In the Iberian Peninsula, an area where several species show high genetic diversity and phylogeographic structure, some snakes are exceptions. There are species such as *Coronella austriaca* or *Vipera latastei* with typical high mitochondrial genetic diversity, but also examples such as *Rhinechis scalaris*, *Hemorrhois hippocrepis* and *Malpolon monspessulanus* with low genetic diversity.

The aim of this work was to apply a multilocus approach, including nuclear markers, to these last three snakes in order to test if the pattern of low mitochondrial genetic diversity observed is due to demographics factors or rather by a selective process.

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Low genetic diversity or selective sweep in mediterranean snakes ?

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Thesis submitted in order to obtain the Master's degree in Biodiversity, Genetics and Evolution

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Co-supervisor: Dr. Daniele Salvi

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And especially to my family, I will always be grateful for all the unconditional love I receive.

Abstract

Mitochondrial DNA has becoming *the marker* per excellence in population genetics and systematic studies. However, its limitations are known, and, there is now a consensus for the need to use a multilocus approach. An important phenomenon is the “selective sweep” on mitochondria. The origin of an advantageous mutation, leads to a fitness advantage, spreading the genetic heritage of the individual to the population, with consequent loss of genetic diversity at the mitochondrial level. This process can mask the natural history of the species, as some demographic phenomenons can be confused with a “selective sweep”. In the Iberian Peninsula, an area where several species show high genetic diversity and substructure, some snakes are notable exceptions. There are species such as *Coronella austriaca* or *Vipera latastei* with typical high mitochondrial genetic diversity, but also examples such as *Rhinechis scalaris*, *Hemorrhois hipocreppis* and *Malpolon monspesulanus* with very low genetic diversity.

The aim of this work was to apply a multilocus approach to these last three snakes in order to test if the pattern of low mitochondrial genetic diversity observed is due to demographics factors or rather by a selective sweep. To do so, for *Rhinechs scalaris* the sampling was extended relative to a previous study using Cytb and included three other markers: ND4 (mitochondrial) and MC1R and BDNF (nuclear) markers. For *Hemorrhois hipocreppis* and *Malpolon monspesulanus* were screened two nuclear markers (MC1R and BDNF) to compare with the mitochondrial data from previous studies. The results show a pattern of low genetic diversity at both the mitochondrial and nuclear DNA levels, which strongly suggests they underwent a recent demographic and range expansion. *Rhinechis scalaris* presents significant statistical support for the occurrence of a severe bottleneck event, while in *Hemorrhois hippocrepis* and *Malpolon monspessulanus* is reinforced the idea of a recent migration from Maghreb to Iberian Peninsula.

These results open interesting questions about the cause for such demographic events, and the reason why we have so distinct phylogeographic patterns within Iberian snakes.

Resumo

O DNA mitocondrial tornou-se o marcador por excelência em estudos de genética populacional e sistemática. Contudo, são conhecidas as suas limitações, e existe hoje um consenso para a necessidade de usar uma abordagem multilocus. Um fenómeno importante é o “selective sweep”, onde o surgimento de uma mutação vantajosa leva a uma vantagem de fitness, espalhando-se o património genético do individuo ao resto da população, com a consequente perda de diversidade genética a nível mitocondrial. Um evento deste tipo, pode mascarar a história natural das espécies, onde fenómenos demográficos podem ser confundidos com um “selective sweep”. Na Península Ibérica, área onde diversas espécies mostram elevada diversidade genética e subestruturação, algumas serpentes são notáveis exceções. Existem espécies como *Coronella austríaca* ou *Vipera latastei* com típica elevada diversidade genética mitocondrial, mas também exemplos *como Rhinechis scalaris, Hemorrhois hipocreppis e Malpolon monspesulanus* com escassa diversidade genética.

O objetivo deste trabalho foi aplicar uma abordagem multilocus a estas três últimas serpentes para testar se o padrão de baixa diversidade genética mitocondrial observada é devido a fatores demográficos ou antes a um “selective sweep”. Para isso, para *Rhinechis scalaris* foi expandida a amostragem relativa a um estudo anterior que usara Cytb e incluídos três novos marcadores: ND4 (mitocondrial) e MC1R d BDNF (nucleares). Para *Hemorrhois hipocreppis* e *Malpolon monspesulanus* analisamos dois marcadores nucleares (MC1R e BDNF) para comparar com os dados mitocondriais de um estudo prévio. Os nossos resultados mostram um padrão de baixa diversidade genética tanto a nível mitocondrial como nuclear, o que sugere que estas espécies sofreram uma expansão demográfica e territorial recente. No caso de *Rhinechis scalaris* encontra-se suporte estatístico significativo para um evento demográfico severo, enquanto em *Hemorrhois hippocrepis* e *Malpolon monspessulanus* é reforçada a ideia de uma migração recente do Magrebe para a Peninsula Ibérica.

Estes resultados abrem questões interessantes sobre a causa de tais eventos demográficos, e o porquê de os padrões filogeográficos variarem fortemente entre diferentes espécies de serpentes na Península Ibérica.

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Introduction

Biodiversity, Genes and Evolution

Biodiversity is not homogenously distributed on Earth (Myers *et al*, 2000, Keppel *et al*, 2012; Médail *et al*, 1999). Why do some particular areas contain such a high number of species and endemism? This question has been over time recurrent among biologists, and is so complex that it needs the integration of different fields (Gaston, 2000). At the species level, genetic variability is an indispensable component. Genetics provide concepts and methodologies that make easier the identification of intraspecific differences among and within populations.

In the last 30 years, a new discipline, phylogeography, began to assess patterns and process of genetic diversity in the interface between population genetics and phylogeny (Avise *et al*, 1987). The number of studies in this field proliferated rapidly, allowing three main inferences: a) Species histories - molecule markers can give insights of the evolutionary history of organisms, phylogeographic patterns reflect past evolutionary forces (migrations, demography, molecular evolution) (fig1) ; b) Major evolutionary and biogeographic process - common phylogeographic patterns among coo-distributed species reflect major events which affected entire biotas; c) Identification of cryptic species – Morphologically indistinct individuals can have identical or higher genetic distances than distinct species. (Avise *et al* 1998, Avise *et al*, 2009: Hickerson *et al*, 2010).

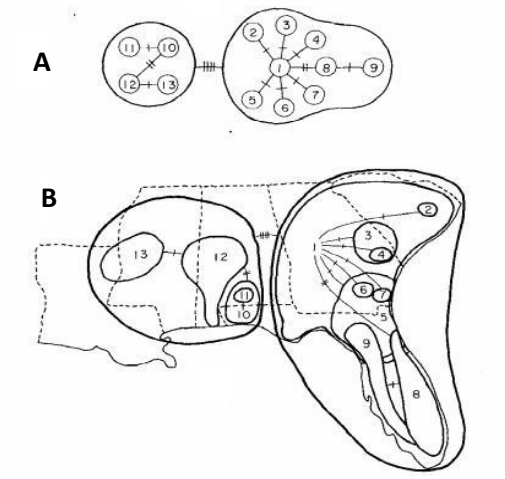


Fig.1 - Network (A) and geographic structuring (B) of 13 mitochondrial DNA (mtDNA) haplotypes of bowfin fish, *Amia cava*, one of the first phylogeographic studies. (From John Avise *et al,* 1987).

Iberian Peninsula Phylogeography

Earth cyclically experiences climatic shifts (glacial and interglacial ages), resulting from oscillations of its orbital properties (Hewitt, 2004, Hewitt, 2011). During the Pleistocene, these shifts became characterized by increasingly severe ice ages and short interglacial ages, resulting in dramatic changes in species distribution and viability (Hampe and Jump, 2011). The information regarding these epoch, decisive to today's biodiversity patterns, has grown in recent years. In particular, phylogeographic studies identify in present populations some genetic signatures: a) isolation in refuges during glacial ages; b) interglacial expansions; c) admixture resulting from contact zones (Hewitt, 1996). In Europe, the Mediterranean basin, especially the three main peninsulas - Iberia, Italy and the Balkans - functioned as glacial refuges from which species re-colonized northern areas during interglacial periods (Hewitt 1996; Hewitt, 2004; Schmitt, 2007). Today, this is reflected in higher levels of species and intraspecific genetic diversity in these three zones, a pattern called "southern richness- northern purity" (Hewitt, 1996).

In the Iberian Peninsula, a more specific pattern was recently proposed. Phylogeographic studies covering a wide number of *taxa*, from vertebrates to invertebrates, revealed similar levels of high genetic intraspecifity, concordant with periods of past population isolation within the Iberian Peninsula (Gomez and Lunt, 2007). Analysing the different biogeography scenarios inferred by these studies, some areas of isolation are shared between different species, meaning that multiple refugia occurred within Iberian Peninsula, in a scenario of "refugia-within-refugia" (Gomez and Lunt, 2007).

This phylogeographic concordance can be explained by a series of characteristics that make this region unique within the biogeographical context of Europe:

i) Localization – Located in Western Europe, undergoing influences from Atlantic Ocean and Mediterranean Sea creating heterogeneity of climates (including, Atlantic, Desert, and Mediterranean). Additionally, being separated from North Africa by only 14 km it acted as a faunistic crossroads between these two continents.

ii) Topography – A series of mountain chains oriented east-west shape Iberian territory allowing a wide spectrum of microclimates along altitude. The localization of the majority

of these mountains chains, relatively close and parallel to the line coast, creates also prominent biogeographical barriers between coastal and interior regions (fig.2).

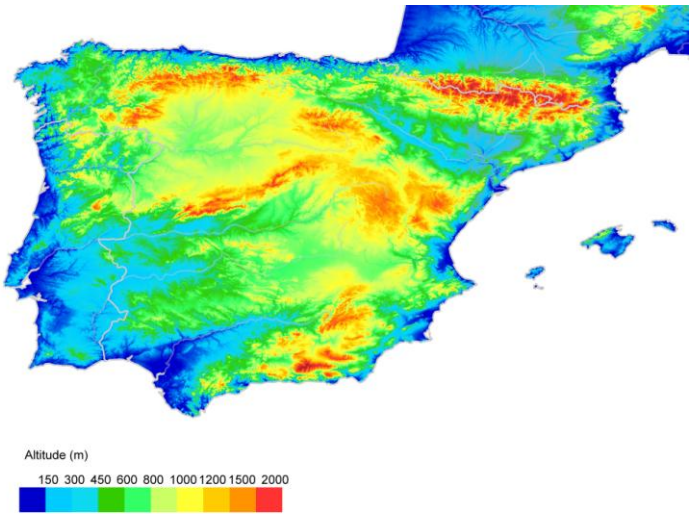
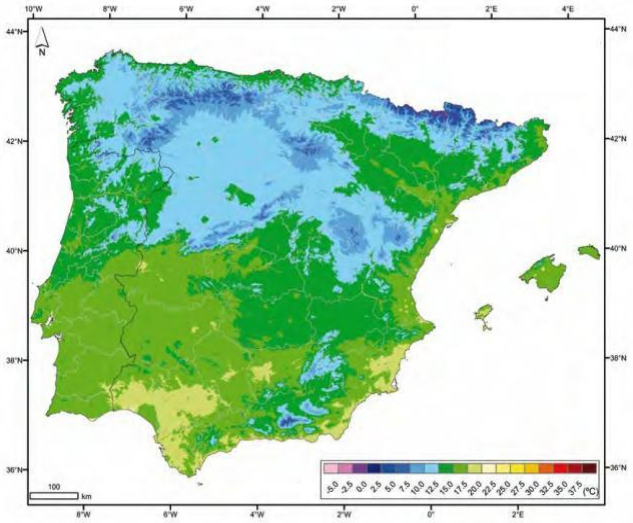


Fig.2 - Altitude distribution (m) in Iberian Peninsula. (From “Atlas Climático Ibérico”, 2011).

iii) Dimension – with an area of 580,000 km^2 and a rectangular shape, combined with the above mentioned features make it host a complex heterogeneity of environmental conditions (Rodriguez-Puebla *et al*, 1998) (fig.3 and 4).



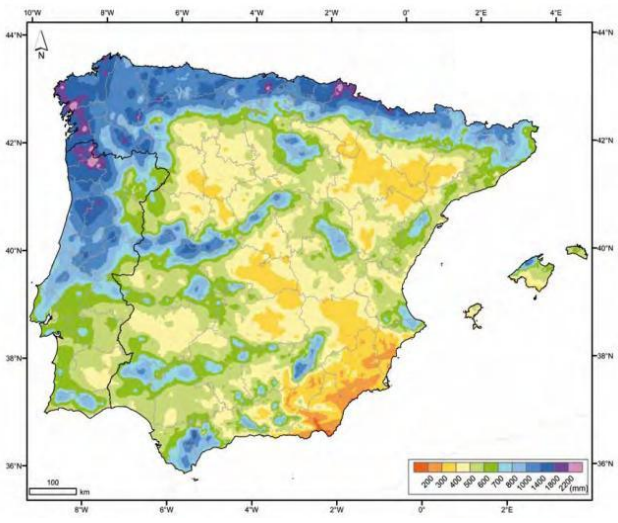


Fig.3 and Fig.4 - Left – Annual distribution of mean temperature (ºC); Right – Annual distribution of precipitation (mm) in Iberia Peninsula. (From “Atlas Climático Ibérico”, 2011).

A case for Iberian Snakes

Reptiles are model organisms for evolutionary studies; their ecological characteristics like low dispersion capacities or a dependency on relatively strict environmental conditions make them very good candidates for studies on phylogeography (Camargo, 2010 and Joger *et al*, 2007). Among this *taxa*, in Iberia Peninsula a considerable amount of information has been gathered about phylogeographic patterns, the *lacertidae* family is a clear example, and contributed to the idea of Iberian Peninsula as an zone of multiple refuges (Gomez and Lunt, 2007).

Contrary to the general pattern observed in Iberian Peninsula, snakes present an interesting distinction in two different general patterns. Some of them present the general high diversity and geographic structuring: *Coronella austriaca, Coronella girondica, Vipera latastei and Natrix maura*; while on the other hand some species such as: *Macroprotodon brevis*, *Malpolon monspessulanus, Hemorrhois hippocrepis* and *Rhinechis scalaris* show no differentiation and lower genetic diversity.

The assessment of *Coronella austriaca*, realized by Santos *et al* (2008), using 814 bp of mtDNA (Cytb, 302 bp; and 16S rRNA, 512 bp) and covering almost all the native distribution of the specie in Iberian Peninsula, revealed the existence of at least three well supported mtDNA lineages exclusive to the region: CaI – Northwest; CaII – Sistema Central, Montes de Toledo and Sierra de Alcaraz ; CaIII – Beceite and Soria up to Pyreneus (fig.5); Lineage CaI was well supported by Bayesian Inference (BI), Maximum likelihood (ML) and Maximum Parsimony (MP) bootstrap values (>0.95, 100, 100), whereas lineages CaII and CaIII where supported only by BI (>0.95) (Santos *et al*, 2008).This three main lineages show relatively high level of genetic differentiation (%) for the portion of the Cytb gene analysed, CaI diverged 5.9 ±1.3 and 4.8 ±1.1 to CaII and CaIII respectively, and CaII and CaIII revealed a differentiation of 6.8 ±1.3 (Santos *et al*, 2008). Within the lineages, again for Cytb, there were low levels of intraspecific variation (%), 0.7 ±0.3, 0.2 ±0.2 and 0.9 ±0.3 for CaI, CaII and CaIII respectively (Santos *et al*, 2008).

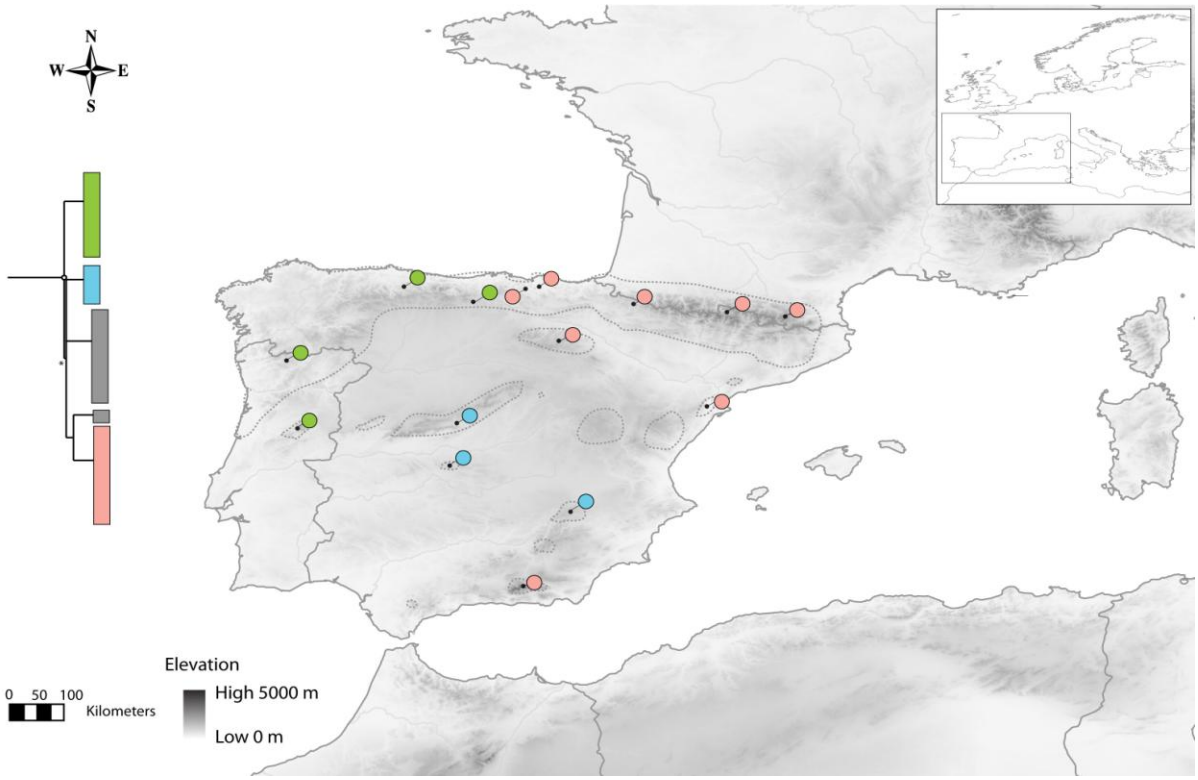


Fig.5 - Maximum-likelihood tree inferred with the mitochondrial Cytb gene for *Coronella austriaca* and geographic localization of the main Iberian lineages (adapted from Santos *et al,* 2008) : Green – CaI ; Blue – CaII; Pink – CaIII; Grey – European lineages. White circle indicate Bayesian, Maximum likelihood and Maximum parsimony bootstrap values equal to 100. Asterisk mean that only Bayesian bootstrap values were higher than 95. Range of the specie in Iberian Peninsula is represented by the dashed lines.

In *Coronella girondica*, Santos *et al* (2012), using 816 bp of mtDNA (Cytb, 303 bp; and 16S, 514 bp) demonstrated the structuring of this specie in three divergent lineages, two of them in Iberia Peninsula: CgI – Betic ranges and CgII – European; supported by BI, and ML bootstrap values equal to 100 (fig.6) (Santos *et al*, 2012). Additionally, a sub-lineage within CgII: SWI – SouthWestern Iberia; revealed some degree of differentiation, but only in BI bootstrap values (0.96) (fig.6) This three lineages revealed a relatively high degree of genetic differentiation (%) for the portion of the Cytb gene analysed, CgI differ by 5.99 ± 1.29 and 6.82±1.43 to CgII and SWI respectively, and CgII and SWI diverge by 3.71±1.1, whereas 16S revealed less divergence, around 1.80 between all lineages (Santos et al, 2012). Intraspecific difference (%) maintained the contrast between the two markers, Cytb gene revealed values of ranging from 0.68 (SWI) to 1.14 (CgI), whereas 16S revealed values ranging between 0.09 (SWI) to 0.26 (CgII) (Santos *et al*, 2012).

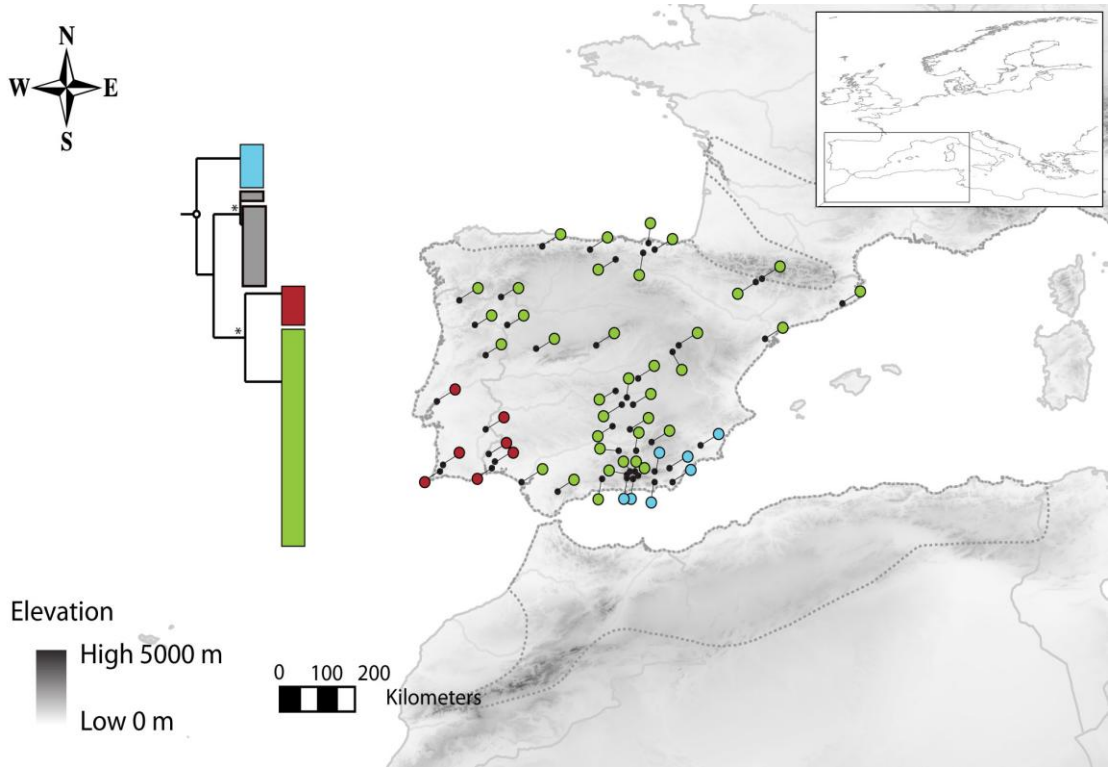


Fig.6 - Maximum-likelihood tree inferred with Cytb and 16s mitochondrial genes for *Coronella girondica* and geographic localization of the Iberian lineages (adapted from Santos *et al,* 2012) : Blue – CgI; Green – CgII ; Red – SW1; Grey – North African lineages. White circle indicate Bayesian and Maximum likelihood bootstrap values equal to 100. Asterisk mean that only Bayesian bootstrap values were higher than 95. Range of the specie in is represented by the dashed lines.

An recent study by Velo Anton *et al* (2012) regarding *Vipera latastei* using a multilocus approach:1003 bp of mtDNA (ND4, 828 bp; and Cytb, 275 bp) and 729 bp of nuDNA (RAG 2); found a very well resolved structure, with three highly divergent main mtDNA lineages in Iberian Peninsula : VlW – West Iberia; VlE – East Iberia; VIS – South Iberia; supported by BI, ML and MP bootstrap values higher than 0.99, 80 and 80 respectively (fig.7) (Velo Anton *et al*, 2012). By the same criteria, lineages VlWest and VlEast also presented two supported sub-lineages: VlWa, VIWb, VIEa and VIEb (fig.7). The genetic differences (%) for the mtDNA lineages was high, VIW diverge by 5.4 ±0.6 and 4.8±0.6 to VIE and VIS respectively, and VIE and VIS diverged by 5.7±0.7 (Velo Anton *et al,* 2012). Values of intraspecific diversity (%) were high in VIW and VIE, 0.3-3.9 and 0.2-4.7, respectively, and lower in VIS, 0.2-1.4 (Velo Anton *et al*, 2012).

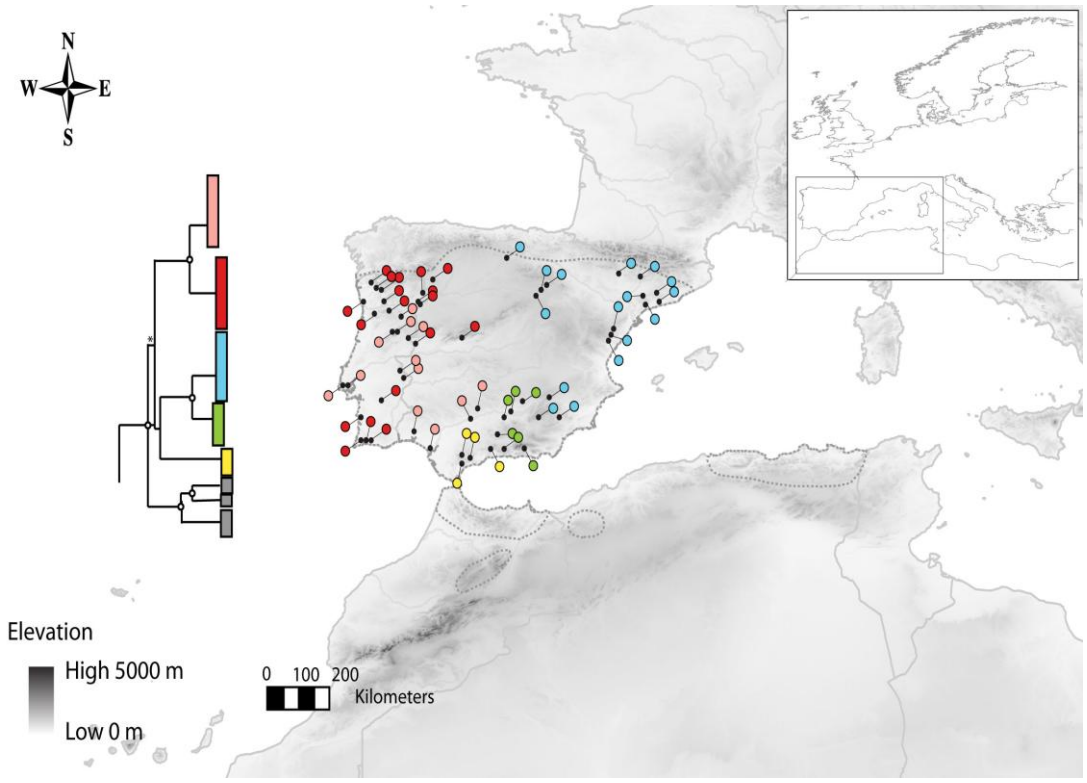


Fig.7 - Bayesian tree inferred with Cytb and ND4 mitochondrial genes for *Vipera latastei* and geographic localization of the Iberian lineages (adapted from Velo-Antón *et al,* 2012) : Pink – VIWa ; Red – VlWb; Blue – VIEa; Green -VIEb ; Yellow - VIS; Grey – North African lineages. White circle indicate Bayesian, Maximum likelihood and Maximum parsimony bootstrap values higher than 99, 80 and 80 respectively. Asterisk means that only Bayesian bootstrap values maintained the significant above mentioned. Range of the specie in is represented by the dashed lines.

The *Natrix maura* phylogeography was verified by Guicking *et al* (2006) using mtDNA data (Cytb, 1117 bp) and genomic inter-simple-sequence-repeats polymerase chain reaction (ISSR-PCR fingerprinting), with Cytb supporting the existence of two lineages in Iberian Peninsula: NmE – Europe linage and NmSS – Southern Spain lineage; supported by BI and MP bootstrap values equal to 100 (fig.8) (Guicking *et al*, 2006). The mitochondrial genetic distance (%) between these two lineages was about 2.4 to 3 (Guicking *et al*, 2006). Even with less structuring comparing to previous snakes, this specie has within the European range a possible lineage exclusive to Iberian Peninsula, and the high genetic diversity found in the south of France and North of Italy point to a possible underestimation of the differentiation within this region due to incomplete sampling (Guicking *et al*, 2006). Indeed, later phylogeographic assessment of *N. maura* uncovered more diversity, albeit in North Africa (Barata *et al*. 2008).

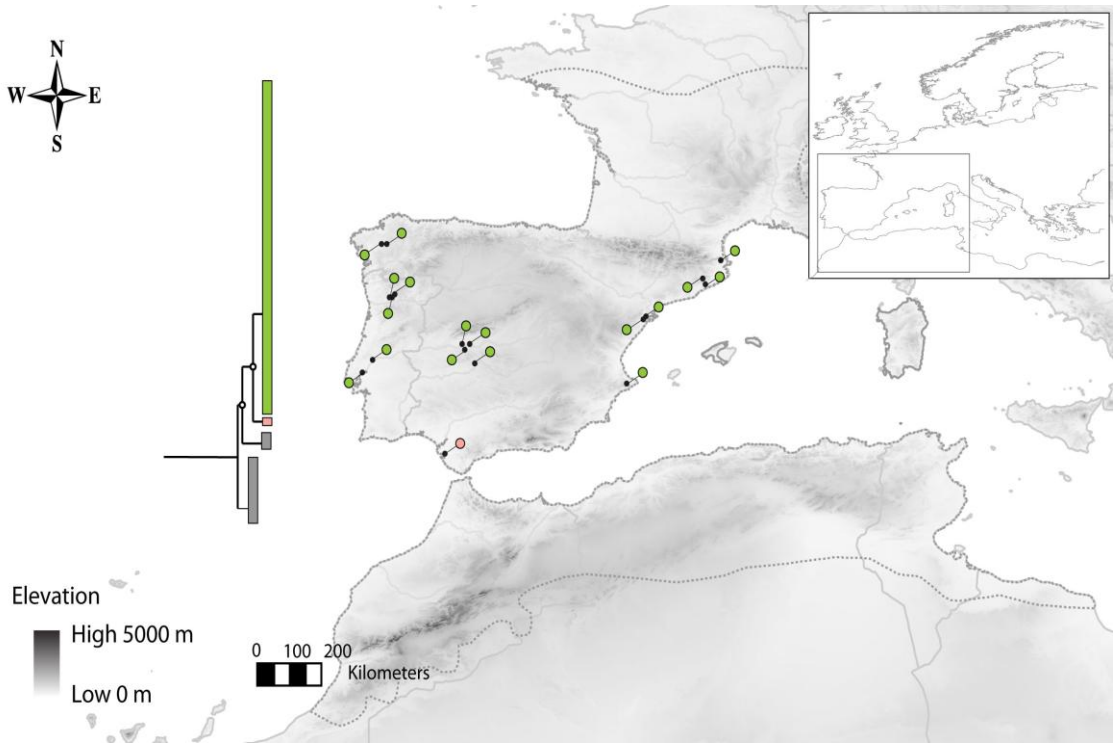


Fig.8 - Maximum likelihhod tree inferred with Cytb mitochondrial gene for *Natrix maura* and geographic localization of the Iberian lineages (adapted from Guicking *et al,* 2008) : Green – NmE ; Pink – NmSS. Grey – North African lineages . White circle indicate Bayesian and Maximum parsimony bootstrap values higher than 95. Range of the specie is represented by the dashed lines.

Carranza et *al* (2004) assessed the phylogeography of the genus *Macroprotodon* using 1075bp of mtDNA (Cytb, 300 bp; 12S rRNA, 393 bp; and 16S rRNA, 382) and verified two lineages within *Macroprotodon brevis*, supported by BI, ML and MP values equal to 100, consistent with the subspecies assumed in literature - *Macroprotodon brevis brevis*, exclusive to Morocco, and *Macroprotodon brevis ibericus* present in the extreme North of Morocco and the Iberian Peninsula (fig9) (Carranza *et al*, 2004). *Macroprotodon brevis ibericus* in Iberian Peninsula didn't reveal any variation in the 1075 bp analysed, differing by one nucleotide from individuals present in Morocco (Carranza *et al*, 2004). For Cytb, the genetic divergence between the two subspecies was 4.7% for, with the *Macropotodon brevis brevis* having higher intraspecific variability (2.6%) comparing to *Macroprotodon brevis ibericus* (0.3%) (Carranza *et al*, 2004).

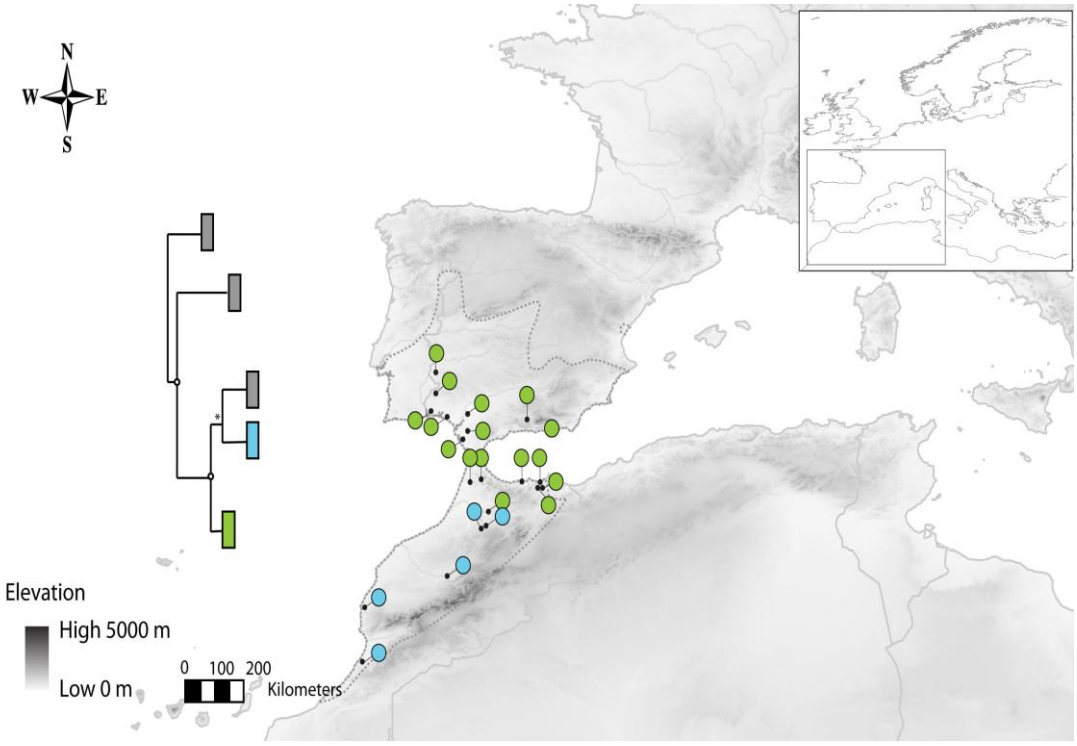


Fig.9 - Maximum likelihood tree inferred with Cytb, 12S rRNA and 16S rRNA mitochondrial genes for *Macroprotodon genus* and geographic localization of the *Macroprotodon brevis* lineages (adapted from Carranza *et al,* 2004):Blue – *Macroprotdon brevis brevis* ; Green – *Macroprotodon brevis ibericus*; Grey – Other *Macropotodon* members. White circle indicate Bayesian, Maximum likelihood and Maximum parsimony bootstrap values equal to 100. Asterisk mean that only Bayesian bootstrap values were significant (98). Range of *Macroprotodon brevis* is represented by the dashed lines.

Rhinechis scalaris was assessed by Nulchis *et al* (2008) in a very preliminary study. This assessment with a limited sampling and 300bp of Cytb gene revealed no single variation in all specimens obtained (fig.10) (Nulchis *et al*, 2008).

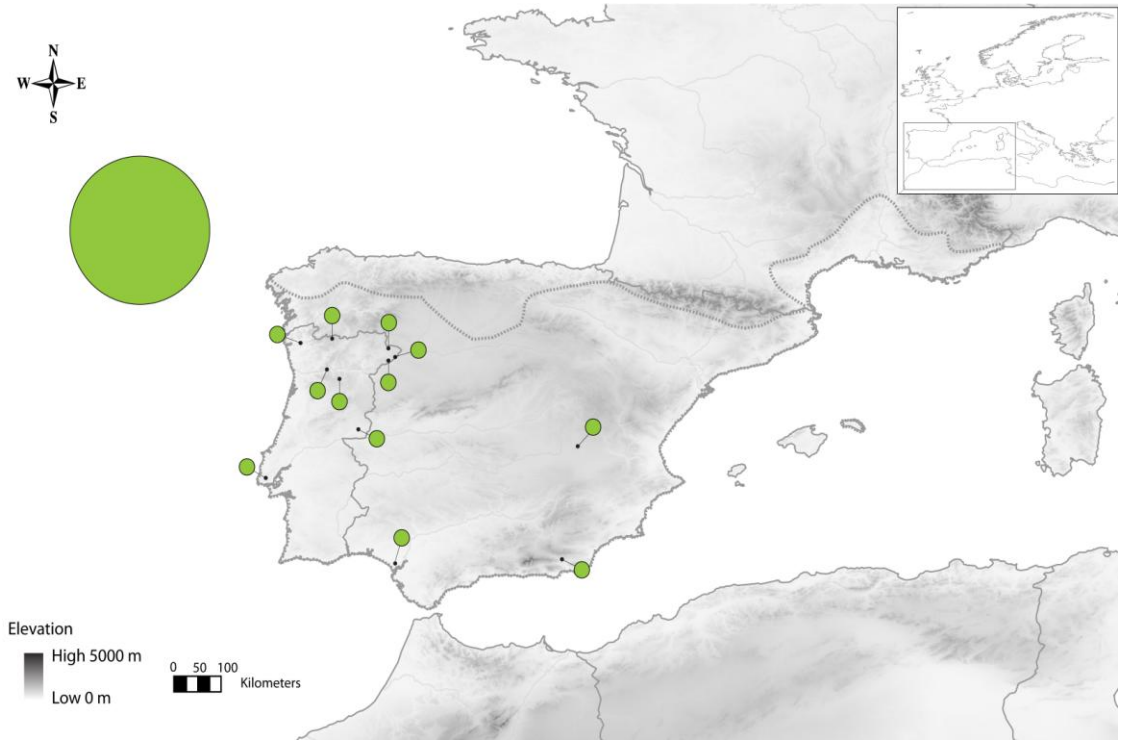


Fig.10 - Results from the phylogeographic study by Nuchis *et al* (2008) for *Rhinechis scalaris*. Representation samples distribution and haplotype network inferred from 300bp of Cytb (adapted from Nulchis *et al* 2008). Range of the specie is represented by the dashed line.

The genus *Malpolon* was assessed by Carranza *et al* (2006), using 815 bp of mtDNA (Cytb, 300bp; and 12S rRNA, 515 bp), and grouped *Malpolpon monspessulanus* in a single clade with Algeria, Morroco and Iberian Peninsula specimens. In this clade, were identified only 45 variable positions, 4 of them parsimony-informative, with 0.6% of genetic differentiation (Carranza *et al*, 2006). Iberian and Maghreb populations were identified, given the significance of the snn test ($p < 0.001$), with both populations having high haplotype diversity values and low value of nucleotide diversity (Carranza *et al*, 2006). The number of exclusive haplotypes and mutation steps between haplotypes was higher in Maghreb populations (fig.11) (Carranza *et al*, 2006).

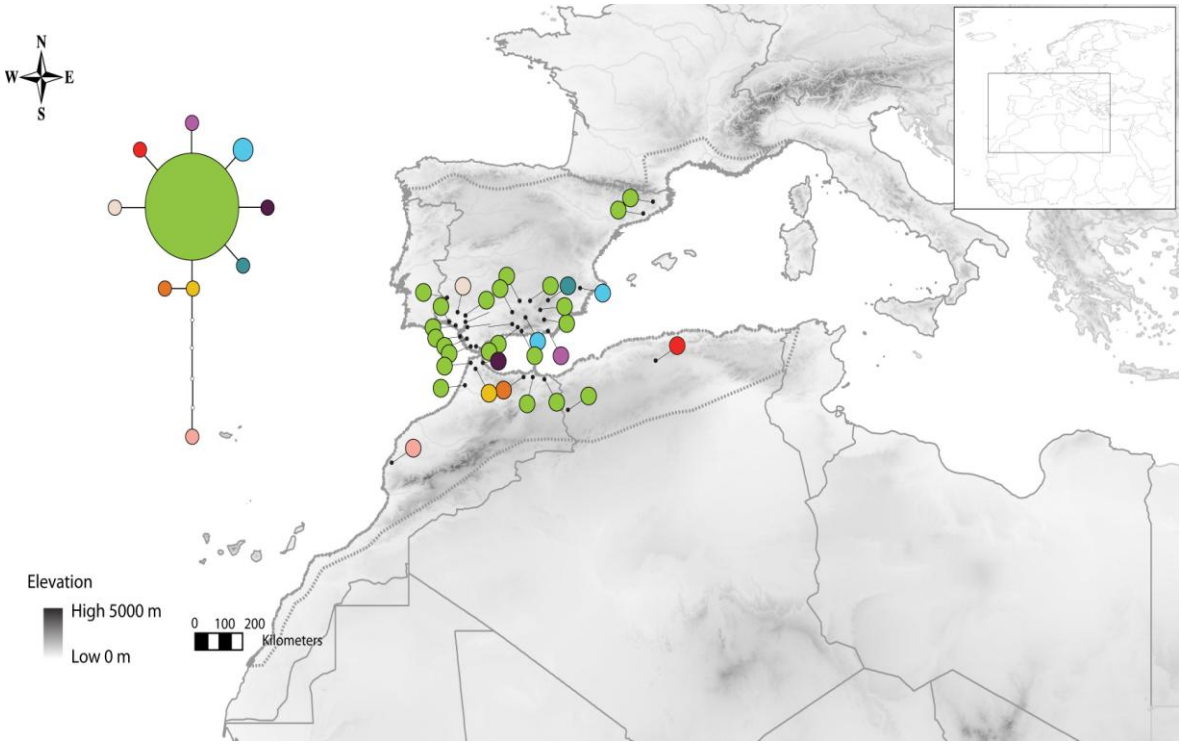


Fig.11 - Results from the phylogeographic study by Carranza *et al* (2006) for *Malpolon monspessulanus*. Representation of samples distribution and haplotype network inferred from 300bp of Cytb and 515bp of 12S rRNA (adapted from Carranza *et al* 2006). White circles represent a mutational step. Range of the specie is represented by the dashed line.

The *Hemorrhois hippocrepis* phylogeographic pattern was also assessed by Carranza *et al* (2006), using 695bp of mtDNA (Cytb, 300 bp; and 12S rRNA, 395 bp). A well supported Tunisian clade was verified by supported ML and MP values equal to 100, with the rest of Maghreb and Iberian individuals being intermixed (Carranza *et al*, 2006). This second large clade revealed 202 variable sites, 145 parsimony informative, with a 1.6% divergence within the clade (Carranza *et al*, 2006). Snn test for population divergence revealed an Iberian and Maghreb population ($p < 0.001$) with both populations showing high levels of haplotype diversity and low levels of nucleotide diversity (Carranza *et al*, 2006). Maghreb population revealed higher number of exclusive haplotypes and mutational steps between haplotypes (fig.12) (Carranza *et al*, 2006).

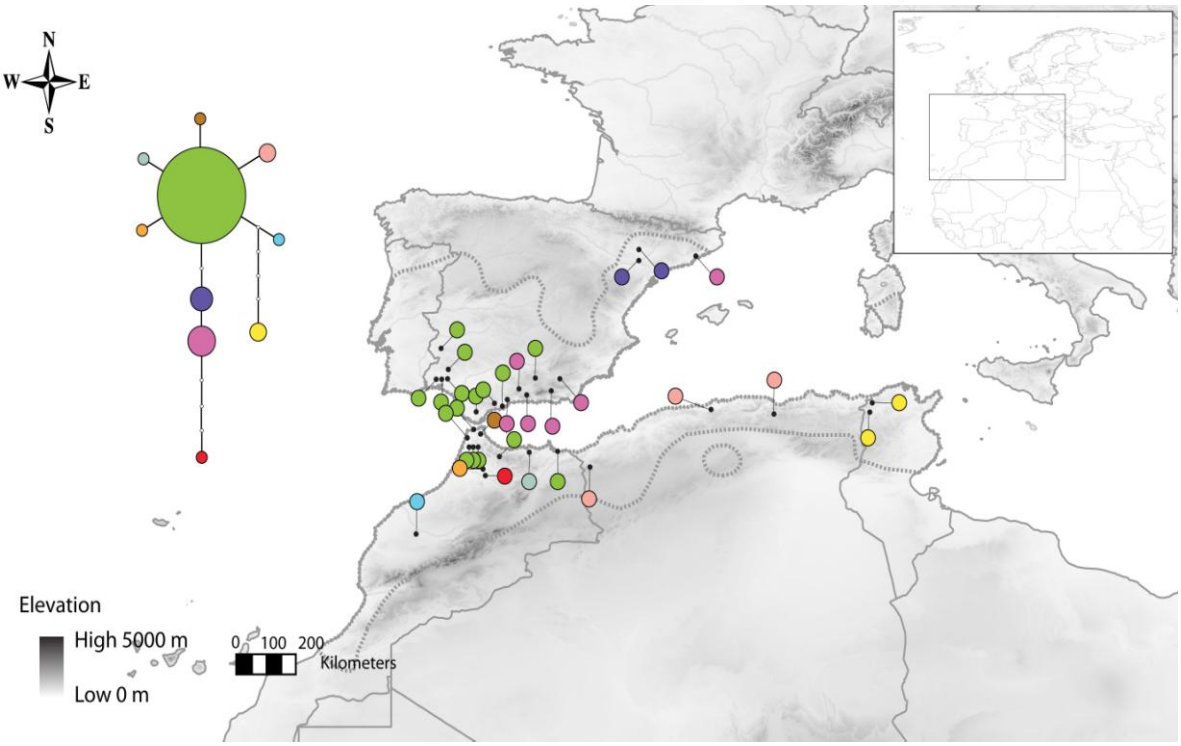


Fig.12 - Results from the phylogeographic study by Carranza *et al* (2006) for *Hemorrhois hippocrepis*. Representation of samples distribution and haplotype network inferred from 300bp of Cytb and 395bp of 12S rRNA (adapted from Carranza *et al* 2006). White circles represent a mutational step. Range of the specie is represented by the dashed line.

The incomplete history of mtDNA markers

Clearly, the mtDNA markers reveal two phylogeographic patterns in Iberian snakes. However an important question should be made - are these mtDNA patterns representing the true species phylogeographic pattern?

Mitochondrial DNA is the standard molecular marker in phylogeographic studies, being maternally inherited and without proven recombination and selection (Gardner and Eyre-Walker, 2004). This means that the variants present are not intermixed and so, the mtDNA characters represent the presumed historical sequence of mutation events accompanying the differentiation of maternal lines (Avise, 1987; Ballard and Whitlock, 2004; Ballard and Rand, 2005). It is widely distributed among organism, and present in each of them with a higher number of equal copies, making this marker comparative and easy to assay (Avise, 1987; Ballard and Whitlock, 2004; Ballard and Rand, 2005; Kocker *et al*, 1989). Despite the fact that some mitochondrial gene codify for protein involved in fundamental metabolic pathways, they have generally higher mutation rates than nuclear DNA, and as consequence most of the mitochondrial markers are polymorphic at the intraspecific level and often nucleotide differences occur among individuals from different populations (Brown *et al*, 1979; Avise, 1987).

However, gene genealogies don't represent the entire organism pedigree. Moreover, mtDNA reflects only the matriarchal lineage of species (Avise, 1998; Hare, 2001; Ballard and Whitlock, 2004). Thus to be representative of male-female genealogies we assume that the life histories/behaviours of males and females at study are the same. Sex biased dispersal behaviours are proven examples that this assumption is not always true (Lyrholm *et al*, 1999; Foitzik *et al*, 2009; Nietlisback *et al*, 2012; Ahrens *et al,* 2005; Rheindt and Edwards, 2011).

Initially, Nuclear DNA (nuDNA) had much less potential to emerge as a standard molecule marker. The sexual transmissition of this kind of markers implicate recombination, which in turn make difficult the assessment of phylogenetic relationships (Avise, 1987; Towes and Breslford, 2012). The lower mutational rates also make it more difficult to verify instraspecific differences among populations (Avise, 1987; Towes and Breslford, 2012). Nuclear molecule markers were more difficult to extract (incomparable less copy and given the more complexity in the nuDNA, the design of appropriate conserved primers was very laborious (Hare, 2001; Towes and Bresfrod, 2012). With the development of genomic databases and laboratory techniques in the last two decades, the nuDNA become easier to assay (Towes and Breslford, 2012). As studies using both

mtDNA and nuDNA increased, cumulative cases of incongruence between the inferences based on these markers emerged (fig.13) (Towes and Breslford, 2012). In most cases, the conflicts between mtDNA and nuDNA are the result of complex histories in which only using a multilocus approach make possible to assess (Leaché and Mcguire, 2006; Spinks *et al*, 2009; Ballard and Whitlock, 2004; Towes and Breslford, 2012).

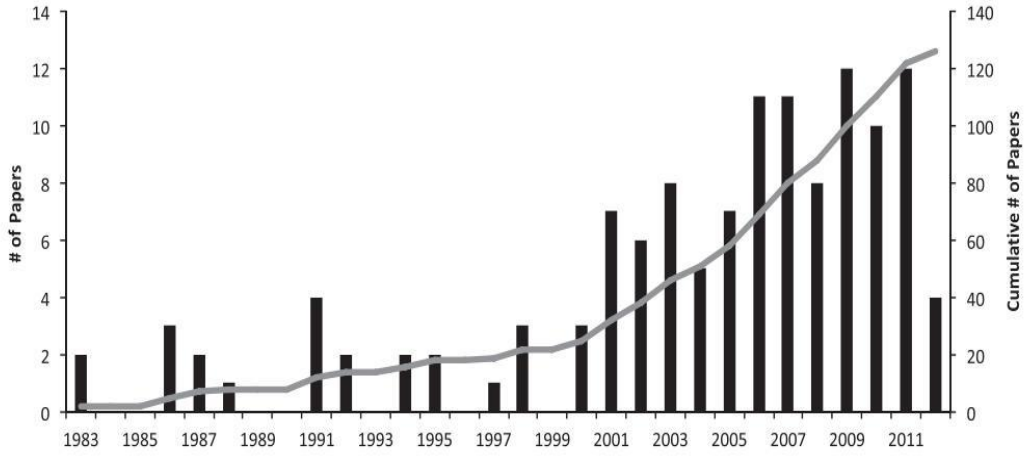


Fig.13 - Number of studies reporting MtDNA and nuDNA conflicts published from 1983 to 2012 (bars). Cumulative number is shown in the right axis (grey line). (From Towes and Brelsford, 2012).

A multilocus approach offers a series of advantages. Conceptually all mitochondrial genes are linked in terms of evolution (Ballard and Whitlock, 2004; Ballard and Rand, 2005; Gautier, 2009). So, as there is no independence between mtDNA molecule markers, and increasing the number of mitochondrial genes analysed does not provide independent genealogies sampled across the genome (Ballard and Whitlock, 2004). On the contrary in the case of nuclear markers we can infer independent gene genealogies by sampling unlinked markers, and increasing the number of such markers will add additional valuable information (Ballard and Whitlock, 2004).

MtDNA being haploid and only maternally inherited has ¼ of the effective population size (*Ne*) of nuDNA (although in real populations, only a fraction of males contribute to reproduction, so nuDNA can have lower Ne than expected); This means that under a neutral model of molecular evolution, these two locus resolve monophylies at different velocities and result in possible incongruence's due to incomplete lineage sorting (Ballard and Whitlock, 2004). Additionally, under a demographic event, due to different effective population sizes, the deviations from neutral model are unequal, lower Ne are more susceptible to lost rare alleles, and so neutrality tests can be biased and given wrong indication of the species evolutionary history when not compared with nuDNA datasets (Ballard and Whitlock, 2004 and Fay and Wu, 1999).

The argument of the mtDNA as the neutral marker *per se*, is based on the assumption that adaptive mutations are very rare, and neutral or deleterious mutations, more frequent, are removed by purifying selection without affecting the diversity at the linked loci, so the observable variation would therefore reflect neutral process only (Ballard and Kreitman, 1994; Hasegawa et *al*, 1998). This assumption is difficult to theoretically accept, because of the important functions of mitochondria metabolism it is legitimate to think that some evolutionary pressures actually act on mtDNA gene (Lee *et al*, 2008; Ballard *et al,* 2007). Other than direct selection, indirect selection due to cyto-nuclear interactions can also be a case (Ballard and Whitlock, 2004). Bazin *et al* 2006 provided more insights by testing the neutral theory argument that genetic diversity is propositional to population size by studying levels of mtDNA and nuDNA diversity along species with marked different population sizes (vertebrates and invertebrates) (Bazin *et al,* 2006). The study showed that mitochondrial diversity basically was not proportional to population size in the species under study, but instead was similar along species (Bazin *et al,* 2006). In contrast, nuclear diversity corresponded to that expected by neutral theory (Bazin *et al*, 2006). This pattern could be explained by recurrent adaptive mutations in mtDNA gene where a new allele spread along the population, hitch-hiking all mtDNA genome, and creating a homonegenization of genetic diversity within the population, a "selective sweep" (Maynard Smith and Haigh, 1974; Jiggins *et al*, 2003 ;

Kaplan *et al*, 1989). This pattern is in agreement with the genetic draft theory of Gillespie, that populations are shaped by genetic drift and stochastic selection sweeps (Gillespie, 2000). MtDNA, having a lower *Ne*, a non-recombining nature and having all the genes neutrally linked is especially prone to this effect (Gillespie, 2000).

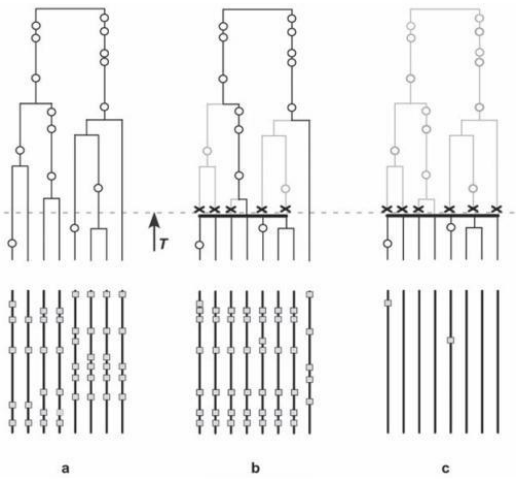


Fig.14 - Schematic representation of selective sweeps (dashed line) consequences on genealogies. (a) – No selective sweep; (b) – selective sweep with recombination ; (c) – selective sweep with no recombination. (From Dmitry Nurminsky, 2005).

Low Genetic Diversity or Mitochondrial Selective Sweep?

In the Iberia Peninsula recently there have been documented two potential cases of selective sweep. Studies using only mtDNA markers for *T. mauritanica* and *H. turcicus* had suggested a pattern of recent expansion from Maghreb to Iberian Peninsula given considerable low levels of nucleotide diversity in Iberian Peninsual compared to North Africa regions (Harris *et al*, 2004a; Harris *et al*, 2004b). Rato *et al* (2010, 2011) reviewed this assumption and found cito-nuclear discrepancies in Iberian Peninsula lineages. For both species, nucleotide diversities infer from nuDNA markers revealed significant higher values than mtDNA markers, suggesting a pattern of selection in the Iberian lineages of both species (Rato *et al*, 2010; Rato *et al*, 2011).

Given that mitochondria exercises critical functions for cellular metabolism, it is reasonable to think that an adaptative allele, could provide higher fitness (Dowling *et al* , 2008; Fan *et al*, 2011; Gershoni *et al*, 2009). The fact that mtDNA is neutral linked and doesn't experience recombination would increase the transmission of a particular favourable mtDNA lineage to the entire population, homogenizing genetic diversity in this locus, a selective sweep by host adaptation. In some case of introgressions, host adaptation had already being the explanation for high degrees of mtDNA invasion in close species reported. An example of this is hares in Iberian Peninsula, where contemporaneous populations present high levels of mtDNA of a Glacial European hare, probably because of massive introgression during glacial periods (Alves *et al*, 2008; Melo-Ferreira *et al*, 2005; Gaultier *et al,* 2009; Rheindt and Edwards, 2011).

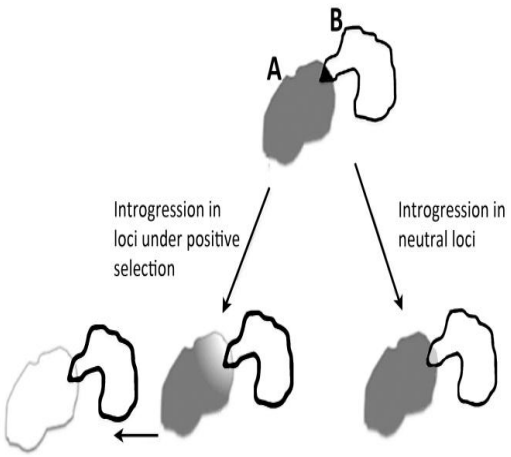


Fig.15 - Different degrees of gene invasion following an Introgression: (a) in loci under positive selection /"introgressive sweep"; (b) in an neutral loci. (From Rheindt and Edwards, 2011).

Can the snakes presenting low levels of mtDNA diversity have similarly experience a mitochondrial selective sweep and so the results obtained mislead the species evolutionary history?

Or, mtDNA indeed reveals a low genetic diversity reflective of the species genomes, in turn reflecting important demographic events such as past regional extinctions or severe bottlenecks with recent demographic expansions, unusual in the pylogeography context of Iberian Peninsula?

The isolated nature of Iberian snake mtDNA phylogeographic patterns within a region assumed to contain high genetic intraspecific differentiation and diversity makes it necessary to assess these patterns using nuclear markers.

The aim of this thesis is to contribute to a better understanding of the contrasting phylogeographic patterns of snakes in Iberia Peninsula. To attain this we develop a multilocus approach, to understand if the low genetic diversity in mtDNA of *Rhinechis scalaris, Malpolon monspessulanus* and *Hemorrhois hippocrepis* was the result of a potential selective sweep.

Several tasks were defined: i) realize the first robust phylogeography study in *Rhinechis scalaris*; ii) add a nuDNA dataset to *Malpolon monspessulanus* and *Hemorrhois hippocrepis* ; iii) compare mitochondrial and nuclear markers patterns in all species.

A large update in the sampling effort, representing all native distribution of *Rhinechis scalaris* and the use of both mitochondrial and nuclear markers will allow a much more clear understanding about the phylogeographic pattern of this specie.

The addition of nuclear markers in *Malpolon monspessulanus* and *Hemorrhois hippocrepis* will allow the confrontation with the previous studies, prompting an acceptance or rejection of the assumptions based on only mitochondrial markers.

A series of statistics analysing diversity and demography patterns in both mtDNA and nuDNA datasets will allow a more much more effective analysis about the evolutionary history of *Rhinechis scalaris, Malpolon monspessulanus and Hemorrhois hippocreps*.

Resolving the phylogeography history of these three species would signify a better understanding of the snakes patterns in Iberia Peninsula, preparing a solid knowledge base for future evolutionary studies regarding these taxa and patterns of phylogeographic concordance.

Materials and Methods

Study species

The ladder snake, *Rhinechis scalaris* Schinz, 1822

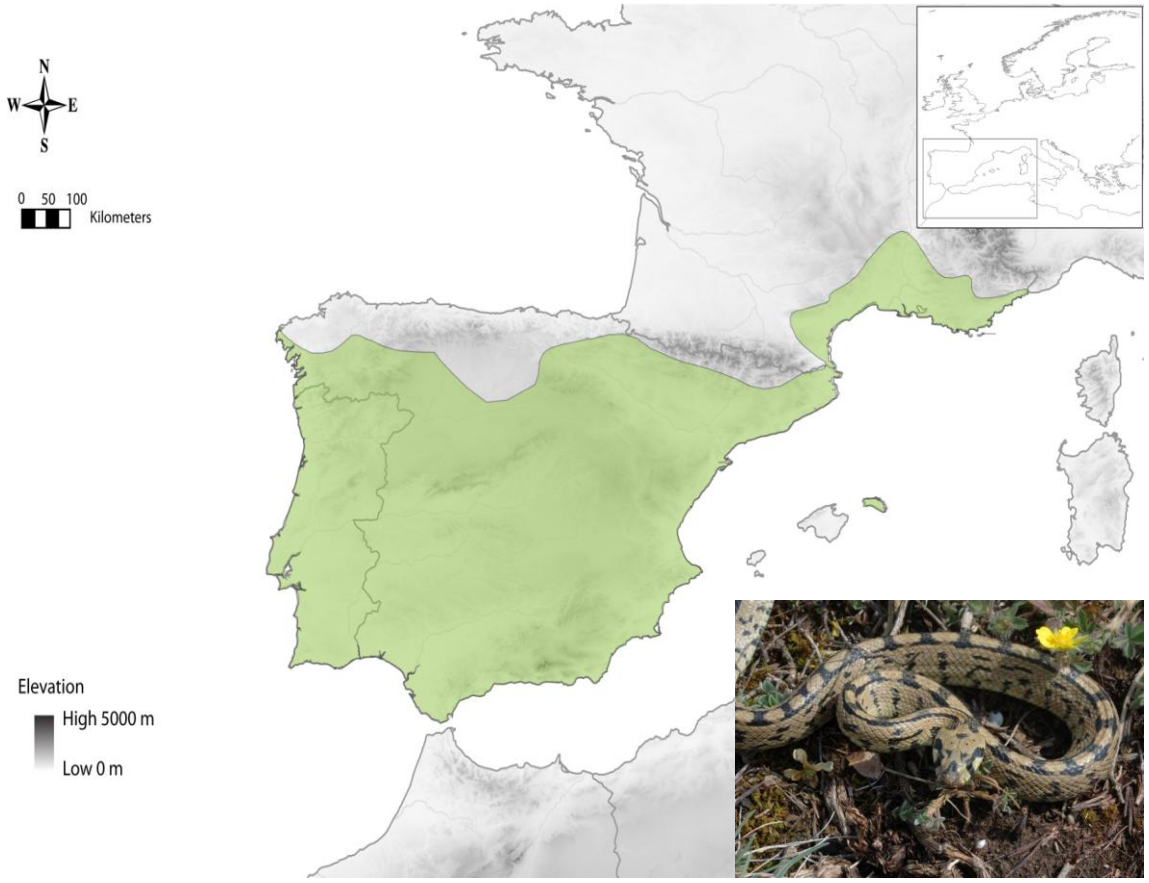


Fig.16 - *Rhinechis scalaris* distribution (drawn based on IUCN, 2012).

A relatively large and robust snake, *Rhinechis scalaris* measures typically from 23 to 150 cm, weighting from 11.2 to 1720 g (Ferrand *et al*, 2001 and Pleguezuelos, 2009). This ophidian is distributed across almost all the Iberia Peninsula, the Mediterranean coast of France, and in a very narrow margin of North-West Italy (fig.16) (based in IUCN, 2012). In Balearic islands this species is considered introduced at different epochs; in Menorca it is classified as an naturalised alien reflecting an introduction parallel to the first human colones, and in Mallorca, Ibiza and Formentera it is classified as a pure alien species, being very recently introduced, probably via the olive industry (Corti *et al*, 1999 and Pynia and Carretero, 2011).

This specie is very distinct from others Iberia snakes. It has a large and pointy head, well differentiated from the rest of the body and small eyes, with a circle pupil and brown iris (Ferrand *et al*, 2001 and Pleguezuelos, 2009). Its dorsal pattern presents two dark longitudinal lines above a brown to yellow pink coloration (Ferrand *et al*, 2001 and Pleguezuelos, 2009). In juveniles the longitudinal lines resembles ladders, which give the specie its common name ((Ferrand *et al*, 2001 and Pleguezuelos, 2009). This snake shows minor sexual dimorphism, with males having more large head and tails (Feriche *et al*, 1993; Ferrand *et al*, 2001 and Pleguezuelos, 2009).

The species occupies a variety of biotopes, from arid zones and open zones, to shrubs, woods or farm fields. Its altitudinal range goes from costal zones to 1500m, but 92% of the occurrence data are below 800m (Loureiro *et al*, 2008). *Rhinechis scalaris* is active during nocturnal hours and from April to October, although in some southern regions can be active all year (Ferrand *et al*, 2001 and Pleguezuelos, 2009).

Fig.17 - High detailed illustration of the head and frontal body of *Rhinechis scalaris*. (from Ferrand et al, 2001) Copyrigh Paulo Ferrand de Almeida.

Its diet is based on small mammals (like mices) and small reptiles (like lizards) having the most stenogafous diet among the Iberian snakes. Additionally, this snake has no ontogenic variation on feeding habits throughout its life (Pleguezuelos *et al*, 2007).

Males become sexual mature when they get to around 450 mm of snoutvent length (SVL) and females around 660 mm (Pleguezuelos and Feriche, 2006 and Pleguezuelos, 2009). This species has an aestival spermatogenesis; the process begins in May, and reaches a peak in July and August before decreasing until autumn (Pleguezuelos and Feriche, 2006 and Pleguezuelos, 2009). Males overwinter the sperm in the deferens ducts until the next spring. Females maximise the ovulation during spring until the end of July, so mating starts around March, with a period of about 20-24 days until 4-14 eggs are laid (Pleguezuelos and Feriche, 2006 and Pleguezuelos, 2009). The incubation is relatively long, around 65 days, with newborns appearing after October (Pleguezuelos and Feriche, 2006 and Pleguezuelos, 2009). This reproduction strategy is typical of snakes of European origin (Pleguezuelos and Feriche, 2006 ; Pleguezuelos, 2009 and Saint-Girons, 1982).

In terms of phylogeny, this specie was previously included in the genus *Elaphe*. Morphologically and ecologically it is different from old and new world ladder snakes, and genetic data confirmed a separate basal position of this species which is now included in the monotypic genus *Rhinechis* (Lenk *et al*, 2001; Nagy *et al*, 2004 and Uttiger *et al*, 2002). The origin of this genus is likely from Asia during Miocene (fossils data from this species only appear in Iberia Peninsula, predominantly along the Mediterranean coast, dating from the Pliocene (Luis *et al* 2009). A previous genetic study by Nulchis *et al* (2008) based on a portion of the Cytb gene showed no genetic variation among populations (fig10).

The horseshoe whip snake, *Hemorrhois hippocrepis* Linnaeus, 1758

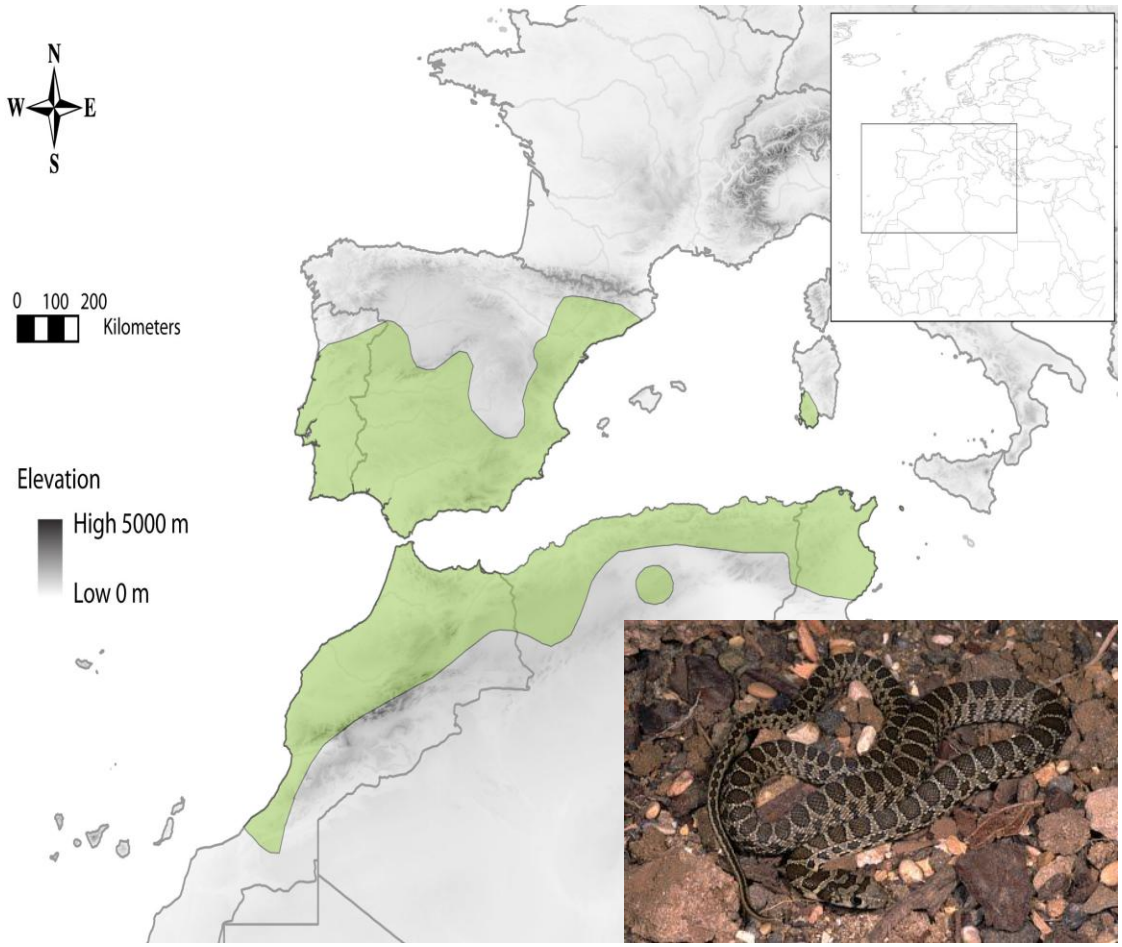


Fig.18 - *Hemorrhois hippocrepis* distribution (drawn based on IUCN, 2012).

A slender snake with a cylindrical shape, *Hemorrhois hippocrepis* can measure between 80 to 150 cm and weigh between 6 to 675 g (Ferrand *et al*, 2001 and Feriche, 2009). This colubrid snake is distributed in the Iberia Peninsula, limited by the Douro and Betic basins, in some Mediterranean islands (Pantelaria and Sardinia), and in Maghreb,

through Morocco, Algeria and Tunisia (fig.20) (based in IUCN, 2012). More recently it has been introduced in Baleares, probably via the olive industry (Corti *et al*, 1999; Pynia and Carretero, 2011).

Fig.19 - High detailed illustration of the head and frontal body of *Hemorrhois hippocrepis*. Copyrigh Paulo Ferrand de Almeida.

This snake has a large and short head well differentiated from the body, has big eyes with a circle pupil and brown or yellow iris and is the only Iberian snake with scales between the eyes and supralabial (Ferrand *et al*, 2001 and Feriche, 2009). It has a dorsal pattern with big circle-elliptical dark zones and white, yellow or grey basal coloration (Ferrand *et al*, 2001 and Feriche, 2009). Males have bigger heads than females, and juveniles have a more contrasted colour pattern (Feriche *et al*, 1993; Ferrand *et al*, 2001 and Feriche, 2009).

This snake is thermophilic specie. It is also very antropophilic, being frequently present in human constructions (Pleguezuelos and Moreno, 1990). It is well represented in rocky environments, frequent in the termo-mediterranean environments and scarce in meso- and supra-mediterranean habitats (Fhad an Pleguezuelos, 2001). It is a diurnal snake; active between March and November and in lower latitudes of its distribution can be active during all the year (Pleguezuelos and Fahad, 2005). It occurs from coastal zones to 1500m above sea level, being more frequent below 700m.

It is an agile and fast snake, and an active forager (Pleguezuelos and Moreno, 1990). It feeds on small mammals, reptiles and small birds (Pleguezuelos and Moreno, 1990). Its feed preferences change during development, only feeding in insects in early ages, and prey's size also undergoes modifications (Pleguezuelos and Moreno, 1990).

Males have a vernal spermatogenesis, starting in the end of March and with a maximum in July, the reproductive cycle finishes within a calendar year, similar to *Malpolon monspessulanus* (Feriche *et al*, 2009). The mating occurs in the end of May, and in June are deposited the eggs (3 to 10) (Feriche *et al*, 2009). The new borns appear from mid August to September. Some indications refer a minimum temperature of 22ºC for the spermatogenesis to occur (Ferrand *et al*, 2001 and Saint-Girons, 1982).

The intraspecific geographic variation in morphology, proteins and genetic characters is limited (Pleguezuelos and Fahad, 2001; Busack, 1986; Carranza *et al*, 2006). Like the rest of the snakes of the genus *Hemorrhois* the origin of this species is thought to be African as inferred by genetic data. The few fossil data available for the Iberian Peninsula date back to the Late Pleistocene supporting a regional extinction, and a recent migration in Iberia similar to *Malpolon monspessulanus* (Luis *et al* 2009; Carranza *et al* 2006).

The western Montpellier snake, *Malpolon monspessulanus,* Herman 1804

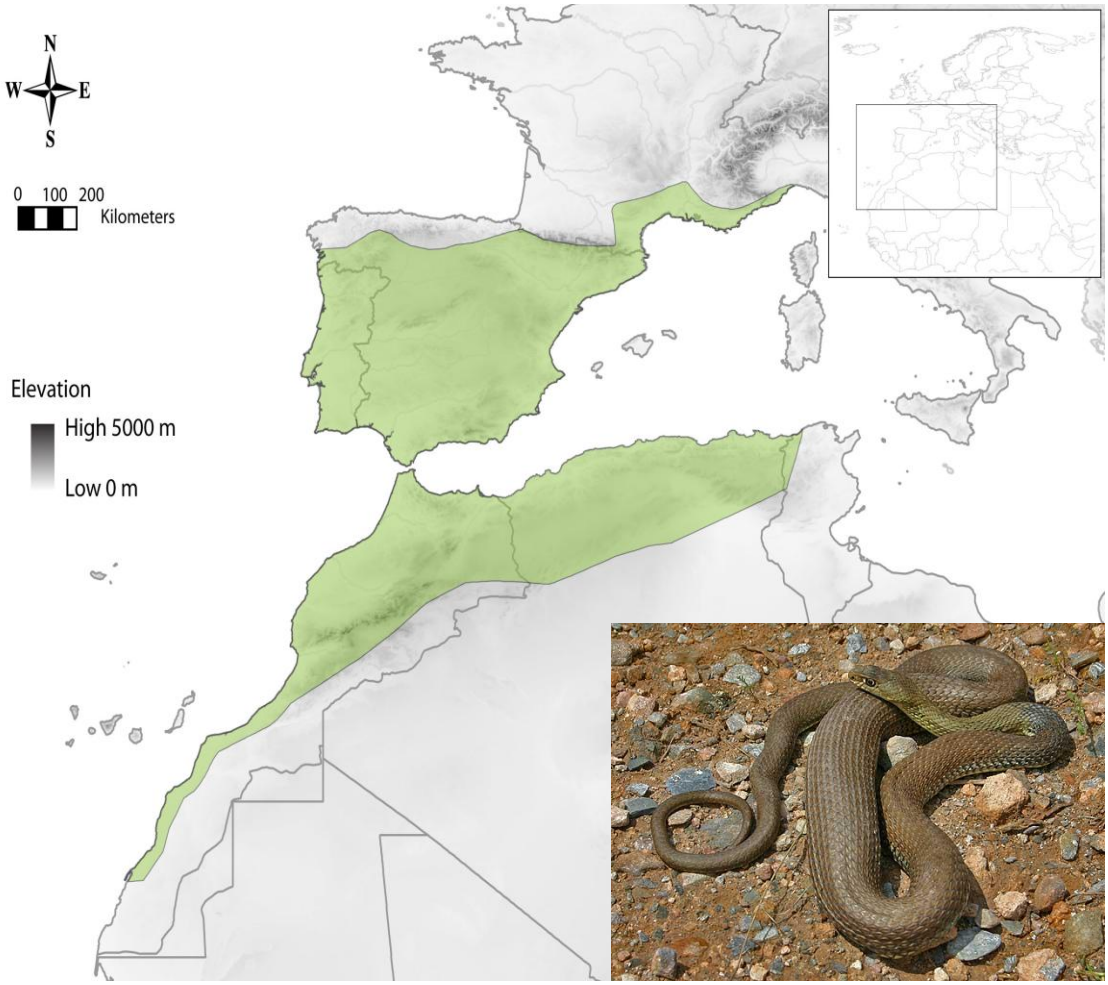


Fig.20 - *Malpolon monspessulanus* distribution (drawn based on IUCN, 2012).

This large ophidian reaches up to 2 m weighting between 60-1340 g (Ferrand *et al*, 2001 and Pleguezuelos, 2009). *Malpolon monspessulanus* is distributed In Iberia Peninsula (limited at North by the Douro and Ebro basins), the Mediterranean coast of France, in the Maghreb regions along the North of Morocco, Algeria and Tunisia and

recently has been introduced to the Baleares islands, probably via the olive Industry (fig.18) (Pleguezuelos 2009; Pynia and Carretero, 2011).

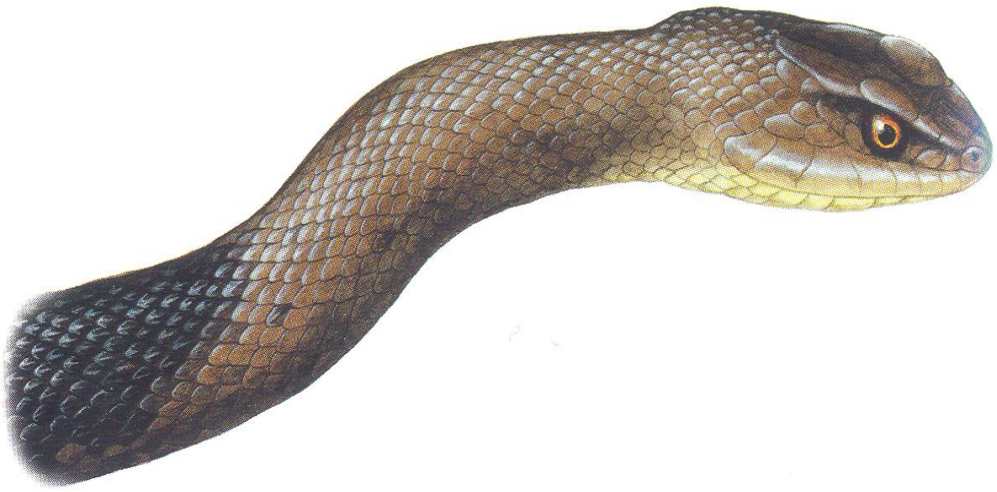


Fig.21 - High detailed illustration of the head and frontal body of *Malpolon monspessulanus* (from Ferrand et al, 2001). Copyrigh Paulo Ferrand de Almeida.

This species has a relatively straight and pointy head and a very long body (Ferrand *et al*, 2001 and Pleguezuelos, 2009). The dorsal coloration varies from olive green to brown or grey; the anterior part of the body has a prominent dark zone and the ventral region is yellow (Ferrand *et al*, 2001 and Pleguezuelos, 2009). Sexual dimorphism in this species is not evident (Feriche *et al*, 1993; Ferrand *et al*, 2001 and Pleguezuelos, 2009).

The preferable habitats are shrubs, rocky areas, agricultural fields or woods (Ferrand *et al*, 2001 and Pleguezuelos, 2009). In North Africa, it occurs in Mediterranean habitats along the Rif and Northern Algeria, as well as desert zones in the southern regions of its range (Fhad and Pleguezuelos, 2001). It occurs from coastal areas to 1500m above sea level (Loureiro *et al*, 2008). It is a diurnal specie normally active from March to November, although in some southern latitudes it can be active all year (Ferrand *et al*, 2001 and Pleguezuelos, 2009). It feeds almost exclusively on vertebrates, but is also euriphagic (Pleguezuelos, 2009). It's an opportunist snake, preying mostly on reptiles, from lizards (especially *Timon lepidus)* to ophidians (even cannibalism), micromammals (*Mus* sp.), and bird eggs (Ferrand *et al*, 2001 and Pleguezuelos, 2009).

It is one of the few Palaearctic colubrids with a vernal reproductive cycle, meaning that it can finish a reproductive cycle within one calendar year (Ferrand *et al*, 2001 and Pleguezuelos, 2009). Spermatogenesis begins in April and finishes in June, with mating realized in May and June, during which some fights occur between males (Ferrand *et al*, 2001 and Pleguezuelos, 2009). The eggs (3 to 11) are laid from 19 to 32 days after the last mating (Pleguezuelos, 2009). New borns appear from 20 August to 10 September (Ferrand *et al*, 2001 and Pleguezuelos, 2009).

It is the only member of the *Psammophinae* family in the Iberia Peninsula, and probably it has an African origin like all the members of this family (Carranza *et al*, 2006). There are no known differences in morphology, protein or genetic diversity between African and European populations of this species (Pleguezuelos and Fahad 1999; Busack, 1986; Carranza *et al,* 2006). Recently a new sub-species was identified in the West Sahara region, *Malpolon monspessulanus saharatlantiicus* based on the scale pattens (Geniez *et al*, 2006). A study by Carranza in 2006, revealed low mtDNA variation, and indicated the Maghreb as the point of differentiation of the genus *Malpolon* (Busack, 1986; Carranza *et al,* 2006).The large collection of fossil data in Iberia Peninsula from the Middle Pliocene; suggests an ancient migration during the Messian Salinity Crisis (Luis *et al,* 2009). A possible regional extinction in the Iberian Peninsula during Pleistocene climatic oscillations, followed by a relatively recent recolonization can explain the shallow differentiation obtained between populations from North and South of the Gibraltar Strait (Carranza *et al*, 2006).

Sampling

i) Rhinechis scalaris

A total of 71 *Rhinechis scalaris* samples were utilized in order to cover entirely the species native range, including areas not sampled by Nulchis *et al* (2008) (fig.10). Compared to this earlier study, the southern part of the Iberian Peninsula is sampled in much more detail, and Menorca Island and southern France are now represented. Specimen codes and geographic locations are detailed in fig: 22, and in table: I. The collection of these specimens was obtained from the CIBIO collection and donations by Dr. Juan M. Pleguezuelos (Universidad de Granada) and Dr. Marck Cheylan (Université Montpelier).

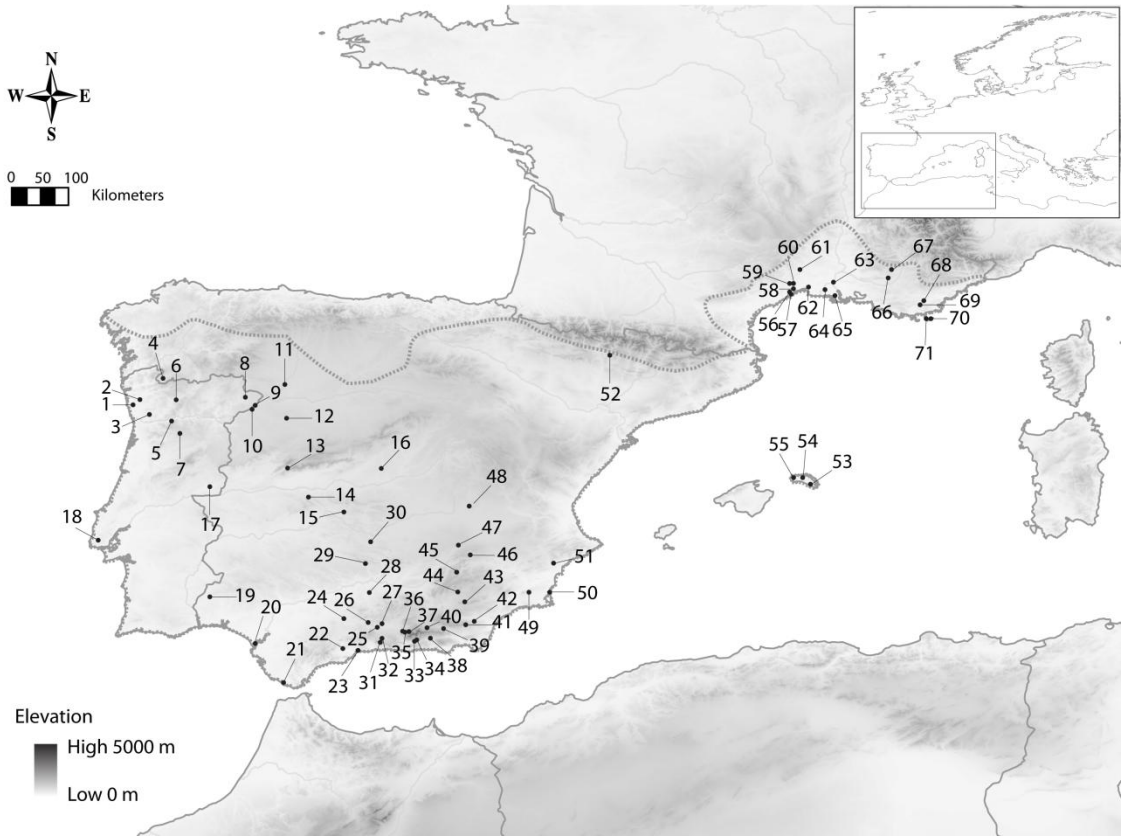


Fig.22 - Geographic localization of *R. scalaris* samples. The dashed line delimits the native range of the specie.

Tab.I (1/2) - Detailed geographic localization of *R. scalaris* samples.

Sample Code	Country	Province	Latitude (°)	Longitude (°)
1	Portugal	Porto	41.338529	-8.681594
2	Portugal	Braga	41.495438	-8.551617
3	Portugal	Porto	41.201433	-8.291497
4	Spain	Ourense	41.928922	-8.113447
5	Portugal	Viseu	41.497206	-6.541330
6	Portugal	Vila Real	41.52139	-7.807200
7	Portugal	Viseu	40.894999	-7.744972
8	Portugal	Bragança	41.497206	-6.541330
9	Spain	Zamora	41.366397	-6.317950
10	Spain	Zamora	41.301350	-6.414717
11	Spain	Zamora	41.811860	-5.778050
12	Spain	Salamanca	41.069327	-5.791439
13	Spain	Cáceres	40.183461	-5.800056
14	Spain	Cáceres	39.514380	-5.347110
15	Spain	Ciudad Real	38.963201	-4.278297
16	Spain	Toledo	40.094246	-4.121192
17	Portugal	Castelo Branco	39.530000	-7.580000
18	Portugal	Lisbon	38.794744	-9.388191
19	Spain	Huelva	37.611434	-7.245838
20	Spain	Sevilha	36.833667	-6.304317
21	Spain	Cadiz	36.087864	-5.766988
22	Spain	Malaga	36.719662	-4.419988
23	Spain	Malaga	36.769035	-4.706431
24	Spain	Cordova	37.561592	-4.655051
25	Spain	Granada	37.288092	-3.877537
26	Spain	Cordova	37.441996	-4.095028
27	Spain	Ourense	37.372020	-3.713825
28	Spain	Jaen	38.035915	-4.055874
29	Spain	Cidade Real	38.573535	-4.071240
30	Spain	Cidade Real	38.986091	-3.927277
31	Spain	Granada	36.937300	-3.857136
32	Spain	Granada	37.030575	-3.829727
33	Spain	Granada	36.949691	-3.325715
34	Spain	Granada	36.996358	-3.268261
35	Spain	Granada	37.116395	-3.485848
36	Spain	Granada	37.131889	-3.501236
37	Spain	Granada	37.127309	-3.432894
38	Spain	Granada	37.007838	-3.014837
39	Spain	Almeria	37.141137	-2.780107
40	Spain	Granada	37.171723	-3.036215

Tab.II (2/2) - Detailed geographic localization of *R. scalaris* samples.

Sample Code	Country	Province	Latitude (°)	Longitude (°)
41	Spain	Almeria	37.293833	-2.328083
42	Spain	Almeria	37.346029	-2.131905
43	Spain	Granada	37.713208	-2.332236
44	Spain	Granada	37.949533	-2.435315
45	Spain	Albacete	38.401651	-2.431216
46	Spain	Albacete	38.744487	-2.031631
47	Spain	Albacete	38.966987	-2.323036
48	Spain	Cuenca	39.567550	-1.920483
49	Spain	Murcia	37.977926	-1.128418
50	Spain	Alicante	37.950061	-0.783467
51	Spain	Alicante	38.478398	-0.639710
52	Spain	Huesca	42.380165	0.313615
53	Spain	Baleares - Menorca	39.927240	4.148270
54	Spain	Baleares - Menorca	40.006500	3.893710
55	Spain	Baleares - Menorca	40.008782	3.936223
56	France	Montpellier	43.487375	3.796054
57	France	Montpellier	43.495800	3.796000
58	France	Montpellier	43.567110	3.849910
59	France	Montpellier	43.648398	3.841094
60	France	Montpellier	43.644300	3.865400
61	France	Nîmes	43.863200	3.989210
62	France	Nîmes	43.562240	4.135820
63	France	Marselha	43.679399	4.609489
64	France	Marselha	43.590700	4.493860
65	France	Marselha	43.407600	4.726000
66	France	Digne-les-Bains	43.909900	5.715230
67	France	Digne-les-Bains	44.059500	5.940690
68	France	Toulon	43.358962	6.396189
69	France	Toulon	43.316605	6.296820
70	France	Toulon	43.004708	6.394525
71	France	Toulon	43.006796	6.394093

ii) *Hemorrhois hippocrepis*

The sampling of *H. hippocrepis* included 38 specimens from all the major geographic localities across its native range. Specimen codes and geographic localizations are reported in fig: 23 and tab: II. The collection of these specimens was obtained using the CIBIO collection.

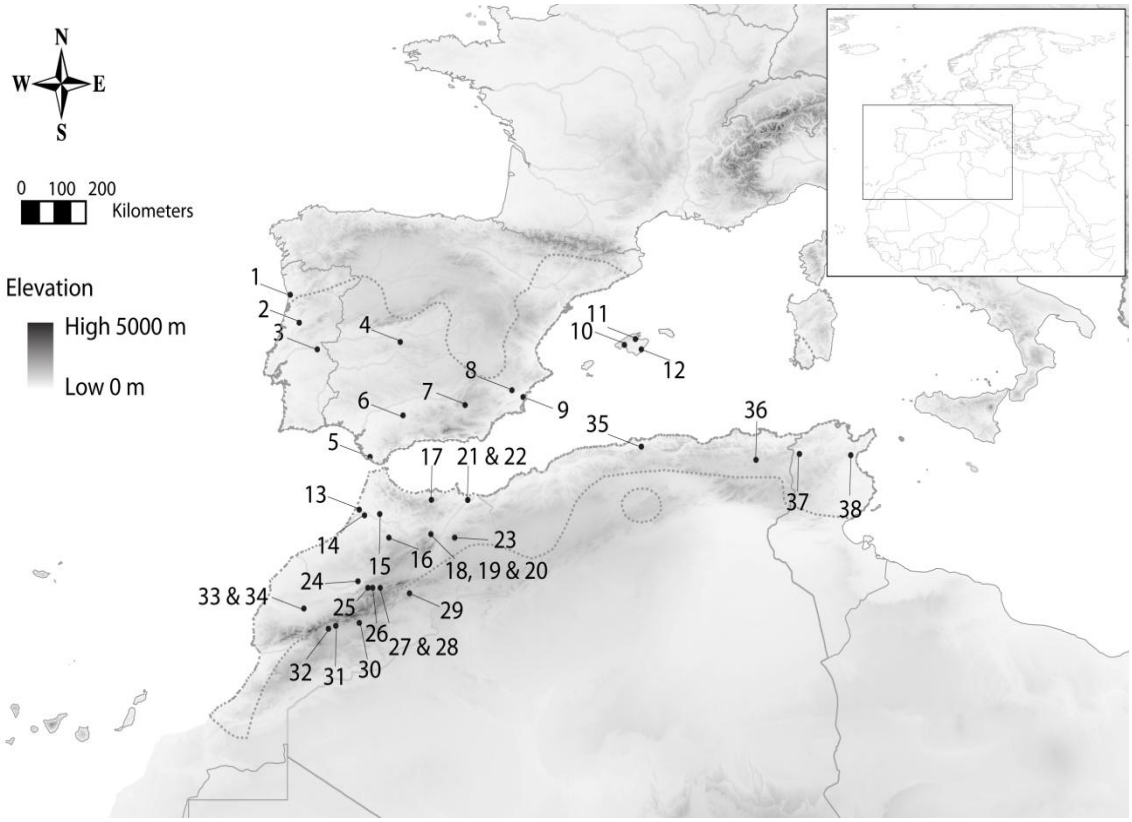


Fig.23 - Geographic localization of *H. hippocrepis* samples utilized in the study. The species native range is represented in dashed lines.

Tab.II - Detailed geographic localization of *H. hippocrepis* samples.

Sample Code	Country	Province	Latitude (°)	Longitude (°)
1	Portugal	Porto	41.147055	-8.579838
2	Portugal	Coimbra	40.194143	-8.388391
3	Portugal	Portalegre	39.432690	-7.578410
4	Spain	Toledo	39.557980	-4.653710
5	Spain	Cadiz	36.218460	-5.859560
6	Spain	Córdova	37.608358	-4.329111
7	Spain	Jaen	38.285690	-2.648500
8	Spain	Múrcia	38.264363	-1.189820
9	Spain	Alicante	38.143989	-0.780968
10	Spain	Baleares- Mallorca	39.692148	3.349073
11	Spain	Baleares. Mallorca	39.696406	3.433352
12	Spain	Baleares- Mallorca	39.699900	3.415300
13	Morroco	Gharb-Chrarda-Béni Hssen	34.872816	-6.294697
14	Morroco	Gharb-Chrarda-Béni Hssen	34.770667	-6.086583
15	Morroco	Gharb-Chrarda-Béni Hssen	34.795983	-5.559050
16	Morroco	Rabat - Salé - Zemmour – Zaer	34.047317	-5.325283
17	Morroco	Taza - Al Hoceima – Taounate	35.040917	-3.821600
18	Morroco	Taza - Al Hoceima – Taounate	34.130483	-4.029183
19	Morroco	Taza - Al Hoceima – Taounate	34.104283	-4.072483
20	Morroco	Taza - Al Hoceima – Taounate	34.128250	-4.033916
21	Morroco	Oriental	35.088750	-2.477483
22	Morroco	Oriental	35.080200	-2.491033
23	Morroco	Oriental	33.872466	-3.038783
24	Morroco	Tadla- Azilal	32.609980	-6.282078
25	Morroco	Tadla- Azilal	32.489416	-5.930766
26	Morroco	Tadla- Azilal	32.460533	-5.785433
27	Morroco	Meknès-Tafilalet	32.262088	-5.146791
28	Morroco	Meknès-Tafilalet	32.262088	-5.146791
29	Morroco	Meknès-Tafilalet	32.221513	-4.676640
30	Morroco	Souss-Massa-Drâa	31.116666	-6.400000
31	Morroco	Souss-Massa-Drâa	30.916517	-7.236367
32	Morroco	Souss-Massa-Drâa	30.808100	-7.583670
33	Morroco	Marrakech-Tensift-El Haouz	31.889432	-7.942285
34	Morroco	Marrakech-Tensift-El Haouz	31.889432	-7.942285
35	Algeria	Tizi Ouzou	36.545433	4.039650
36	Algeria	Batna	35.404933	6.394767
37	Tunisia	Jendouba	36.464600	8.740420
38	Tunisia	Béja	36.545900	9.413750

iii) Malpolon monspessulanus

In the case of *M. monspessulanus*, 25 samples were included, representing the major geographic regions of the species range (fig: 24). Detailed information is presented in table III. All these specimens were obtained from the CIBIO collection.

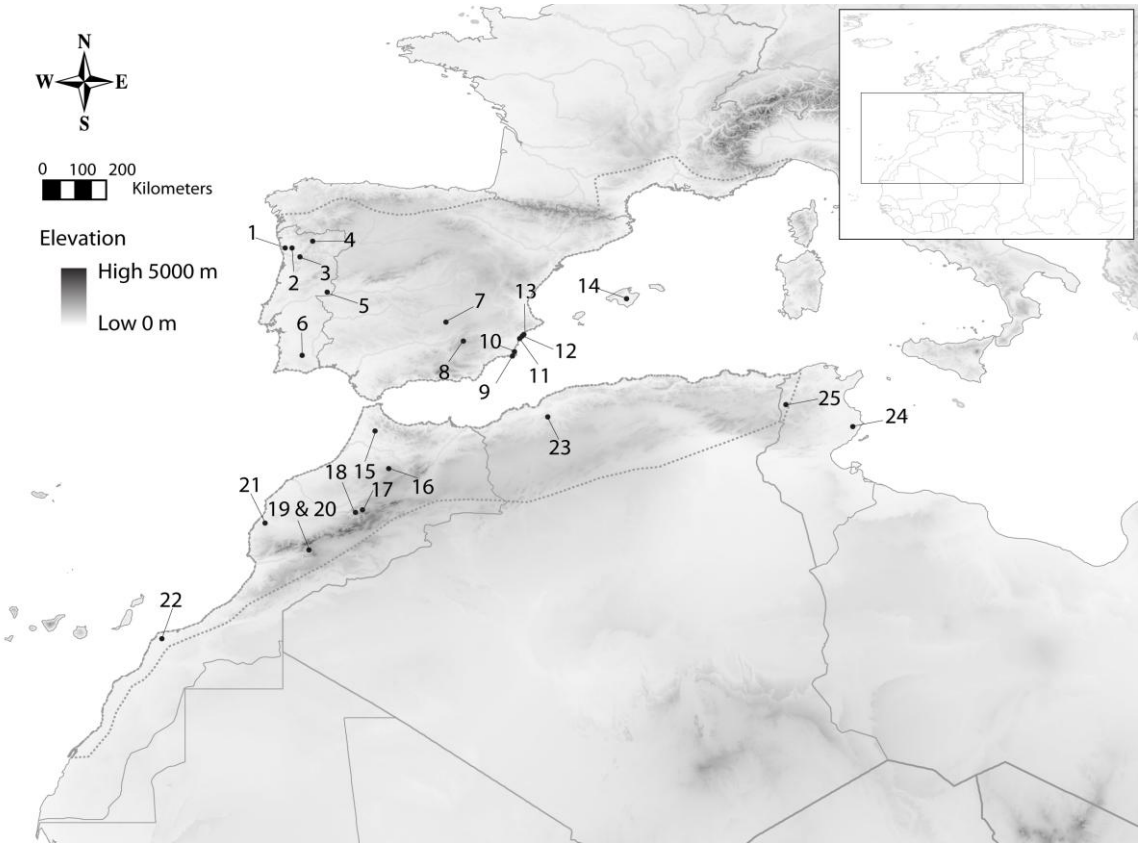


Fig.24 - Geographic localization of *M. monspessulanus* samples utilized in the study. The species native range is represented by the dashed lines.

Tab.III - Detailed geographic localization of *M. monspessulanus* samples.

Sample Code	Country	Province	Latitude (°)	Longitude (°)
1	Portugal	Porto	41.339030	-8.465549
2	Portugal	Braga	41.351670	-8.296386
3	Portugal	Viseu	40.978112	-8.014489
4	Portugal	Vila Real	41.492380	-7.621288
5	Portugal	Castelo Branco	39.814882	-6.998665
6	Portugal	Beja	37.589878	-7.957582
7	Spain	Ciudad Real	39.050140	-2.959360
8	Spain	Albacete	38.168340	-2.271834
9	Spain	Murcia	37.627485	-1.157081
10	Spain	Murcia	37.727773	-0.707503
11	Spain	Alicante	38.244080	-0.524851
12	Spain	Alicante	38.413130	-0.445547
13	Spain	Alicante	38.422699	-0.403104
14	Spain	Baleares – Mallorca	39.834422	3.101820
15	Morroco	Gharb-Chrarda-Béni Hssen	34.768370	-5.516183
16	Morroco	Meknès-Tafilalet	33.439070	-5.166500
17	Morroco	Tadla-Azilal	32.442140	-5.988647
18	Morroco	Tadla-Azilal	32.271330	-6.501583
19	Morroco	Souss-Massa-Drâa	30.799400	-7.527300
20	Morroco	Souss-Massa-Drâa	30.808100	-7.583670
21	Morroco	Marrakech-Tensift-El Haouz	31.539770	-9.504620
22	Morroco	Saara Ocidental	27.500870	-12.97305
23	Algeria	Tiaret	35.383330	1.350000
24	Tunisia	Jendouba	36.965970	9.035100
25	Tunisia	Mahdia	35.409300	10.95378

DNA extraction, amplification and sequencing

Following a standard saline method (Sambrock *et al*, 1989), total genomic DNA was extracted from snake's tail muscle preserved in alcohol 99%. This method starts with the addition of 600 µL of lysis buffer (0.5M tris, 0.1M EDTA, 2% SDS, pH 8.0) and 15 µL of proteinase K to small portion of tissue (previously cute into piecies of about 25 mg) and placed into 2 mL eppendorf tubes. With this step, is inducted tissue lysis which is maximized by incubation at 56ºC, overnight and with agitation. When the tissue is digested, the eppedorf tubes are placed in the freezer for 30 min. After, 300 µL of ammonium acetate are added, followed by centrifugation at 14,000 rotations per minute (rpm) for 15 minutes, at -4ºC. The resulting supernatant is transferred to a new 2 mL eppendorf tube and 600 µL of cold isopropanol are added, followed by a briefly agitation and overnight in the freezer. In the next step, tubes are centrifuge at 14,000 rpm for 30 min at -4ºC and the supernatant is discarded. Next, 1000 µL of cold ethanol are added to the pellet and the eppendorf tubes are centrifuged a last time at 14000 rpm for 15 minutes at -4ºC. Again, the supernatant is discarded, and all ethanol is allowed to evaporate for a few hours at room temperature. To finalize the process, 50 to 200 µL of ultrapure water or buffer are added, and the eppendorf tubes stay hydrating overnight.

From the total genomic DNA extracted, 4 gene regions were amplified: two mitochondrial gene fragments : NADH dehydrogenase subunit 4 (ND4-tRNA^{Leu}) and Cytochrome b (Cytb*);* and two nuclear gene fragments : Melanocortyin receptor 1 (MC1R) and Brain-derived neurotrophic factor (BDNF); For *R. scalaris* both mtDNA and nuDNA markers were used given that available mitochondrial data from Nulchis *et al* (2008) were not sufficient for depicting the phylogeographic pattern of the species. For *M. monspessulanus* and *H. hippocrepis* only nuDNA markers were used as an exhaustive mtDNA dataset was already analysed in Carranza *et al* (2006). See table: IV for more details.

Tab.IV - Detailed information of the genetic markers and primers used in this study. Rs – *R. scalaris*; Mm – *M. monspessulanus* and Hh – *H. hippocrepis.*

Gene	Primer	Primer sequence	Reference	Species
Cytb	GLuDG	5' TGA CTT GAA RAA CCA YCG TTG 3'	Palumbi *et al*. (1991)	Rs
	Cytb2	5' CCC TCA GAA TGA TAT TTG TCC TCA 3'		
ND4	ND4	5' CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC 3'	Arévalo *et al*. (1994)	Rs
	Leu	5' CAT TAC TTT TAC TTG GAA TTT GCA CCA 3'		
MC1R	MC1R F	5' GGCNGCCATYGTCAAGAACCGGAACC 3'	Pinho *et al*. (2009)	Rs, Mm & Hh
	MC1R R	5' CTC CGR AAG GCR TAG ATG ATG GGG TCC AC 3'		
BDNF	BDNF_DRV_F	5' ACC ATC CTT TTC CTK ACT ATG G 3'	Vieteis *et al.* (2007)	Rs, Mm & Hh
	BDNF_DRV_R	5' CTA TCT TCC CCT TTT AAT GGT C 3'		

The 4 target gene fragments were amplified by standard Polymerase Chain Reaction (PCR). PCR mixes for MC1R/BDNF were performed to 25 µL volumes, containing 2.5 µL of 10X PCR buffer (Invitrogen), 4 mM $MgCl_2$, 0.1 mM each dNTP, 0.2 mM each primer, 0.01% 1%W-1, 0.5 µL Boverine Serin Albumine (BSA), 1 U *taq* DNA polymerase (Invitrogen) and approximately 50 ng of DNA. For ND4/Cytb, PCR mixes were performed to 25 µL volumes, containing 2.5 µL of 10X PCR buffer (Invitrogen)/ 5 µL of 10X PCR buffer (GOTAQ), 4mM/ 2mM $MgCl_2$, 0.1mM/0.05mM each dNTP, 0.2 mM each primer, 0.01% 1%W-1 and 0.5 µL BSA (ND4), 1 U *taq* DNA polymerase (Invitrogen - ND4) / 1 U *taq* DNA polymerase (GOTAQ - Cytb) and approximately 50 ng of DNA.

PCR cycling for all genes was initiated with a pre-denaturing step of 3 min at 94ºC, 35 cycles of denaturing step of 30 s at 94ºC, annealing step of 45 s at 50ºC (30 s in MC1R case) and a extension step at 72ºC of 45 s or 1 m or 2 m for mitochondrial genes, MC1R, and BDNF respectively, followed by a final extension step of 10 or 5 min at 72ºC for mitochondrial genes and BDNF, and MC1R, respectively.

PCR results were examined in 2% agarose gel, with the ladder M5. The sequencing of the PCR products was realized by an external service (Macrogen) with the same primers used for amplification.

Genetic Analysis

Sequences were examined manually and aligned using the Clustal W (Larkin *et al*, 2007) application in BioEdit7.0.9.0 (Hall, 1999). In the case of nuclear fragments, the aligments were phased using DNAsp5 (Librado and Rozas, 2009). Only mitochondrial markers were concatenated, the nuclear markers were analysed individually, corresponding to a total of three datasets for *R. scalaris* (“mtDNA”, “MC1R” and” BDNF”), and two datasets for *M. monspessulnus* and *H. hippocrepis* (“MC1R” and “BDNF”). Variable and conserved sites for each dataset were calculated using Mega5 (Tamura *et al*, 2011).

For each dataset, phylogenetic relationships were inferred using statistical parsimony haplotype networks (Templeton *et al*, 1992) implemented in the program TCS 1.2.1 (Clement *et al* 2000). This method can be highly informative in patterns of low genetic diversity. For each haplotype network a map with the sampled specimens localization and correspondent haplotype composition was produced.

Genetic diversity estimates were carried out with the software DNAsp5 (Librado and Rozas, 2009). In the case of *M. monspessulanus* and *H. hippocrepis* the two groups established by Carranza *et al* 2006 were maintained: ”Iberia Peninsula” and “Maghreb”; Haplotype diversity (*H*d*)*, the probability of two randomly haplotypes beign different in the dataset, and Nucleotide diversity (π), the average number of differences per site between two samples in each dataset, were calculated to estimate haplotipic and gene diversity.

Demographic history was accessed using three classes of statistics: I – inference from mutation frequency; II – inference from haplotype distribution; III – inference from the distribution of the pairwise sequence differences; Within Class I we realised the Tajima’s D test (Tajima, 1989) and R_2 test (Ramons-Onsins and Rozas, 2002). Tajima’s D test is based on the differences between the number of segregating sites and the average number of nucleotide differences, if the population follows neutrality, the result should be $D = 0$; in scenarios that don’t follow neutrality the result can be $D > 0$, suggesting either a recent population expansion or some form of balancing selection or $D < 0$ suggesting either population expansion or purifying selection. R_2 test is based on the difference between the number of singletons mutations and the average number of nucleotide differences, lower values of R_2 are expected under a demographic expansion. This is a very conservative test and especially powerfull in cases of small cample sizes.

Significance of both tests was assessed by performing 10,000 coalescent simulations in DNAsp5 (Librado and Rozas, 2009).

Within Class II we realised the Fu's F_s test (Fu, 1997) that verifies neutrality based on the haplotype frequency distribution conditional the value of Θ. In scenarios that don't follow neutrality the result can be $F_s > 0$, expected from population bottleneck or dominant selection, or $F_s < 0$ suggesting population expansion or genetic hitchhiking. Significance for this test was accessed by performing 10.000 coalescent simulations in DNAsp5 (Librado and Rozas, 2009).

Within Class III, mismatch distributions were used for each dataset, with the models of Rogers and Harpending (1992) in ARLEQUIN (version 3.11; Excoffier *et al*, 2005). The pairwise differences between sequences are expected to generate different distributions depending on the demographic history: unimodal – recent growth; bimodal – stable (Rogers and Harpending, 1992). The observed distributions are compared with simulated distributions (representing models of sudden expansion), and the fit of these two distributions is accessed by the Harpending's raggedness index statistic (Hr), a measure of the smoothness of the two distributions, and the Sum of Square Deviations (SSD) that represent the probability of the simulated distribution being larger or equal to the observed one.

Results

Sequence diversity

i) *Rhinechis scalaris*

Three datasets were generated: MtDNA (Cytb + ND4), MC1R and BDNF. The MtDNA dataset resulted in 60 sequences of 1046 bp (353 bp from Cytb and 693 bp from ND4), consisting of 1028 constant nucleotides (98% of the total set) and 18 variable nucleotides (2% of the total set). The MC1R dataset was obtained from 66 sequences of 559 bp, among which 556 nucleotides were constant (99.5% of the total set) and 3 nucleotides were variable (0.5% of the total set).The BDNF dataset consisted in 80 sequences of 638 bp, presenting 637 constant nucleotides (99.8% of the total set) and 1 variable nucleotide (0.2% of the total set).

The MtDNA and nuclear (MC1R and BDNF) datasets presented similar levels of low haplotype diversity, with the MC1R dataset showing the lowest values. All the three datasets presented low levels of nucleotide diversity (tab.V).

Tab.V – Haplotype and nucleotide diversity statistics for MtDNA, MC1R and BDNF datasets of *Rhinechis scalars.*

Rhinechis scalaris			
Marker	MtDNA	MC1R	BDNF
Haplotype diversity	0.48757±0.0807	0.17300±0.0619	0.46804±0.0307
Nucleotide Diversity	0.00066±0.00056	0.00032±0.000468	0.00073±0.00072

ii) Hemorrhois hippocrepis

Two nuDNA datasets were built: MC1R and BDNF. The MC1R dataset consisted in 46 sequences of 574 bp, resulting in 569 constant nucleotides (99.2% of total set) and 5 variable nucleotides (0.8% of total set). The BDNF dataset presented 60 sequences of 563 bp, among which 559 nucleotides were constant (99.3% of total set) and 4 nucleotides were variable (0.7% of total set).

The MC1R dataset presented no variation in the Iberian Peninsula, and low haplotype and nucleotide diversity in Maghreb. The BDNF dataset presented similar values between the two regions (details in tab.VI).

Tab.VI – Haplotype and nucleotide diversity statistics for mtDNA, MC1R and BDNF datasets of Iberian and Maghreb *Hemorrhois hippocrepis* populations. * - MtDNA data from Carranza *et al* (2006).

Hemorrhois hippocrepis			
Iberian Peninsula			
Marker	MtDNA*	MC1R	BDNF
Haplotype diversity	0.617 ±0.0.076	0	0.54113 ± 0.094
Nucleotide diversity	0.00514 ± 0.0054	0	0.00111 ± 0.001014
Maghreb			
Marker	MtDNA*	MC1R	BDNF
Haplotype diversity	0.815 ±0.070	0.20635 ± 0.1	0.48506 ± 0.094
Nucleotide diversity	0.00875± 0.0054	0.00073± 0.00076	0.00132 ± 0.001108

iii) Malpolon monspessulanus

Two nuDNA datasets were obtained: MC1R and BDNF. The MC1R dataset combined a total of 34 sequences of 614 bp, among which 610 nucleotides were constant (99.4% of the total set) and 3 nucleotides were variable (0.6% of the total set). The BDNF dataset resulted in 32 sequences of 536 bp, consisting of 532 constant nucleotides (99.3% of total set) and 4 variable nucleotides (0.7% of total set).

Both datasets presented higher haplotype and nucleotide diversity in Maghreb than in Iberian populations, with the MC1R dataset showing no variation in the Iberia Peninsula (details in tab.VII).

Tab.VII – Haplotype and nucleotide diversity statistics for mtDNA, MC1R and BDNF datasets of Iberian and Maghreb *Malpolon monspessulanus* populations. * - MtDNA data from Carranza *et al* (2006).

Malpolon monspessulanus			
Iberian Peninsula			
Marker	MtDNA*	MC1R	BDNF
Haplotype diversity	0.269 ±0.109	0	0.36601 ± 0.1124
Nucleotide diversity	0.00095 ± 0.0011	0	0.00068 ± 0.000769
Maghreb			
Marker	MtDNA*	MC1R	BDNF
Haplotype diversity	0.727 ±0.144	0.575 ± 0.1150	0.78022 ± 0.0846
Nucleotide diversity	0.00630± 0.0043	0.00225± 0.001637	0.00207 ± 0.001613

Phylogeographic patterns

i) Rhinechis scalaris

The MtDNA dataset resulted in 15 haplotypes diverging by a maximum of 6 mutational steps. 72% of the sequences (43) represent the most common haplotype. Twelve haplotypes were represented by a single sequence and the remaining two haplotypes by 3 or 2, sequence. The haplotype network and geographic distribution of haplotypes are shown in fig.25.

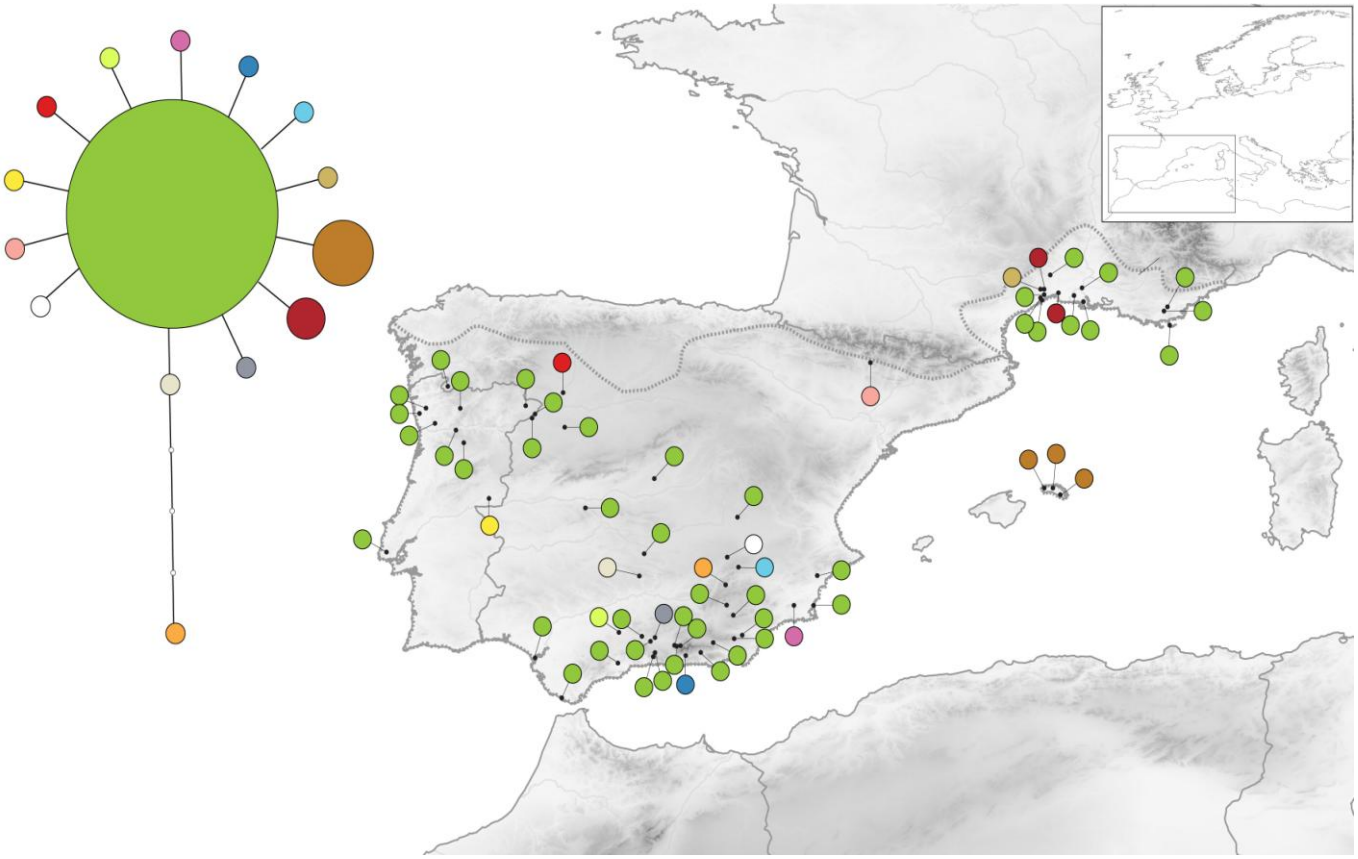


Fig.25 - Statistical parsimony network based on mitochondrial haplotypes (ND4 and Cytb combined) observed in 60 samples of *Rhinechis scalaris*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; open circle represent missing haplotypes, circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

The MC1R dataset consisted in 4 haplotyes diverging by a maximum of 2 mutational steps, 91% of the sequences (60) represent the most common haplotype.The remaining haplotypes were represented by, 3, 2 and 1 sequences. The haplotype network and geographic structure can be seen in detail in fig.26.

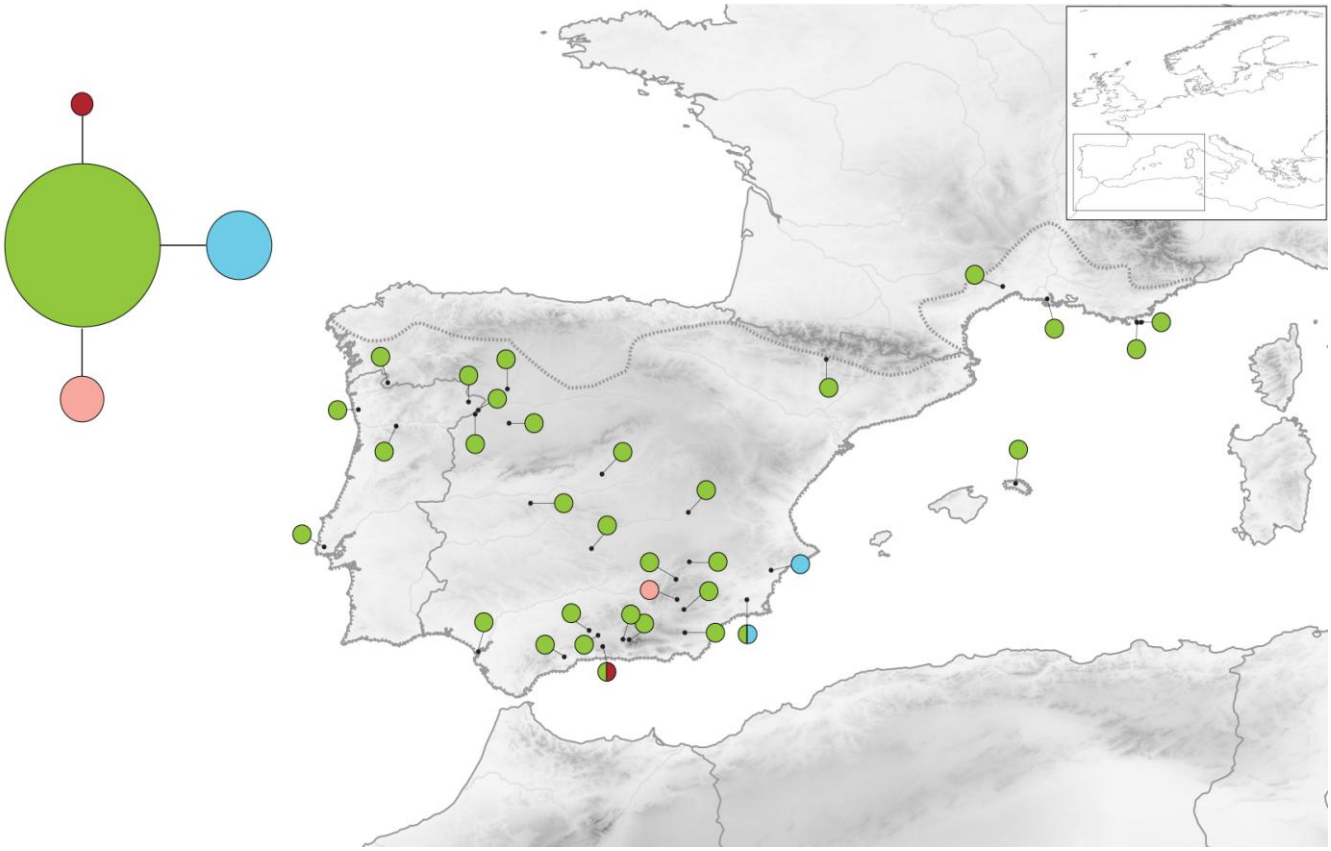


Fig.26 - Statistical parsimony network based on MC1R haplotypes, observed in 33 samples of *Rhinechis scalaris*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

The BDNF dataset combined 2 haplotypes, diverging by a maximum of 1 mutational step, 64% of the sequences represented the most common haplotype. The haplotype network and geographic structure can be seen in detail in fig.27.

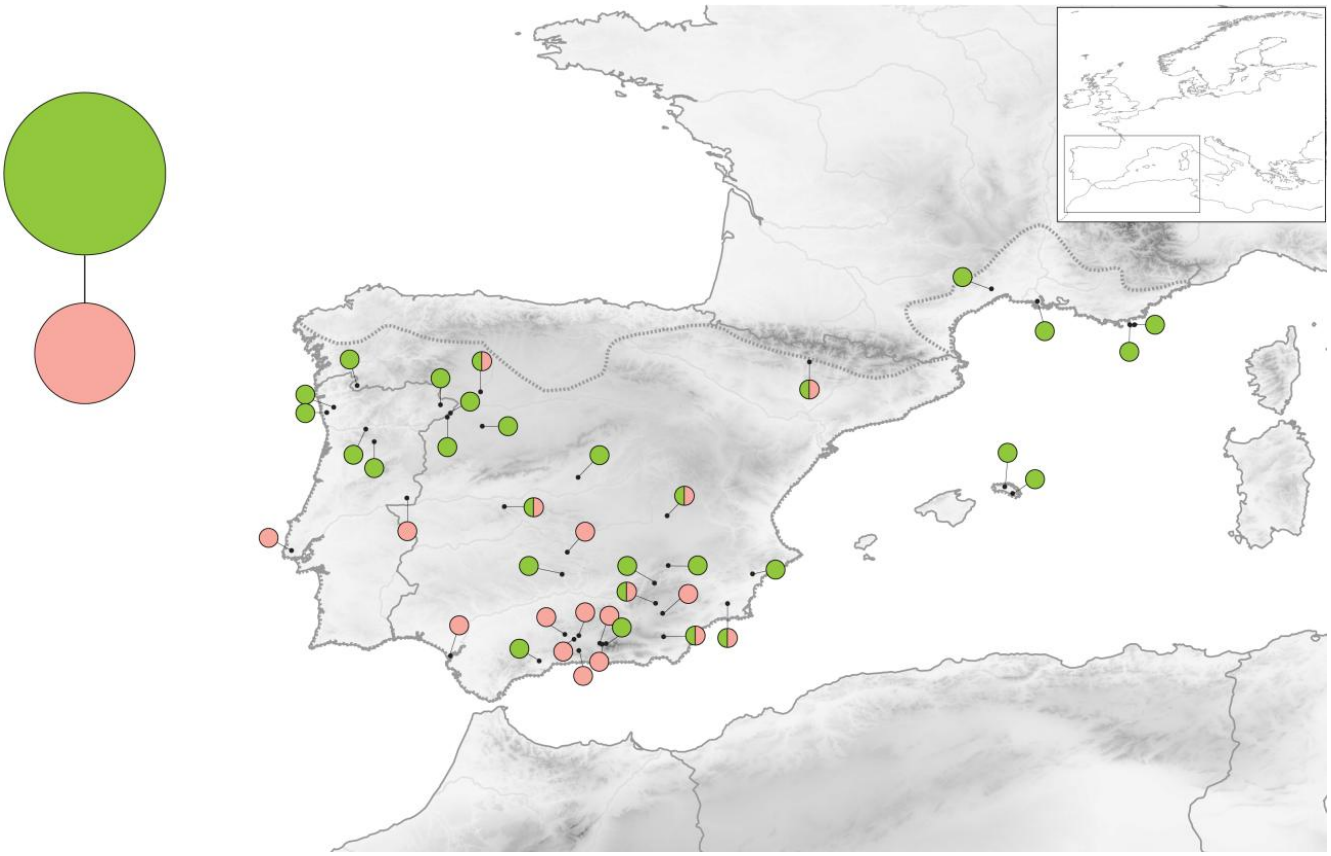


Fig.27 - Statistical parsimony network based on BDNF haplotypes observed in 40 samples of *Rhinechis scalaris*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

Tab.VIII – Characterization of haplotype composition in *Rhinechis scalaris* for mtDNA, MC1R and BDNF datasets.

Rhinechis scalaris			
Marker	MtDNA	MC1R	BDNF
Number of samples	60	33	40
Number of haplotypes	15	4	2
Max. Number of differences between haplotypes	6	2	1
% (sequences) of the most common haplotype	72	91	64

ii) *Hemorrhois hippocrepis*

The MC1R dataset resulted in 5 haplotypes, diverging by a maximum of 4 mutational steps. 89.1 % of the sequences (41) represented the most common haplotype. Three haplotypes were represented by a single sequence, and the remaining by 2 sequences. The haplotype network and geographic structure can be seen in more detail in fig.28.

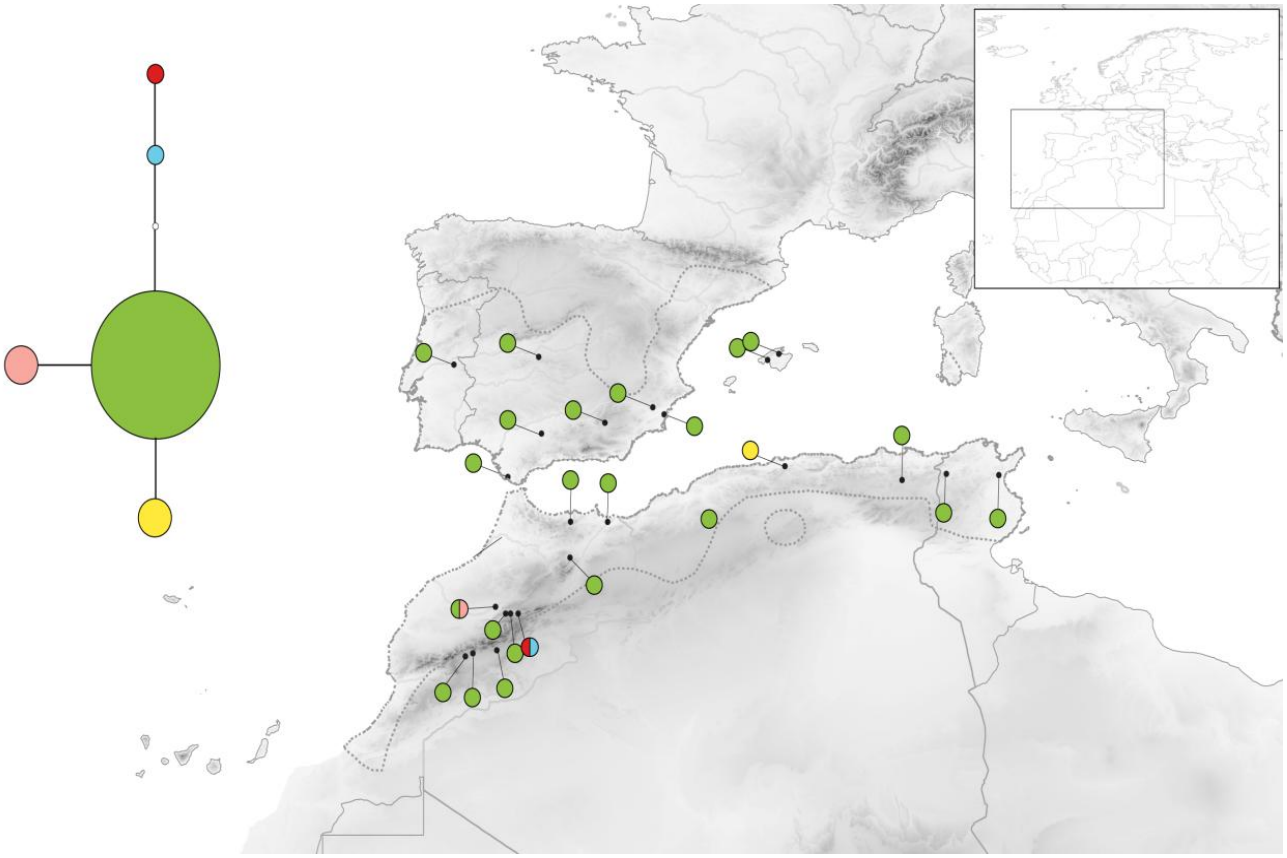


Fig. 28 - Statistical parsimony network based on MC1R haplotypes observed in 23 smaples of *Hemorrhois hippocrepis*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; open circle represent missing haplotypes, circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

The BDNF dataset resulted in 6 haplotypes, diverging by a maximum of 3 mutational steps. 68.3% of the sequences (35) represent the most common haplotype. The remaining haplotypes were represented by 18, 3, 2, 1 and 1 sequences. The haplotype network and geographic structure can be seen in more detail in fig.29.

MC1R revealed a singular haplotype for Iberian Peninsula populations, shared with Magreb population, in contrast in the later population presented 3 exclusive hapotypes. In BDNF case, the number of shared and exclusive haplotypes was equal in both populations, and similar in terms of divergence between haplotypes (tab.IX).

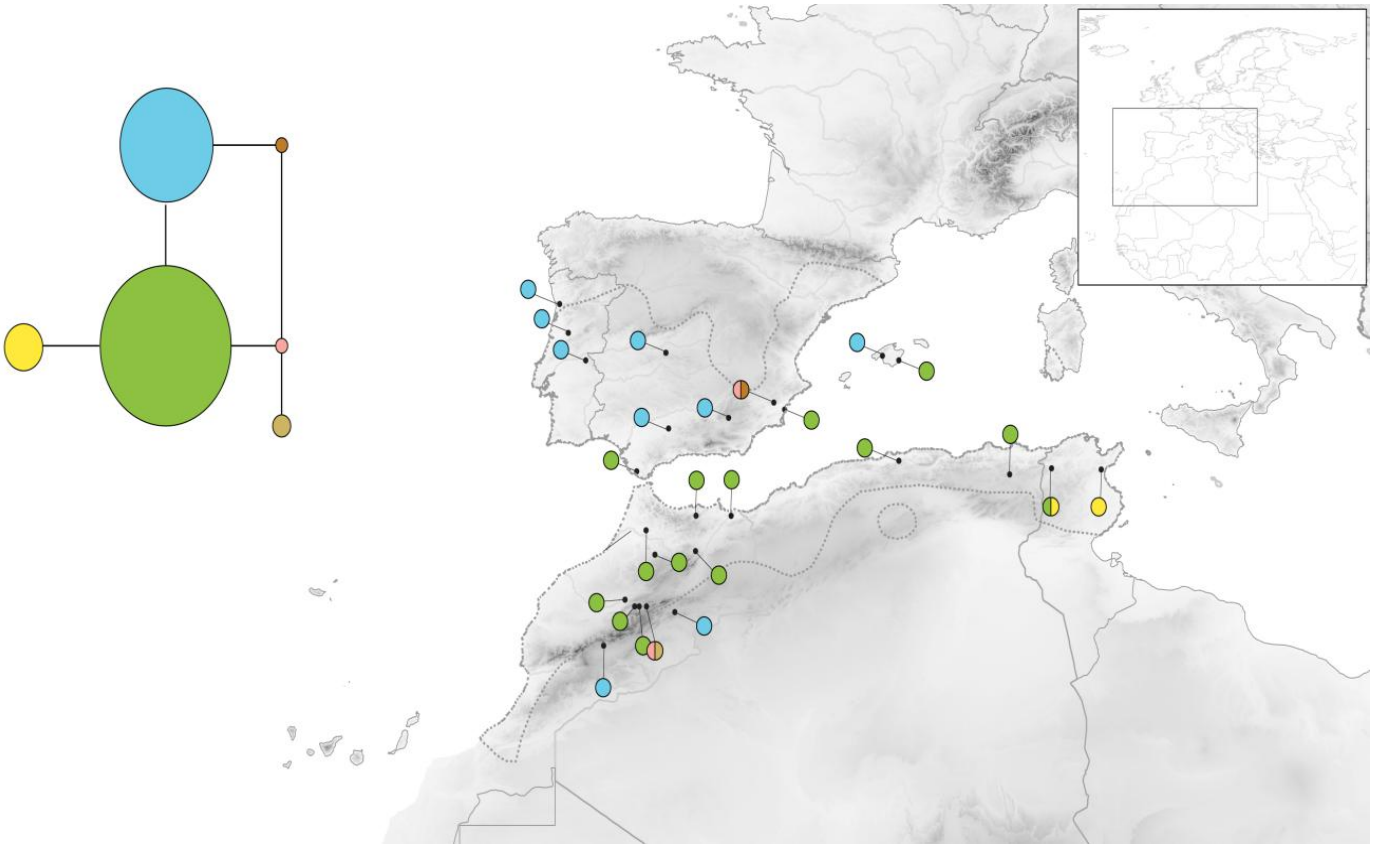


Fig.29 - Statistical parsimony network based on BDNF haplotypes observed in 30 samples of *Hemorrhois hippocrepis*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

Tab.IX – Characterization of haplotype composition in *Hemorrhois hippocrepis* from the MC1R and BDNF datasets in Iberia Peninsula and Maghreb. * - mtDNA Data from Carranza *et al* (2006).

Hemorrhois hippocrepis			
Iberian Peninsula			
Marker	MtDNA*	MC1R	BDNF
Number of samples	17	12	12
Haplotypes shared between both areas	1	1	2
Haplotypes exclusive to region	2	0	2
Max. Number of differences between haplotypes	3	0	3
Maghreb			
Marker	MTDNA*	MC1R	BDNF
Number of samples	20	26	26
Haplotypes shared between both areas	1	1	2
Haplotypes exclusive to region	7	3	2
Max. Number of differences between haplotypes	12	4	4

iii) *Malpolon monspessulanus*

The MC1R dataset resulted in 4 haplotypes, diverging by a maximum of 3 mutational steps. 82% of the sequences (28) represent the most common haplotype. Two haplotypes were represented by a single sequence and the remaining haplotype by 4 sequences. The haplotype network and geographic structure can be seen in detail in fig.30.

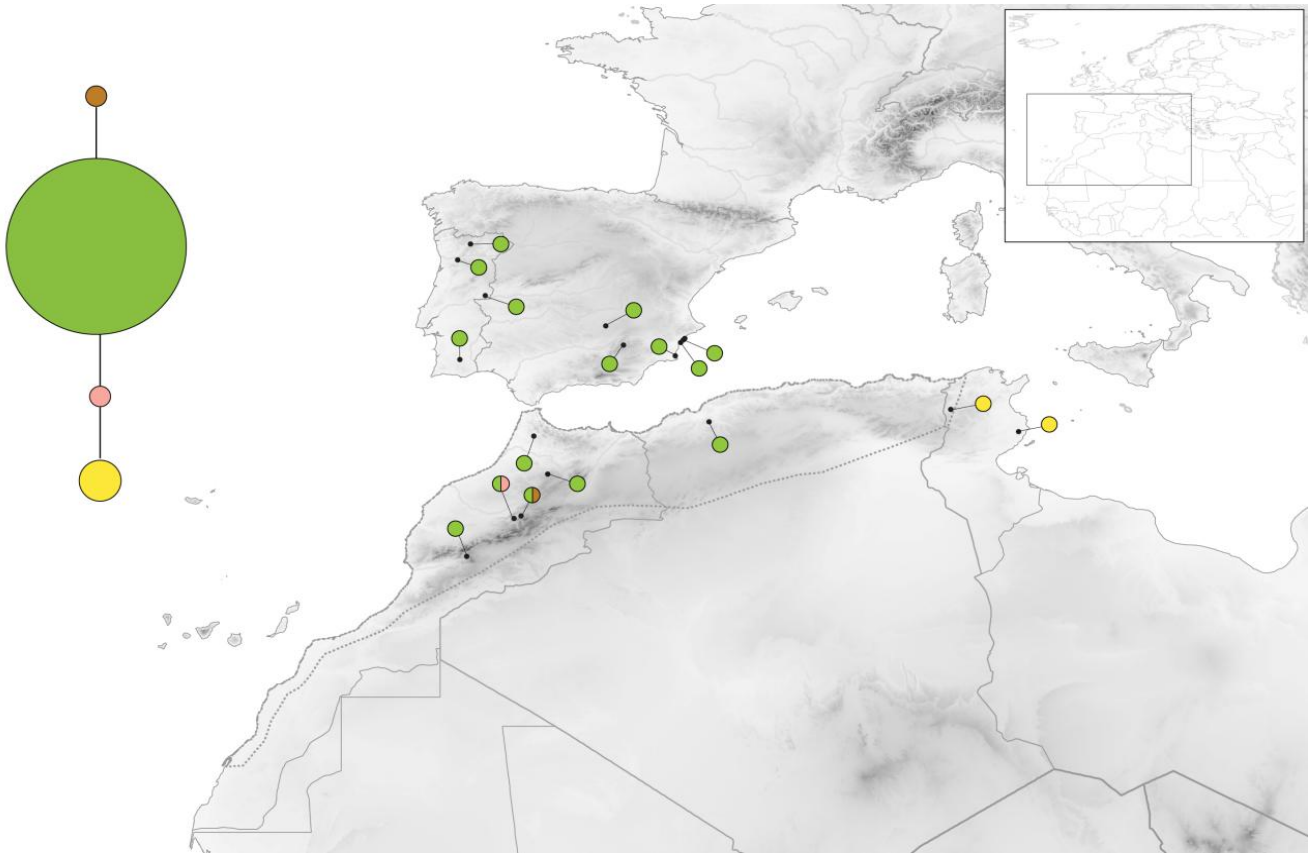


Fig.30 - Statistical parsimony network based on MC1R haplotypes, observed in 17 samples of *Malpolon monspessulanus*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

The BDNF dataset resulted in 5 haplotypes, diverging by a maximum of 4 mutational steps. 53% of the sequences (17) represent the most common haplotype. The remaining 4 haplotypes were represented by, 10, 2, 2 and 1 sequence. The haplotype network and geographic structure can be seen in detail in fig.31.

For both nuDNA markers, all haplotypes in Iberian Peninsula were shared with Maghreb populations, in contrast, Maghreb populations presented exclusive haplotypes for both markers. Distances between haplotypes were always higher in Maghreb populations, and in the MC1R dataset, only one haplotype was detected in Iberian populations (tab.X).

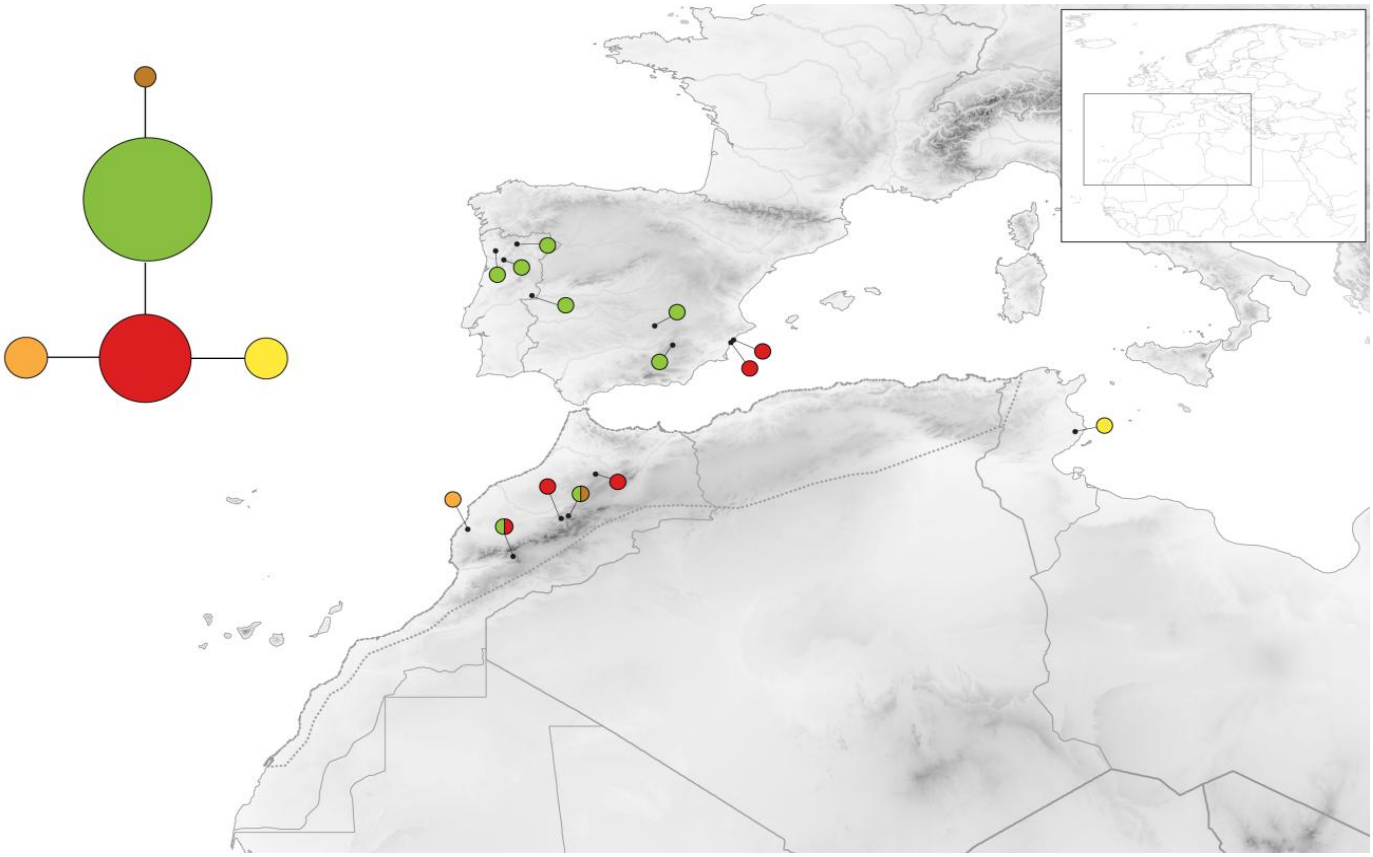


Fig. 31 - Statistical parsimony network based on BDNF haplotypes observed in 16 samples of *Malpolon monspessulanus*. The geographical distribution of haplotypes is shown in the map. Each colour represents a distinct haplotype; circles sizes are proportional to the haplotype frequency; connections between haplotypes represent a single mutational step.

Tab.X – Characterization of haplotype composition in *Malpolon monspessulanus* from the MC1R and BDNF datasets in Iberia Peninsula and Maghreb. * - mtDNA Data from Carranza *et al* (2006).

Malpolon monspessulanus			
Iberian Peninsula			
Marker	MtDNA*	MC1R	BDNF
Number of samples	28	14	14
Haplotypes shared between both areas	1	1	2
Haplotypes exclusive to region	4	0	0
Max. Number of differences between haplotypes	2	0	1
Maghreb			
Marker	MtDNA*	MC1R	BDNF
Number of samples	11	11	11
Haplotypes shared between both areas	1	1	2
Haplotypes exclusive to region	5	3	3
Max. Number of differences between haplotypes	7	3	3

Demographic inferences

i) Rhinechis scalaris

In the mismatch distribution realised, all datasets revealed a good fitting betweem observed and simulated distributions under models of sudden expansion, in MtDNA and MC1R supported statistically by non-significant values of SSD and Hr (fig.32) (tab.XI). Regarding Tajima's D and Fu's F_s, those statistics were significantly negative in MtDNA and MC1R datasets and positive and not significant in BDNF (tab.XI). R_2 statistic was small and significant in MtDNA dataset, small and not significant in MC1R and large and not significant in BDNF dataset (tab.XI).

Tab. XI – Resume of demography inference statistics (Tajima's D ; Fu's F_s and R_2) and mismatch distribution parameters (Tau; Theta0; Theta1; SSD , and Hr index) obtained for *Rhinechis scalaris* in mtDNA, MC1R and BDNF datasets.

Rhinechis scalaris			
	MtDNA	MC1R	BDNF
Tajima's D	-2.45672	-1.3953	1.58371
p value	<0.001	<0.05	Ns
Fu's F_s	-16.81047	-3.10134	2.04974
p value	<0.001	<0.01	Ns
R_2	0.0368	0.0476	0.234
p value	< 0.05	Ns	Ns
Tau	0.648	3	0.701
Theta0	0	0	0
Theta1	99999	0.219	99999
SSD	0.00576	0.000823	0.00576
p value	Ns	Ns	<0.05
Hr index	0.12492	0.46105	0.22314
p value	Ns	Ns	<0.05

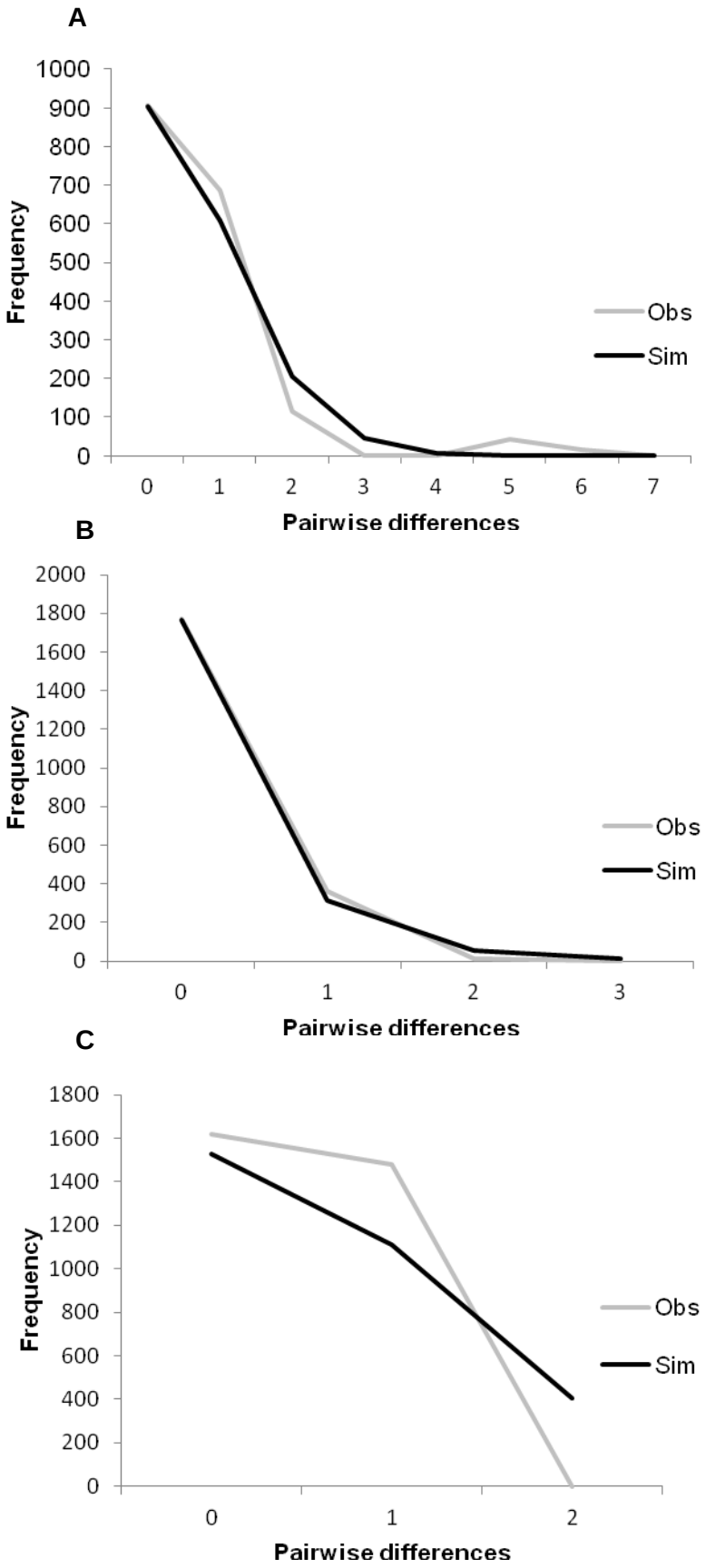


Fig.32 - Mismatch distribution of *Rhinechis scalaris* considering mtDNA (A), MC1R (B) and BDNF (C) datasets. In grey, observed relative frequencies between pairs of individuals; in black distribution under the model of population expansion of Rogers and Harpending (1992).

ii) Hemorrhois hippocrepis

In the mismatch distribution realised for Iberian populations, BDNF dataset revealed a good fitting between observed and simulated distributions under models of sudden expansion, supported statistically by non-significant values of SSD and Hr index (fig. 33) (Tab.XII). Also, Tajima's D was positive and not significant, Fu's F_s negative and not significant and R_2 large and not significant (Tab.XII). In the case of Maghreb populations, both MC1R and BDNF datasets revealed a good fitting between observed and simulated mismatch distributions under models of sudden expansion, supported statistically by non-significant values of SSD and Hr index(fig.34) (Tab.XII). Tajima's D and Fu's F_s were significantly negative for the MC1R dataset, and positive and not significant for the the BDNF dataset (Tab XII). Regarding R_2, this statistic was small and not significant for both MC1R and BDNF datasets (Tab XII).

Tab.XII– Resume of demography analysis (Tajima's D ; Fu's F_s and R_2) and mismatch distribution parameters (Tau; Theta0; Theta1; SSD , and Hr index) obtained for *Hemorrhois hippocrepis* in MC1R and BDNF datasets, for Iberia and Maghreb populations. Ns – No significance; * - mtDNA data from Carranza *et al*, (2006).

Hemorrhois hippocrepis						
	Iberia			Maghreb		
Marker	MtDNA*	MC1R	BDNF	MtDNA*	MC1R	BDNF
Tajima's D	2.09605		0.32649	-1.2244	-1.5525	-0.9713
p value	Ns		Ns	Ns	<0.05	Ns
Fu's F_s	2.14426		-0.966	-1.2771	-1.675	-1.18
p value	Ns		Ns	Ns	<0.05	Ns
R_2			0.1569		0.073	0.0798
p value			Ns		Ns	Ns
Tau	3.559		0.773	6.529	3	1.539
Theta0	0.002		0	0.002	0	0.004
Theta1	2.930		99999	3.480	0.21	1.096
SSD	0.0988		0.01219	0.0530	0.01250	0.00201
p value	< 0.05		Ns	<0.05	Ns	Ns
Hr index			0.14291		0.52982	0.08934
p value			Ns		Ns	Ns

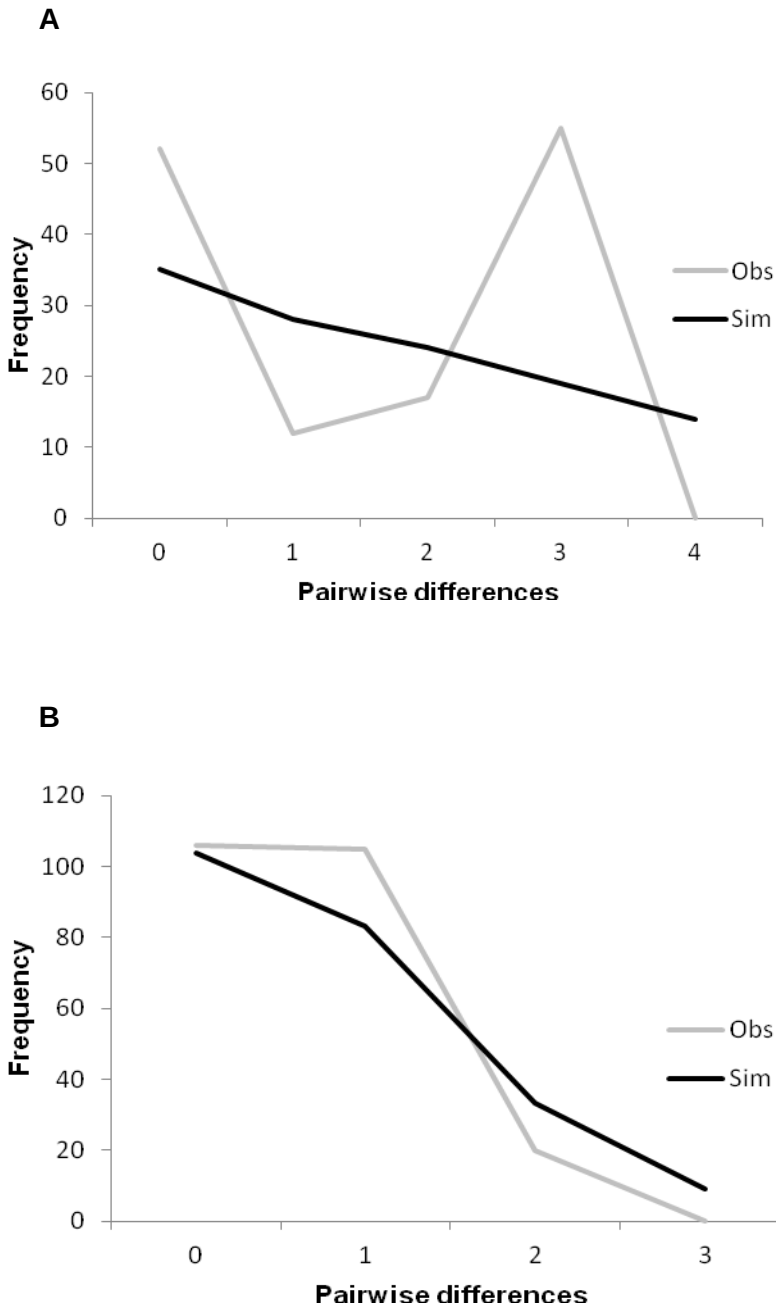


Fig.33 - Mismatch distribution of Iberian *Hemorrhois hippocrepis* populations considering mtDNA (A) – from Carranza *et al*, (2006); and BDNF (B) datasets. In grey, observed relative frequencies between pairs of individuals; in black distribution under the model of population expansion of Rogers and Harpending (1992).

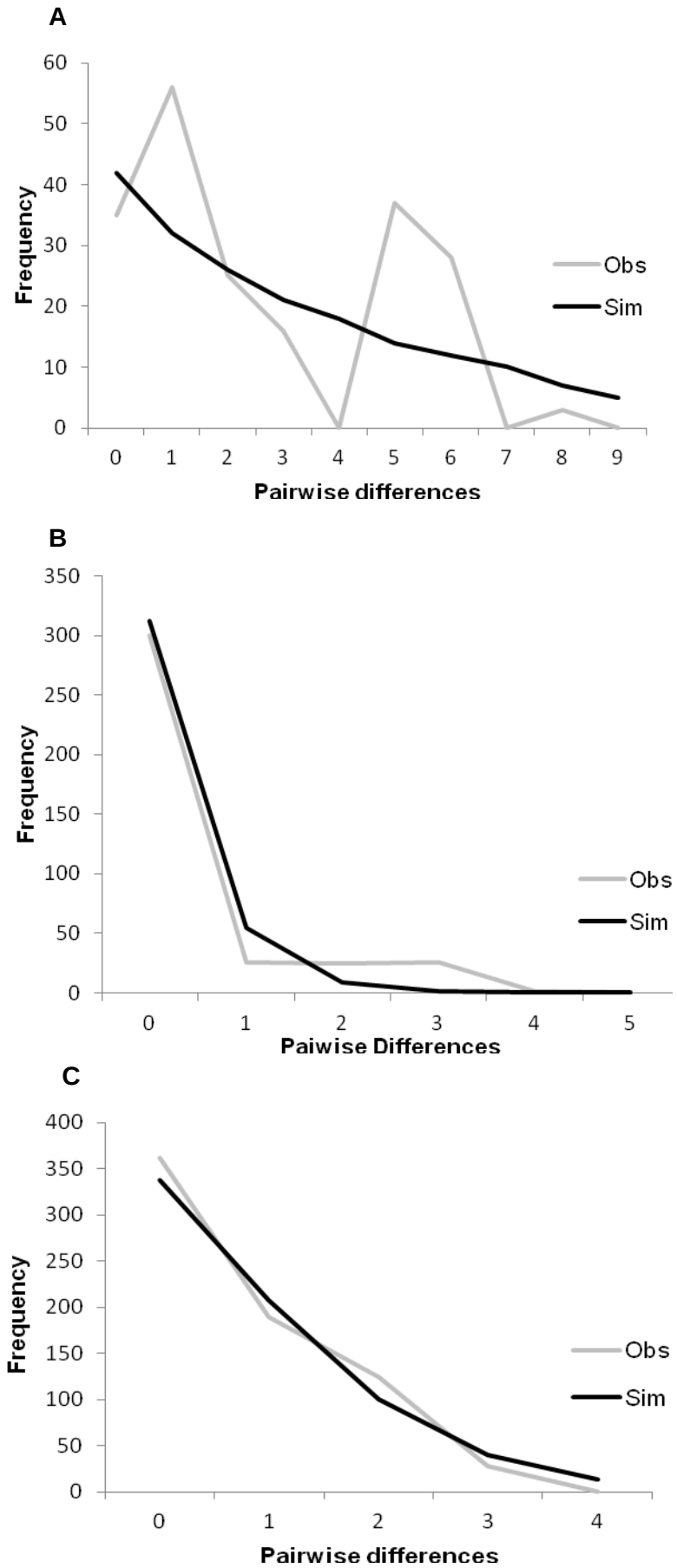


Fig.34 - Mismatch distribution of Magreb *Hemorrhois hippocrepis* populations considering mtDNA (A) - from Carranza *et al* (2006); MC1R (B) and BDNF (C) datasets. In grey, observed relative frequencies between pairs of individuals; in black distribution under the model of population expansion of Rogers and Harpending (1992).

iii) Malpolon monspessulanus

In the mismatch distribution realised for Iberian populations, BDNF dataset revealed a good fitting between observed and simulated distributions under models of sudden expansion, supported statistically by non-significant values of SSD and Hr index (fig. 35) (Tab.XIII). Also, Tajima's D was positive and not significant, Fu's F_s positive and not significant and R_2 large and not significant (Tab.XIII). In the case of Maghreb populations, MC1R mismatch distributions revealed a good fitting between observed and simulated mismatch distributions under models of sudden expansion, supported statistically by non-significant values of SSD and Hr index(fig.36) (Tab.XIII). In contrarly, BDNF mismatch distributions didn't fit with the simulated mismatch distributions under models of sudden expansion. Tajima's D and Fu's F_s were positive and not significant for the MC1R dataset, and negative and not significant for the the BDNF dataset (Tab XIII). Regarding R_2, this statistic was large and not significant for both MC1R and BDNF datasets (Tab XIII).

Tab.XIII – Resume of demography analysis (Tajima's D; Fu's F_s and R_2) and mismatch distribution parameters (Tau; Theta0; Theta1; SSD , and Hr index) obtained for *Malpolon monspessulanus* in MC1R and BDNF datasets, for Iberia and Maghreb populations. Ns – No significance. * - mtDNA data from Carranza *et al*, (2006).

Malpolon monspessulanus						
	Iberia			Maghreb		
Marker	MtDNA*	MC1R	BDNF	MtDNA*	MC1R	BDNF
Tajima's D	-1.8892		0.48809	-1.6164	0.49163	-0.3874
p value	<0.05		Ns	Ns	Ns	Ns
Fu's F_s	-4.2708		0.796	-1.7068	0.483	-1.371
p value	<0.001		Ns	Ns	Ns	Ns
R_2			0.2211		0.1998	0.1529
p value			Ns		Ns	Ns
Tau	0.803		0.502	1.172	0	1.281
Theta0	0		0	0	0	0
Theta1	0.388		99999	313.75	99999	99999
SSD	0.4390		0.00468	0.0242	0.47236	0.02563
p value	Ns		Ns	Ns	< 0.001	Ns
Hr index			0.20578		0.25854	0.18041
p value			Ns		Ns	Ns

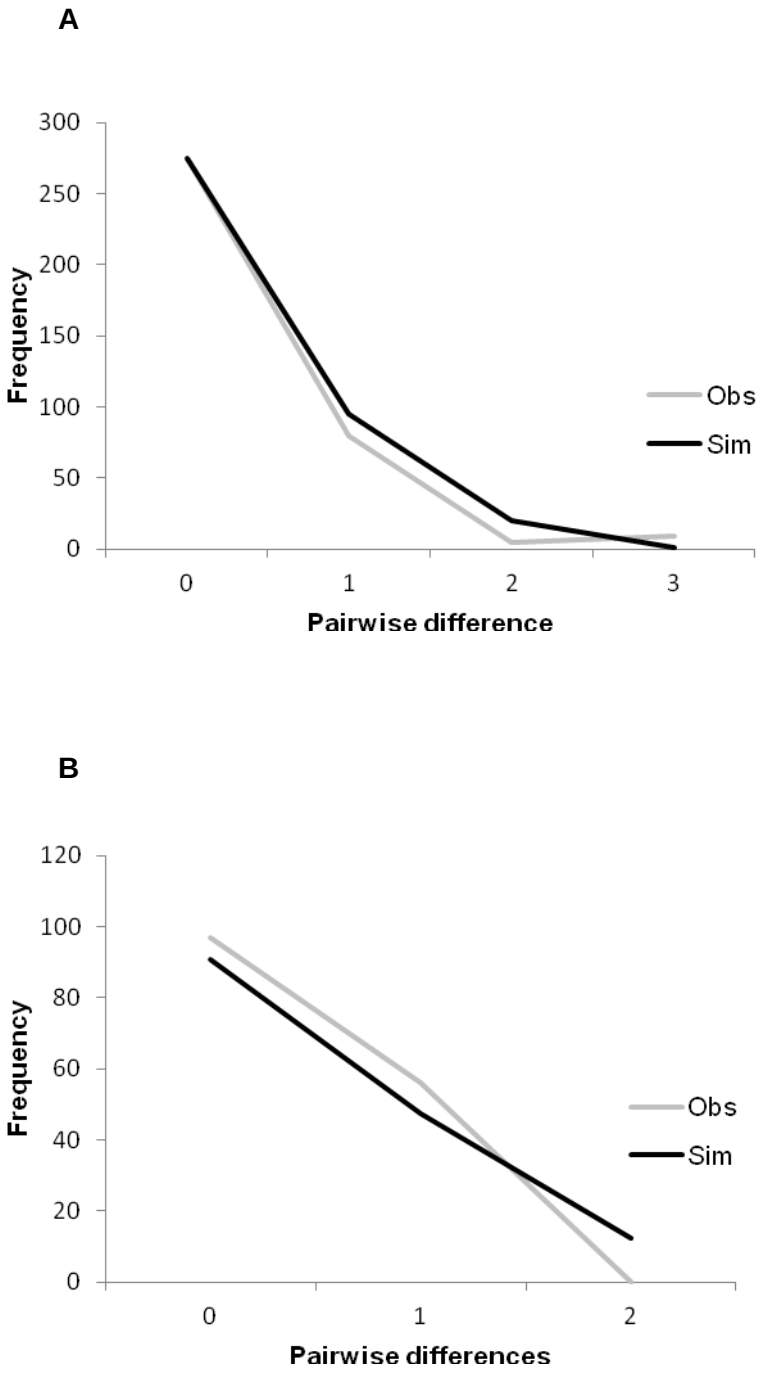


Fig.35 - Mismatch distribution of Iberian *Malpolon monspessulanus* populations considering mtDNA (A) – from Carranza et al, 2006; and BDNF (B). In grey, observed relative frequencies between pairs of individuals; in black distribution under the model of population expansion of Rogers and Harpending (1992).

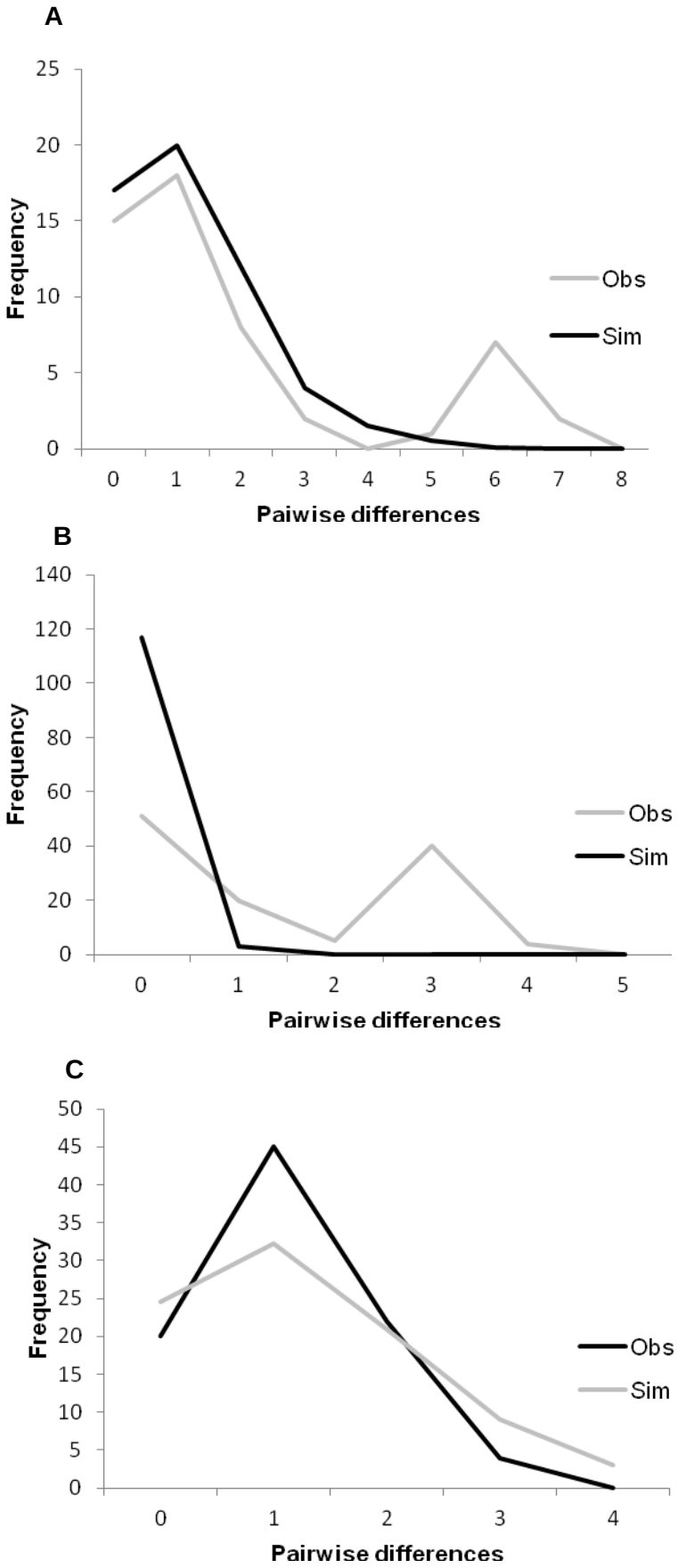


Fig.36 - Mismatch distribution of Magreb malpolon monspessulanus populations considering mtDNA (A) - from Carranza et al 2006; MC1R (B) and BDNF (C) datasets. In grey, observed relative frequencies between pairs of individuals; in black distribution under the model of population expansion of Rogers and Harpending (1992).

Discussion

Low genetic diversity or mitochondrial selective sweep?

Insights from *Rhinechis scalaris*

The analysis of mtDNA and nuDNA revealed well supported evidences of effective low genetic diversity for *Rhinechis scalaris*. A concordant pattern of genetic homogeneity is supported by the extremely rare percentage of variable sites, and the low level of haplotype and nucleotide diversity index in both locus (tab.V).

Additionally, the mtDNA dataset revealed a dense star-shaped network, characteristic of a recent expansion (fig.25). A recent expansion is characterised by a high frequent allele - an old allele, with a broad distribution and multiple direct connections to a series of rare alleles, therefore recent, and with a specific localization, resulting in the mentioned star-shaped network that is perfectly accessed in fig.25. In the case of the nuclear datasets, as expected the MC1R and BDNF dataset show less resolution. The MC1R haplotype network, revealed a commons and disperse allele, located in all native range, and a few more recent alleles directly connected, located along the Betic ranges (fig.26). In BDNF case, there is two almost equivalent haplotypes with no clear geographic structure (fig. 27).

Regarding demographic inferences, in the mtDNA dataset, the observed mismatch distribution is statistical concordant with the model of sudden expansion, Tajima's D (< 0.001) and Fu's Fs (<0.001) present significantly negative values and R_2 is significantly small (<0.05). In the case of nuclear markers we have two distinct results: in MC1R dataset, observed mismatch distribution is statistical concordant with the model of sudden expansion, Tajima's D (< 0.05) and Fu's F_s (< 0.01) are significantly negatives and R_2, small but not significant, on the other hand, in BDNF, observed mismatch distribution is statistical concordant with model of sudden expansion and the rest of statistics didn't show any significance.

Given the pattern observed above, this species was possibly on the brink of extinction, *Rhinechis scalaris* diverged in Miocene and has a fossil record dating from Pliocene in Iberian Peninsula, so a severe demographic event is the most plausible

explanation for such shallow genetic diversity (Luis *et al*, 2009; Lenk *et al*, 2001; Nagy *et al*, 2004 and Uttiger *et al*, 2002).

Comparing with other phylogeographic studies in Iberian Peninsula, *Rhinechis scalaris* present a very interinsting case, without a clear geographic structure. In our study, a vast improvement on sampling representativeness, especially in southern Spain and southern France, allowed us to detect variation, in contrast to Nuchis *et al* (2008), however, with the current data we are not yet able to detect a geographic pattern in this species. There is not a specific region harvesting a significant amount of haplotype diversity (i.e a potencial refuge), and even if the most divergent haplotype is found in southern Spain, most specifically the Betic ranges, this region is also sampled with significant more detailed that the rest of the species range (fig.22).

Important, the haplotype network in the mtDNA dataset, give us an important empirical sign of expansion that is statistically supported by demographic inferences in both mitochondrial and nuclear locus. Neutrality tests can produce selection and demographic interpretations, and only with a multilocus dataset we can better identify the cause for the desviation from neutrality. Having in our *Rhinechis scalaris* dataset, for mtDNA, statistical significance for all tests, and in MC1R, statistical significance for all but $R_{2,}$ we have a strong argument for a recent expansion as the cause for the desviation for neutrality.

Rhinechis scalaris constitutes a very intriguing case, his life traits (p.e reproduction cycle) is similar with snakes likely with a European origin, snakes that present high genetic differentiation and geographic structure (*Coronella girondica*, *Coronella austriaca*, *Natrix maura* and *Vipera latastei*), however this species most probably suffer a severe demographic event. This contrast makes even more important to explore more sophisticated techniques, like demographic reconstructions with multilocus data, identify the time of expansion or explore modelling techniques to project the species distribution under different climate scenarios in order to explain this conflict.

Another important objective should be the improvement of sampling detail in the northen regions of the species range, to produce a more definitive formulation to this singular phylogeographic scenario within Iberian Peninsula.

Insights from *Hemorrhois hippocrepis*

The nuclear datasets produced important corroboration of the evolutionary history of *Hemorrhois hippocrepis* inferred by Carranza *et al* (2006) solely based in mtDNA data.

In terms of sequence diversity, Maghreb population in the mtDNA dataset of Carranza *et al* (2006), revealed slightly more haplotype and nucleotide diversity comparing to Iberian population (tab.VI). The nuclear dataset generated in this study for both BDNF and MC1R revealed the same pattern, a contrast of diversity: Magreb – relative higher diversity; Iberian Peninsula – lower diversity. This pattern suggests a movement from Maghreb to Iberian Peninsula.

Haplotype distribution and parsimony networks were also coincident between the two loci. In Carranza *et al* (2006), mitochondrial data revealed in Maghreb populations, more haplotypes and more divergence between them in relation to Iberian populations, additionaly the haplotype network revealed a sign of expansion. Both MC1R and BDNF datasets maintain this contrast between the regions, with MC1R presenting only one haplotype in Iberian Peninsula, shared with Maghreb population, which instead showed variation. In MC1R a common haplotype is distributed along all over the range of the specie with few rare alleles directly linked, on contrary, in BDNF, the haplotype nerwork present a duality between two haplotypes in Iberian Peninsula and Maghreb that however are represented in both regions (fig.29). Between, mtDNA and nuDNA was also maintained the existence of a specific haplotype in east regions of Maghreb (figs. 12, 28 and 29).

Demographic reconstruction based in several methods revealed some discordance between loci. In Iberian population, mismatch distributions of mtDNA in Carranza *et al* (2006) registed a bimodal distribution, expected from a stable demography and Tajima's D and Fu's F_s were positive and not significant, where in our study, BDNF presented a statistical concordance with the sudden expansion model, Tajima's D positive and not significant, Fu's F_s negative and not significant and R_2 large and not significant. In the Maghreb region, mismatch distributions of mtDNA in Carranza *et al* (2006) registed a bimodal distribution, expected from a stable demography and Tajima's D and Fu's F_s were negative but not significant, where in our study, both MC1R and BDNF revealed a statistical concordance with the sudden expansion model, with MC1R having negative and significant values of Tajima's D and Fu's F_s and a small but not significant R_2, and BDNF having negative but not significant values of Tajima's D and Fu's F_s and small but not significant R_2 (tab.XII and fig.34).

The presence of *Hemorrhois hippocrepis* and it's sister species, *Hemorrhois algirus*, in Maghreb, makes more plausible, as mitochondrial and nuclear DNA suggests, a recent colonization of Iberian Peninsula as pointed by Carranza *et al* 2006. Additionaly, the analysis of diet habits of this species in both regions of the strait revealed more plasticity in Maghreb, suggesting also this region as source (Pleguezuelos and Fahd, 2004). Fossil data, dating from Late Pleistocene points to a earlier presence of this species in Iberian Peninsula, that possibly experienced regional exctinction (Luís *et al*, 2009). This species has a reproductive strategy that needs long springs and summers with hot temperatures, has been suggested 22ºC as the minimum temperature in July to occur reproduction, so during glacial ages the survival in Europe would be extremely difficult (Saint-Girons, 1982).

One interesting point found with the nuclear data, it's the conflict in Tajima's D and Fu' Fs tests between mtDNA data from Carranza *et* al (2006) and MC1R data. The significant negative results obtained in this study are intriguing, and MC1R, a genetic marker widely used, could have an important role in this species evolutionary history. But, we have to take in to account, that for this neutrality tests to be fully accountable we need to have a very robust population data, that in the future can be addressed.

Insights from *Malpolon monspessulanus*

The nuDNA added in this study, reveal information that in complement with mtDNA results in Carranza *et al* (2006) gives additional insights about the evolutionary history of the species. Carranza *et al (*2006) identified a pattern similar to *Hemorrhois hippocrespis*, *ie* a higher haplotype and nucleotide diversity in Maghreb when compared with Iberian Peninsula (tab.VII). The same pattern is observed in nuDNA, with similar differences between regions, ie, a tenfold difference between nucleotide diversity in each area (tab.VII). Important to a better understanding of this phenom, MC1R didn't report a singular variation in Iberian Peninsula, with the only allele being also present in Maghreb, suggesting a movement from Maghreb to Iberian Peninsula.

The geographic localization of haplotypes and specifities of each region gives also important informations. Mitochondrial data from Carranza *et al* (2006) revealed a haplotype network with a typical sign of expansion, and Maghreb populations presented more haplotype variability and divergence between them in relation to Iberian populations. Our nuclear data, in both MC1R and BDNF datasets maintain this scenario, MC1R reported only one haplotype in Iberian Peninsula, with this being also present in Maghreb, whereas Maghreb presented more haplotype diversity and more divergence between haplotypes (tab.X); a common haplotype is distributed along all over the rangeof the specie, with few rare alleles directly linked. In BDNF there was less discontinuity in comparation with MC1R, but again, all Iberian haplotypes were shared with Maghreb, and only Maghreb had exclusive haplotypes (tab.X).

Another signal of congruence between the two locus was found with the demographic inferences. In Iberian Peninsula, BDNF presented a unimodal distribution with statistical concordance with the sudden expansion model, in concordance with the mtDNA dataset from Carranza *et al* (2006), but the latter registed also significance for a negative Tajima's D and Fu's Fs tests (fig.35 and tab.XIII). In Maghreb, all datasets reveal a bimodal distribution non concordant with the sudden expansion model (fig.36).

Together, the data suggest a more stable population in Maghreb and emprirical and statistical signs of recent expansion in Iberian Peninsula, and phylogeny support the idea of this movement form Maghreb to Iberian Peninsula. All *Psammophinae* family has roots in Africa, and his sister species, *Malpolon moilensis* has likely also an afrincan origin (Carranza *et al*, 2006). *Malpolon monspessulanus* had an earlier colonization in Iberian Peninsula, as his reported by a large fossil data dating from Pliocene; these specimens became exctint, probably during Pleistocene oscillations (Luís *et al*, 2009).

This species reproductive strategy, which needs long, and hot spring and summers, some studies suggesting 22ºC as the mean temperature during Juy, probably made insustanaible the survival of this species during Pleistocene oscillations in Iberian Peninsula (Saint-Girons, 1982). Carranza *et al* (2006) suggested 82000-168000 years has the time this specie is present in Iberian Peninsula, the re-colonization was suggested to take place by oscillations in water depth that made possibly to this specie to make the transition along the Strait of Gibraltar (Carranza *et al*, 2006).

In the future, however, is recommended a sampling even more representative of the specie distribution. This species occupies a vast area, and in some countries due to political reasons, namely West Sahara and Argelia, sampling is very difficult (fig.20) In Carranza *et al* (2006), was found more divergence in southern regions of the species range (fig.11); with a more comprehensive sampling in the zone, probably more important information could be accessed.

Conclusions

The results from mtDNA and NuDNA genes in relation to sequence diversity, phylogeographic inferences and demographic inferences are in agreement, suggesting effective low genetic diversity in *Rhinechis scalaris, Hemorrhois hippocrepis and Malpolon monspessulanus*, therefore reducing drastically the possibility of a selective event, a selective sweep.

The major improve in the sampling quality, and the introducement of 3 new markers, make possible to conclude with great statistic confidence, the occurrence of a severe bottleneck event in *Rhinechis scalaris*, both marker suggest a very important moment shaping today genetic diversity patterns at both locus.

In relation to *Hemorrhois hippocrepis*, the nuclear genes complemented the information already obtained in previous studies for the specie. Was found a interesting conflict between MC1R and previous mtDNA data regarding some neutrality tests, but the concept a recent migration from Maghreb to Iberian Peninsula was reinforced with the nuDNA data.

The analysis of *Malpolon monspessulanus* nuDNA revealed the same patterns reported in previous studies, a recent migration from Maghreb to Iberian Peninsula, A relatively short sampling, even covering the major geographic regions, could be a limitation pointed to the inferences (mtDNA and nuDNA) relatively to this specie.

Together, the inferences from this three snakes, forms a solid knowledge. The comparasion of nuDNA with mtDNA reinforced the contrast existent in Iberian Peninsula regarding snakes genetic diversity, a conflict that reflects the complexity of animals' evolutionary history.

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