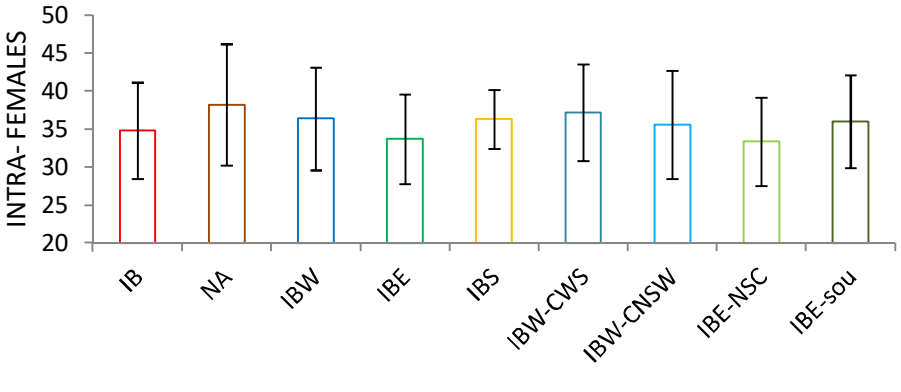
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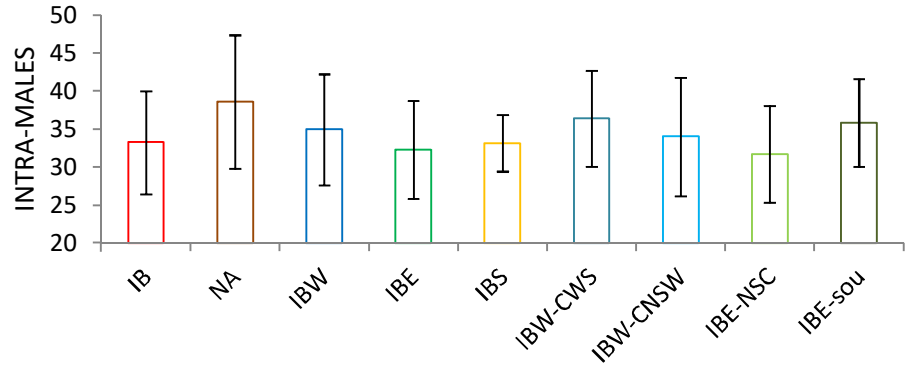
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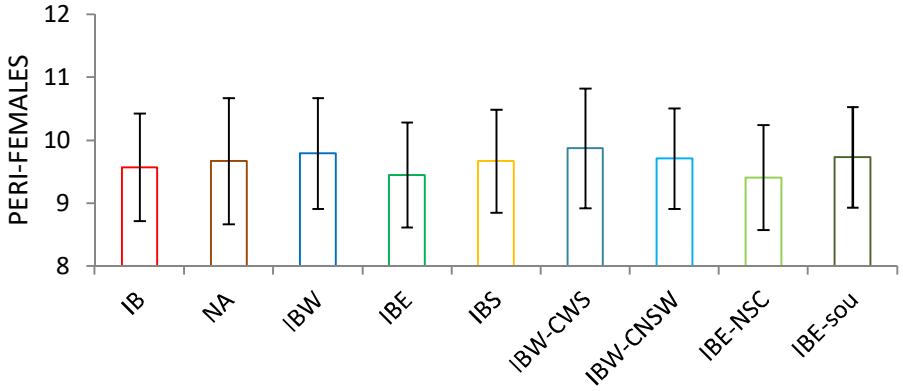
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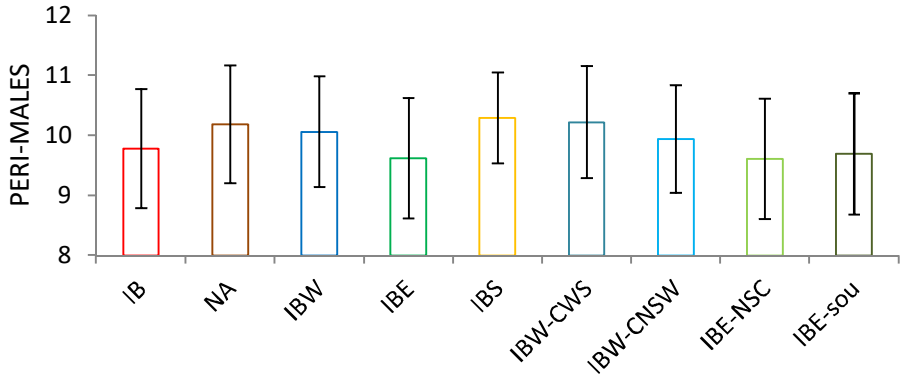
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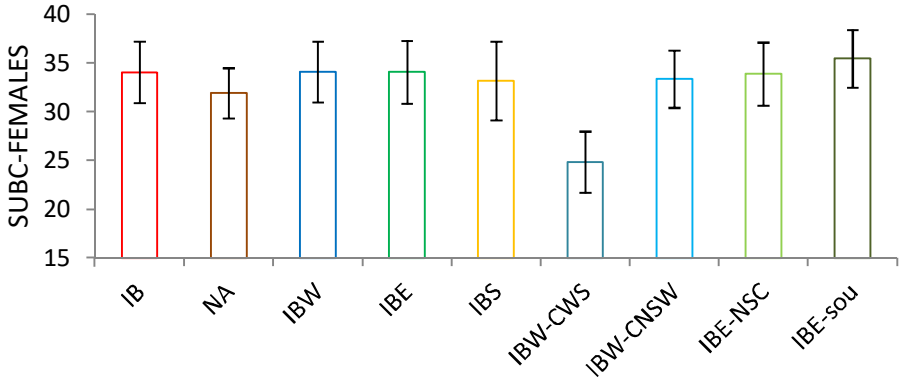
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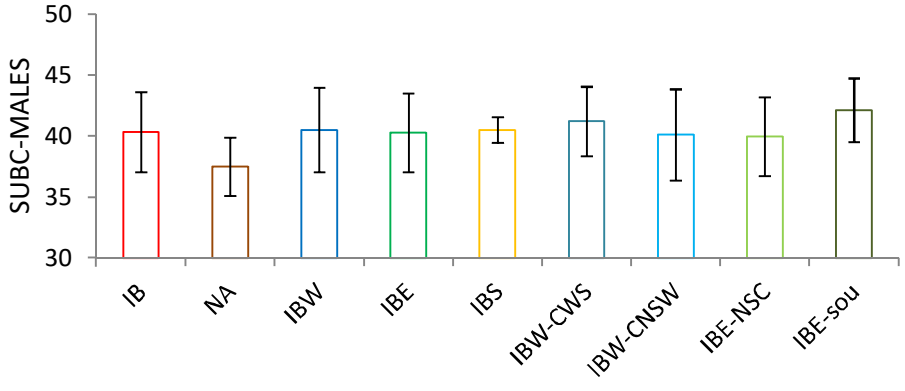
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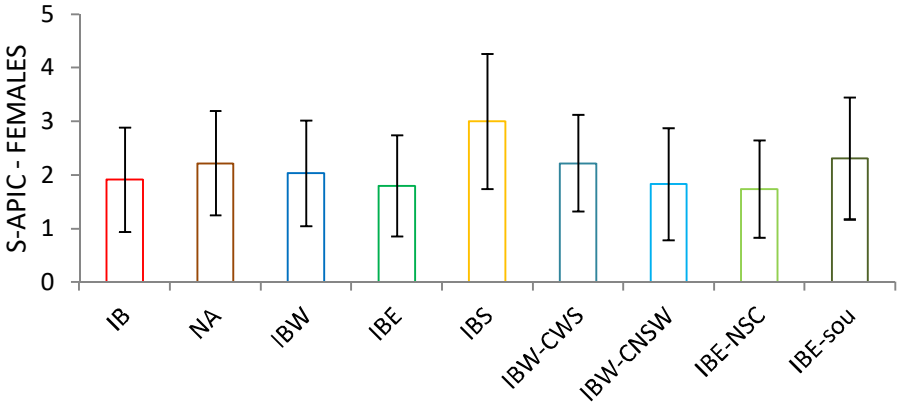
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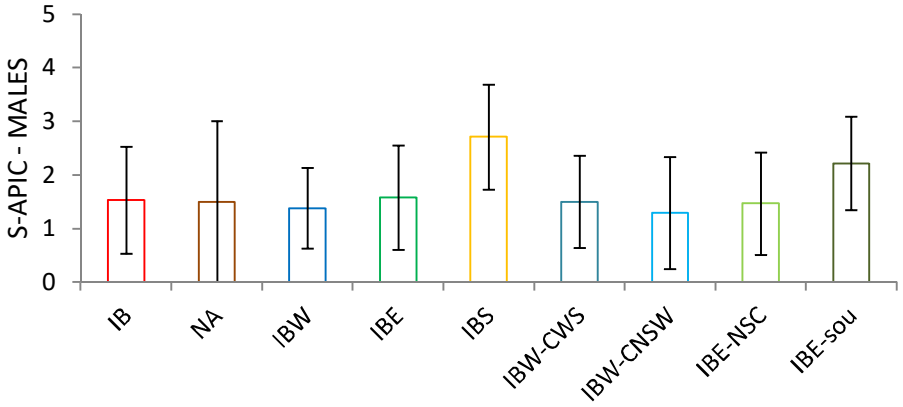
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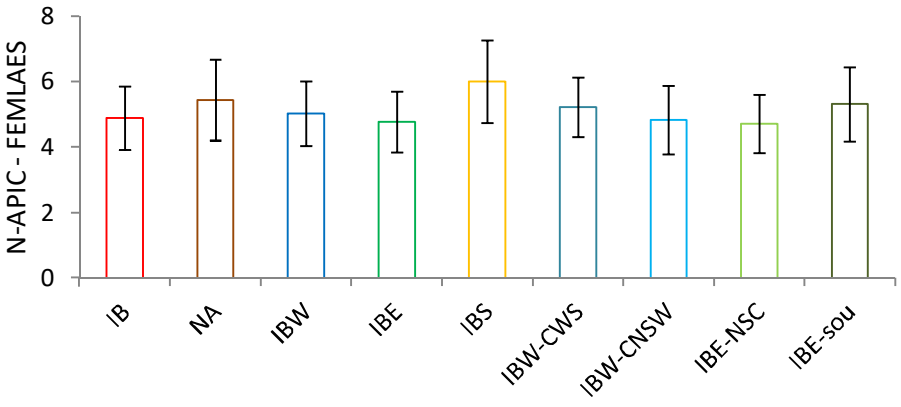
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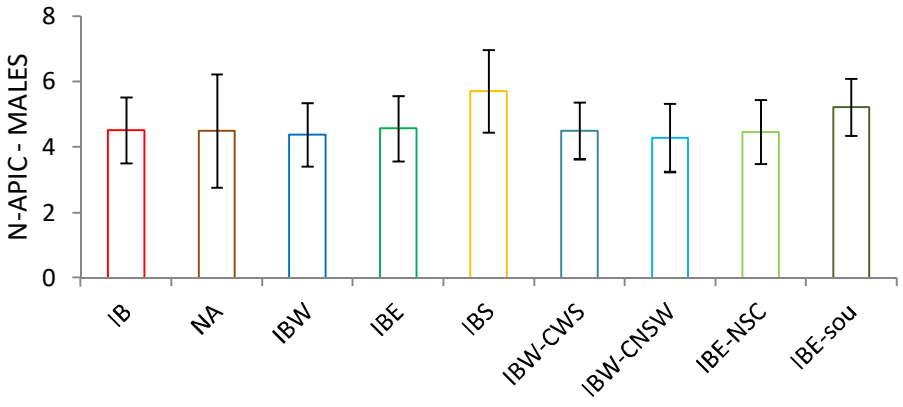
N-APIC - MALES
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