Evolutionary history and genetic structure of the spined toad *Bufo spinosus*, Daudin 1803

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

The spined toad, *Bufo spinosus*, is a widespread species occurring in southern France, the Iberian Peninsula, and Maghreb region, in north Africa, occupying in a great variety of habitats across its range. The species was formerly assigned as subspecies of *B. bufo* species group, but recent studies have clarified its phylogenetic affinities and characterized its contact zone with *B. bufo* in France. Despite that, little is known about its intraspecific evolutionary history. The great heterogeneous physiography, complex climatic and geological history within Iberia and Maghreb played a major role influencing species evolution. Because of that, expecting to find significant associations between patterns of genetic diversity and geography, in this study, we combined both historical (*cyt b* gene of mtDNA) and contemporary (microsatellite) genetic markers to access the evolutionary history and genetic structure of *Bufo spinosus.* To do so, we used samples from populations in Iberia, south France, Morocco, and Tunisia. Mitochondrial genealogy, and characterization of genetic diversity and structure were also performed.

High overall genetic diversity indices and high levels of connectivity among genetic clusters was detected. Moreover, our genetic markers revealed discordant patterns of relationships between European and African populations, and among Morocco and Tunisia populations. On the one hand, mtDNA recovered Africa and European, and Moroccan and Tunisian as fully separated populations. On the other hand, microsatellite data uncovered a connection of European and African samples along a Mediterranean corridor in the European portion of the study area.

Our study provides a comprehensive consideration regarding historical and contemporary process shaping biodiversity across Iberia and Maghreb. The genetic diversity encountered within European (especially within Iberia) populations did not experienced considerable amounts of fragmentation before and during Pleistocene glaciations, suggesting potential multiple glacial refugia. Further, we do not discard the presence of new cryptic species across Maghreb.

Keywords: Iberia; Maghreb; gene flow; *cyt b* gene; biogeography; cyto-nuclear discordances; allopatric speciation.

Resumo

O sapo comum, *Bufo spinosus*, é uma espécie generalista que ocorre no sul da França, na Península Ibérica e região do Magrebe, no norte da África, ocupando uma grande variedade de habitats em toda a sua distribuição. Anteriormente, era designada como subespécie do grupo *B. bufo*, mas estudos recentes esclareceram suas afinidades filogenéticas e caracterizaram sua zona de contato com *B. bufo* na França. Apesar disso, pouco se sabe sobre sua história evolutiva intraespecífica. Dentro da Península Ibérica e do Magrebe, a grande fisiografia heterogênea, clima complexo e história geológica, desempenharam um papel importante na evolução das espécies. Desse modo, esperando encontrar associações significativas entre padrões de diversidade genética e geografia, neste estudo, combinamos marcadores genéticos de escalas histórica (gene cyt b do mtDNA) e contemporânea (microssatélites) para acessar a história evolutiva e estrutura genética de *Bufo spinosus* . Para isso, usamos amostras de populações da Península Ibérica, sul da França, Marrocos e Tunísia. A genealogia mitocondrial e a caracterização da diversidade e estrutura genética também foram realizadas.

Detectou-se altos índices gerais de diversidade genética e altos níveis de conectividade entre os agrupamentos genéticos. Além disso, nossos marcadores genéticos revelaram padrões discordantes de relacionamento entre as populações européias e africanas, e entre as populações do Marrocos e da Tunísia. Por um lado, o mtDNA recuperou as populações da Africa e Europa, e do Marrocos e Tunísia como populações totalmente separadas. Por outro lado, dados de microssatélites revelaram uma conexão de amostras europeias e africanas ao longo de um corredor na porção mediterrânea da Península Ibérica.

O presente estudo fornece uma consideração abrangente sobre processos históricos e contemporâneos que moldam a biodiversidade na Península Ibérica e Magrebe. A diversidade genética encontrada nas populações europeias (especialmente na Península Ibérica), não experimentou quantidades consideráveis de fragmentação antes e durante as glaciações do Pleistoceno, sugerindo múltiplos refúgios glaciais em potencial. Além disso, não descartamos a presença de novas espécies crípticas em todo o Magrebe.

Palavras chave: Peninsula Ibérica; Magrebe; fluxo genético; biogeografia; discordancia cyto-nuclear; especiação alopátrica.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

MYA	Million Years Ago
MSC	Messinian Salinity Crisis
LGM	Last Glacial Maximum
mtDNA	mithocondrial DNA
nDNA	nuclear DNA
POMC	Proopimelanocortin
RPL3	Ribosomal Protein L3
RFLP	Restriction Fragment Lenght Polymorphism
Cytb	Cytochrome b
PCR	Polymerase Chain Reaction
NGS	Next generation sequencing
MCMC	Markov Chain Monte Carlo
HWE	Hardy-Weinberg Equilibrium
AMOVA	Analysis of Molecular Variance
DAPC	Discriminant Analysis of Principal Components
PCA	Principal Components Analysis
PC	Principal Components
BIC	Bayesian Information Criterion

1.Introduction

1.1 Biogeographic process within Mediterranean Basin

Palaeogelogical events such as the Messinian Salinity Crisis (MSC; ~5.9-5.3 Mya) played a major role in explaining the onset of the Mediterranean's heterogeneity evolutionary history (Thompson, 2005). The MSC is considered as one of the most dramatic paleoceanographic crisis in Earth's history (Hsü, Ryan and Cita, 1973), initiated after the closure of the Atlantic-Mediterranean Betic and Riffan corridors. This process led to the desiccation of the Mediterranean sea (Duggen et al. 2003; Krijgsman et al. 1999), and ended when the strait breached (Garcia-Castellanos et al. 2009; Krijgsman et al. 1999). Although this is the classic view regarding the Gibraltar breaching, there is no evidence of clear tectonic pulse supporting this hypothesis (Booth-Rea, Ranero and Grevemeyer, 2018). Recent works suggest the strait of Gibraltar was always open as watergate during the MSC and that Mediterranean was a deep basin on this period (Simon and Meijer, 2017), requiring a land bridge as an alternative to exchange terrestrial fauna between Africa and Iberia (Booth-Rea, Ranero and Grevemeyer, 2018). In this scenario, Booth-Rea et al. (2018) proposed that magmatism created a land bridge (~10 - 6 Mya) that possibly emerged as an archipelago (Alborán volcanic archipelago, located few hundreds of km east of Gibraltar strait, see fig. 1) allowing the exchange of terrestrial biota between Iberian and Africa (~7 - 3 Mya), determining species' paths and allowing species' differentiation. The cooling of the crust caused lithospheric thickening and thermal subsidence, ending MSC and gradually limiting the terrestrial faunal exchange across the archipelago. Molecular studies focused on faunal exchange between Iberia and Africa provided evidence of terrestrial biota exchange before (*e.g. Podarcis*, Kaliontzopoulou et al. 2011; *Pleurodeles poireti*, Hassine et al. 2016; *Buthus occitanus,* Gantenbein and Largiadèr 2003) during (*e.g. Vipera latastei/monticola*, Velo-Antón et al. 2012; *Natrix,* Kindler et al. 2018; *Trechus fulvus* in Faille et al. 2014) and after the MSC (e.g. *Hyla*, Recuero et al. 2007; *Mauremys leprosa,* Veríssimo et al. 2016, *Pelobates cultripes*, Gutierrez-Rodríquez et al. 2017; *Emys orbicularis*, Velo-Antón et al. 2015 and *Crossidura russula* Cosson et al. 2005), suggesting the Alborán volcanic archipelago played an important role in connecting European and African taxa and that the Strait of Gibraltar acted as semi-permeable barrier to dispersal for some taxa.

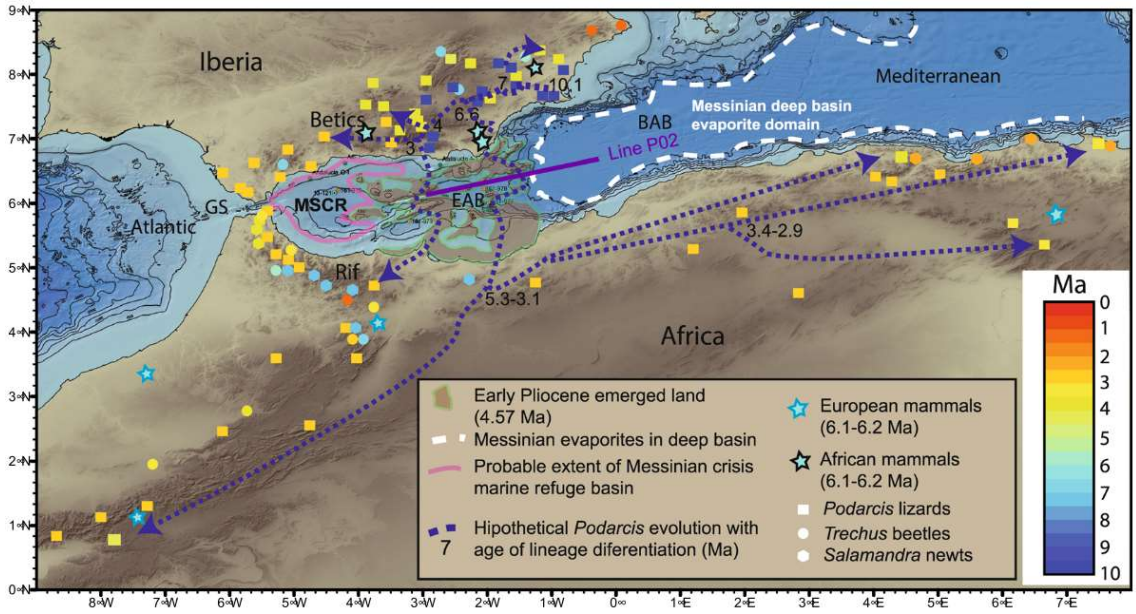

Figure 1: Patterns of faunal exchange between North Africa and South Iberia. EAB: East Alborán Basin. BAB: Balearic-Algerian Basin. MSCR: Messinian Salinity Crisis Refugee. GS: Gibraltar Strait. Adapted from Booth-Rea et al. 2018.

1.2 Climatic processes

Climatic changes during the Quaternary forced species to cyclic retractions and expansion of their ranges. In the Pleistocene, combination of climatic and paleogeographic events have led Mediterranean species to respond in different manners to such events, whose reaction could vary on their ecological constraints (Veríssimo et al. 2016). This process resulted in population extinctions, persistence in suitable climatic refugia, mainly in southern European peninsulas (Iberian, Italian and the Balkans), and the current high levels of intraspecific genetic differentiation and multiple secondary contact zones (Araújo et al. 2008; Hewitt, 2000; Nicolas et al. 2018; Velo-Antón et al. 2012; Steven and Ferrand, 2007).

Although several paleogeographic events have been identified as potential triggers in speciation process (***e.g.*** Betic Crisis, end of the MSC, land bridge connections between Africa and Iberia), climatic events (specially climate change during Plio-Pleistocene) might have major influence in amphibian's evolution that occur in Iberian Peninsula and North Africa (Ehl, Vences and Veith, 2019). Affinities in climatic and ecological conditions during the Pliocene and Pleistocene in southern Europe and North Africa led to similarities in species composition in both regions. In the Last Glacial Maximum (LGM), the Sahara Desert retracted to the south, and vegetations in lower elevations of North Africa were remodeled into warm mixed forests at post-glacial stages commencement (Jedoui et al.

2002). During the Late Pleistocene, these forests were common in North Africa and southern Europe, providing analogous habitats for temperate species in both regions (Husemann et al. 2013). Maghreb's (here identified as Northern Morocco, Algeria and Tunisia), location at the junction of two different biogeographic regions (Mediterranean climate in the north and Saharan climate in the south) generates a unique species composition (Dobson and Wright, 2000). For Mediterranean lineages, aridity has been playing an important role in fragmentation and consequent allopatric divergence, especially when humid and hyper-arid phases regularly alternated from the mid-Pliocene to the Pleistocene (Cosson et al. 2005).

1.3 Diversification in Mediterranean basin

The Mediterranean Basin is one the world's major centres of diversity (i.e. hotspots) and it has been identified as conservation priority because it shelters high levels of endemism and species richness (Myers et al. 2000; Beddek et al. 2018). High levels of biodiversity is generally associated with the paleogeographic and climatic process affecting this region since the Miocene (Duggen et al. 2003).

In Iberia, topographic features such as mountains (*e.g.:* Central System, Pyrenees, Cantabrian mountains and Betic System) and rivers are largely associated with barriers to gene flow. Species with different life history traits are expected to respond differently to topography, and as outcome, will show divergency in genetic structure across landscapes (Steele, Baumsteiger and Storfer, 2009). Mountains are not an absolute barrier, acting sometimes as a permeable obstacle or filter to gene flow, and as current refugial areas for species that were more widespread during colder periods in the Pleistocene (Abellán and Svenning, 2014; Sánchez-Montes et al. 2018). In special, the orientation of mountain ranges along east-west axis, that might have limited populations expansion or contraction because of the climatic oscillations during Pleistocene (Sánchez-Montes et al. 2018). This characteristics led to a refugia-within-refugia pattern, resulting in a complex history of population fragmentation, turning this area an important centre of diversification that promoted high rates of endemism and complex population structures such as in *Chioglossa lusitanica* (Alexandrino et al. 2000), *Rana iberica* (Teixeira et al. 2018) and *Acanthodactylus erythrurus* (Harris, Belliure and Cuervo, 2018) (Gómez and Lunt, 2007; Weiss and Ferrand, 2007; Abellán and Svenning, 2014). Alike, the Maghreb, harbour high mountain ranges (*e.g*. Atlas and Rif) and high diversity of landscapes and bioclimatic regions (Beddek et al. 2018; Sampaio et al. 2015), also served as climatic refugia for many

taxonomic groups during Quaternary climatic oscillations. For instance, arid zones functioned as main driver of population fragmentation and subsequent allopatric divergence in Mediterranean lineages *(e.g.* Veríssimo et al. 2016; Martínez-Freiría et al. 2017; Freitas et al. 2018; Dinis et al. 2019).

Amphibians, as ectotherms with permeable skin, are sensitive to environmental changes (Muths et al. 2017). This taxonomic group is strongly affected by climatic changes and topographic elements. Their physiology, behaviour, ecological attributes and retention of a strong philopatric character may influence the reproductive behaviour leading to high levels of genetic structure within species (Blaustein et al. 2003; Cushman, 2005). Furthermore, their sensitiveness to climate change makes them great organisms to discriminating the effect of environmental changes upon their genetic structure and evaluate their biogeographic histories (Zeisset and Beebee, 2008).

Generalist species can tolerate a variety of environments, possessing adaptive features for surviving in several ecological environment as part of their overall niche breadth (Kassen, 2002; Kirkpatrick and Barton, 1997). Likewise, species both locally abundant and widespread might provide information on the most efficient barriers to gene flow, since low levels of genetic structure are expected for these organisms (Seppä and Laurila, 1999; Spear et al. 2005). When populations become geographically isolated, mechanisms of gene flow are disrupted and isolated populations can suffer genetic, ecological and phenotypical divergence (Cushman, 2005; Dufresnes et al. 2019; Stoelting, Measey and Drewes, 2014).

1.4 *Bufo bufo* species group and *Bufo spinosus*

The genus *Bufo* was the most specious genus of amphibia in the world, containing 238 described species until recently (Frost et al. 2006). As a result of the paraphyly of *Bufo spp.*, Frost et al. (2006) split this group into several taxa, moving most species of former "*Bufo*" to other genera, and restricting *Bufo* to members of *Bufo bufo* species group (*Bufo ailaoanus, B. aspinius, B. bankorensis, B. bufo, B. cryptotympanicus, B. eichwaldi, B. gargarizans, B. japonicus, B. luchunnicus, B. menglianus, B. pageoti, B. spinosus, B. stejnegeri, B. torrenticola, B. tuberculatus, B. tuberospinius, B. verrucosissimus*), a widespread taxonomic group, with an Euro-Asiatic distribution (Frost, 2019).

The parallel studies of Recuero et al. (2012) and García-Porta et al. (2012) played a major role regarding the phylogeny of *Bufo bufo* species complex. Recuero et al. (2012) made use of mitochondrial and four nuclear genes, while García-Porta et al. (2012), integrated analysis of allozyme data, mitochondrial DNA, and species distribution models. Displaying similar results, their phylogenetic tree recovered five major clades: Caspian, European, Caucasian, Iberian and African. The phylogenetic relationships show Caspian as the basal-most clade, corresponding to *B. eichwaldi* species, European and Caucasian clades are sister groups regarding *B. bufo* and *B. verrucosissimus* respectively, and Iberian and African clades are sister groups and includes specimens of *B. b. spinosus*. This species occurs in all Iberia Peninsula, southern and western France, and in humid areas and mountain ranges of Morocco, Algeria, and Tunisia. The African clade includes two subclades: Moroccan and Tunisian subclades. Both studies revealed a long and isolated evolutionary history between African and Iberian clades of *B. spinosus*. In one hand Recuero et al (2012) recognized *B. spinosu*s as an independent and well differentiated lineage of *B. bufo*. In the other hand, García-Porta et al (2012), suggested that the African specimens might in fact, represent two different subspecies, one in the western, other in eastern Maghreb. Although the arid conditions of the Mouluya river basin in Maghreb could explain the dichotomy present in their phylogeny, the lack of sampling in this area prevented further interpretations about its role of putative barrier to gene flow (García-Porta et al. 2012; Recuero et al. 2012). The gap in Algeria is mainly due to political and safety issues, leading to a large deficiency of sampling that disrupts a better understanding of the origin of the genetic lineages within Maghreb (Beddek et al. 2018).

Studies investigating the contact zone between *Bufo bufo* and *B. spinosus* located in France and using different sampling strategies supported *B. spinosus* as a valid species. Arntzen et al. (2013a) used allozyme data to test for hybridization between *B. bufo* and *B. spinosus*. Based on the deep genetic differentiation found in García-Porta et al. (2012) and on absence for hybridization and introgression for *B. bufo* and *B. spinosus*, they proposed elevating *B. spinosus* to the species level. In the same year, Arntzen et al. (2013b) presented morphological and genetic differentiation between *B. bufo* and *B. spinosus* (using two fragments of nuclear DNA (nDNA) (POMC and RPL3) and a mtDNA RFLP), suggesting that they are best considered different species. Genetic admixture was reported in a morphologically intermediate population (Moyoux) from the contact zone by Trujillo et al. (2017). This studied demonstrated Moyoux as a hybrid population, where microsatellites, mitochondrial and nuclear DNA revealed a clearly admixed genetic pool, unveiling interspecific gene flow, granting thus, a solid evidence of hybridization bounding *B. bufo* and *B. spinosus.* Arntzen et al. (2017), in turn, proposed an origin for the hybrid

zone documenting the local distribution of interspecific mtDNA lineages, proposing a pendulum movement. In the LGM, about 22 000 years before present, an isolated population of *B. bufo* resides in Provence, and when climatic conditions ameliorated, this lineage expanded its range moving northwards. In the post-glaciation period, *B. spinosus* expanded its range southwards from a refugium in southern France or northern Spain. In this period, *B. bufo* reached southwestern Europe came from the Balkans, traced a route north and south of the Alps, originating the current position of *B. bufo – B. spinosus* hybrid zone.

The morphological discrimination of these two species is done with characters related to the parotoid glands and metatarsal tubercle shape and size (Figure 2). Despite morphological contrasts are not abundant, the genetic differentiation between them is outstanding (global *Fst*: 0,79 over 13 polymorphic loci in Arntzen et al. 2013b; and 7,0% at the combined cytb and 16S genes in Recuero et al. 2012). The separation of these two species was estimated in about 5.3 million years ago, at the end of the Messinian Salinity Crisis, and might be associated with the different phases of the uplift of the Pyrenees (Recuero et al. 2012).

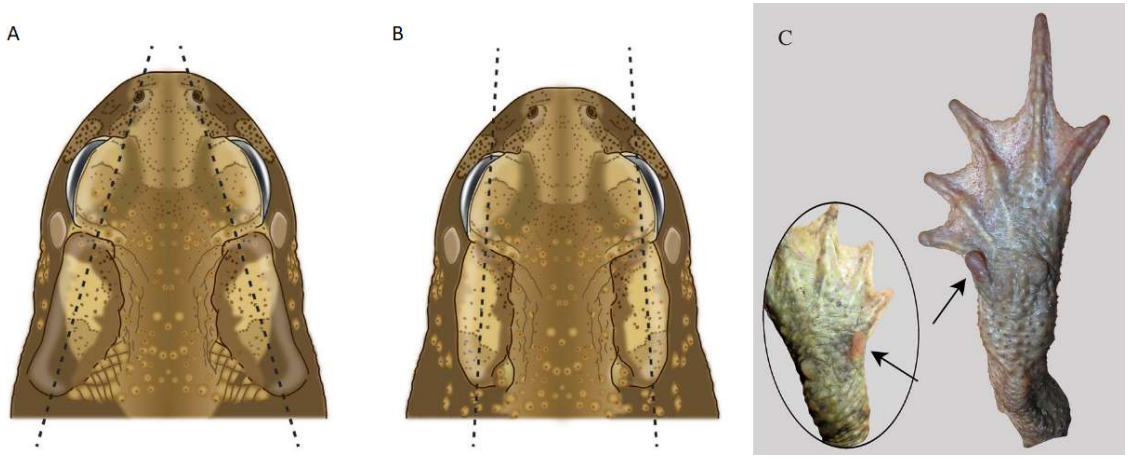

Figure 2: Key characters in morphological differentiation between *Bufo spinosus* and *Bufo bufo*. A: divergent positions of parotoid gland in *B. spinosus*. B: parallel positions of parotoid glands in *B. bufo*. C: inner metatarsal tubercle shape in *B. bufo* (left) and *B. spinosus* (right). This image was adapted from Arntzen et al. 2013b.

The spined toad *Bufo spinosus* Doudin 1803 (Figure. 3) is a generalist anuran occurring in southwestern France, Iberian Peninsula, Jersey Islands and north of the Maghreb region (Ortiz-Santaliestra, 2014) (Figure.4). The species occupies termomediterraneans and eurosiberian areas, with terrestrial habits, using aquatic environments during the reproductive season for mating and egg-laying (Brischoux et al. 2018; Reading and Clarke, 1983).

Figure 3: The spined toad *Bufo spinosus* Doudin 1803. Photo by G. Velo-Antón.

Bufo spinosus occurs in a variety of habitats, from environments with high diversity of arboreal vegetation in Parque Nacional de Peneda-Gerês, to degraded zones with poor soil in Galicia from sea level to 2600 m altitude in Pyrenees (Ortiz-Santaliestra, 2014). Its population densities across the Iberian Peninsula decrease when altitude is higher than 1500 m, and this negative effect is also noted in Parque Nacional Peneda-Gerês (Soares and Brito, 2007) and Sierra de Gredos (Ortiz-Santaliestra, 2014; Soares and Brito, 2007). Adults diet is very diverse, being composed by ants, small arthropods and coleopterans, and its habits are mostly terrestrial, migrating to permanent ponds (natural or man-made) and streams to breed (Ortiz-Santaliestra, 2014). The reproductive period depends on climatic conditions and correspond to the end of winter when nocturnal temperatures are higher than 0ºC. Differently from other amphibians, *B. spinosus* has one annual reproductive period, conferring a competitive advantage, since its tadpoles present certain development when embryos of other species appear in the water (Ortiz-Santaliestra, 2014). Unfortunately, information about this species vagility is scarce, detecting 470m of maximum distance in reproductive periods in Parque Natural de Peñarla in Madrid (Daversa, Bosch and Muths, 2012). In counterpart, maximum average of dispersal rates for *B. bufo/ B. spinosus* is 1.32 km (ranging 0.12−3.62km) (Arntzen et al. 2016). Curiously, migrations are well studied for *B. bufo,* whitch migrations exceeding 500m are common, reaching 500-5000m (Kovar et al. 2012).

In North Africa, the species is restricted to humid and temperate zones, mostly occupying mountain areas, and reaching 2750m in High Atlas (Morocco) (Vialas and Boned, 2016). In Tunisia, the species is distributed in the northwest, it is very hygrophilous and occurs only in very particular bioclimatic zones and environments, with oak forests character (Hassine and Nouira, 2012).

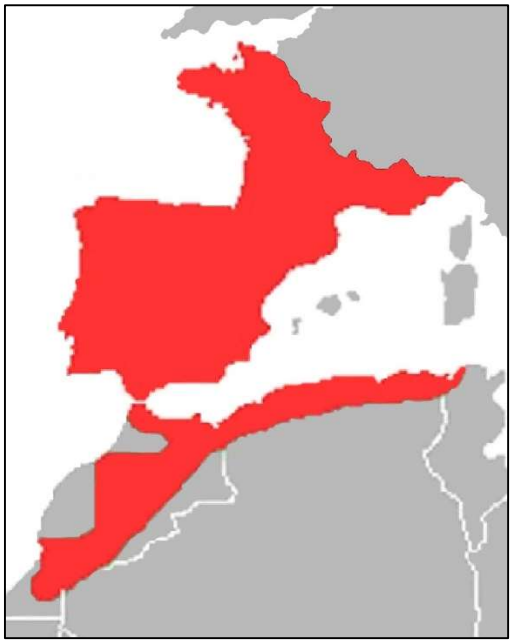

Figure 4: A: Distribution of *Bufo spinosus* across Western Europe and northern Africa. Adapted from Arntzen et al. 2019.

Spanish populations of *B. spinosus* are considered in decline (Ortiz-Santaliestra, 2014). It is listed as least concern in Portugal, and as vulnerable in Morocco and Algeria (Mateo, Geniez and Pether, 2013) with low population densities (Schleich, Werner and Klaus, 1996), and rare in Tunisia (Hassine and Escoriza, 2014). Main threats include deforestation, fragmentation, contamination and desiccation of aquatic environment and trampling during migration in reproductive season (Hassine and Escoriza, 2014; Ortiz-Santaliestra, 2014; Martínez-Freiría and Brito, 2016). In fact, the presence of roads is one of the main factors leading to fragmentation and isolation in populations of *B. spinosus*. For the record, mortality by roadkill can reach incredible 43% of vertebrates' road killed in a highway connecting Lerida and Gerona in Spain (Eizaguerri et al. 2001). Additionally, invasive species in both Europe and Maghreb, such as *Gambusia sp*. fishes can lead to local extinction of *B. spinosus* populations, as reported in Iberian Peninsula (Galán, 1997).

1.5 Molecular markers and genetic patterns

Advances outcoming from improvements in computing technology and laboratory techniques allowed molecular studies supplementing approaches in biogeographic research (Parker and Jorgensen, 2003; Selkoe and Toonen, 2006). The invention of polymerase chain reaction (PCR) techniques and introduction of sensitive molecular markers has modified the way researchers understand molecular data in the last century (Zhang and Hewitt, 2003). Nowadays, next generation sequencing (NGS) techniques are the spotlight of molecular studies because of the easy generation of multilocus data, fast and cost-effectiveness (Desalle, Schierwater and Hadrys, 2017). Despite studies using NGS techniques are increasing in the last years, sanger sequencing is still a gold standard method for sequencing short fragments of DNA (<1000bp) (Totomoch-Serra, Marquez and Cervantes-Barragán, 2017), mainly due to its 10x higher base guard precision in relation to NGS. In addition, due to elevate cost of NGS, many laboratories do not have access to this technique, therefore, sanger sequencing-based methods are still used very often.

With many different types of genetic markers being available, is essential to comprehend the nature of information that each one can provide, avoiding mistakes interpreting the results. Two classes of DNA markers are clearly predominant in works employing molecular markers for sanger sequencing: mitochondrial DNA and microsatellites (Zhang and Hewitt, 2003) . In one hand, mitochondrial DNA (mtDNA) markers were the most common method in most of phylogeographic studies (Avise, 1998), are relatively easy to amplify mtDNA because it appears in multiple copies in the cell, lack of recombination and possess fast rates of base substitution (Zhang and Hewitt, 2003; Galtier et al. 2009). Although it has been proved to be a great mechanism for genealogy, and evolutionary studies of animal genetic population, it provides a limited view of population history or can be biased to female-mediated process due to its maternal inheritance (Sequeira et al. 2008; Zhang and Hewitt, 2003). In the other hand, microsatellites present co-dominant nature, high levels of mutation rates (100-1000 times faster than nDNA), are highly polymorphic and biparentally inherited (Muniz et al. 2019; Zhang and Hewitt, 2003). These fast-evolving rates are useful in revealing fine-scale population genetic structure, inferring recent evolutionary process and provide insights about biogeographic histories (Gonçalves et al. 2009; Vieira et al. 2016). Despite NGS allows the rapid and cost-effectively development of markers including non-model species (Muniz et al. 2019), combining mtDNA and microsatellites is still a valid and useful option to investigate biogeographic histories (Dufresnes et al. 2016; Gutiérrez-Rodríguez,

Barbosa and Martínez-Solano, 2017), hybrid zones or parentage analysis (e.g. Dufresnes et al. 2016; Gutiérrez-Rodríguez et al. 2017; Sequeira et al. 2019; Trujillo et al. 2017).

It is fundamental to investigate alternative evolution scenarios by extending the inclusion of multiple and independent markers (Gonçalves et al. 2009). Thus, microsatellites and mtDNA are complementary molecular markers because they reveal different aspects of story at different depths of perspectives (Sequeira et al. 2008; Wang, 2010; Zhang and Hewitt, 2003). Additionally, incorporating these both markers might increase the comprehension on historical and contemporary demographic events shaping population structure of a species.

1.6 Objectives

The Strait of Gibraltar was an important migration corridor for terrestrial organisms between southern Iberia and Northern Maghreb during the MSC, and ecological relatedness in this area provided the availability of similar habitats in both regions (Brito et al. 2008). When the Strait of Gibraltar flood and the Mediterranean Sea was refilled, the terrestrial biota suffered a huge barrier to gene flow and was already reported in several ectotherm species. In addition, environmental factors such as climate and landscape already has shown a potential role in shaping patterns of genetic diversity and structure in the Mediterranean Basin (Ehl, Vences and Veith, 2019; Steele, Baumteiger and Storfer, 2009; Veríssimo et al. 2016) Studies with species occurring in Iberian Peninsula and Maghreb helped to solve systematic issues and unveil biogeographic patterns regarding this region. In counterpart, a widespread and generalist species, which can dispute previous biogeographic patterns and landscape barriers, as well as can access potential allopatric (Iberia vs. Africa) and cryptic speciation was not evaluated yet.

This study aims to assess the evolutionary history of *Bufo spinosus* combining mtDNA (historical processes) and microsatellite markers (contemporary processes). Specifically, this study expects to: 1) evaluate previously detected major genetic groups of *B. spinosus*; 2) infer the evolutionary history of the species.

Understanding the evolutionary history of a generalist amphibian as *Bufo spinosus* can shed light to evolutionary process and biogeographical patterns shaping of widespread species throughout the Mediterranean Basin.

2. Material and Methods

2.1 Study area and sampling

Sampling was performed in suitable habitat areas for the species encompassing the entire range of *Bufo spinosus*, except Algeria, obtaining 42 individual toad samples from 17 localities. Additionally, a set of 189 individual samples from 45 localities were obtained from the Tissue and DNA collection of the Museo Nacional de Ciencias Naturales (MNCN, Madrid). Thus, the complete dataset included samples from 231 individuals from 62 localities (Table S1, Figure 5), with 1 to 18 individuals per locality. Tissue samples were collected from larval or adult individuals (roadkills and living animals) and preserved in ethanol prior to DNA extraction.

2.2 Laboratory procedures

Total DNA extraction was performed using Genomic DNA Tissue Kits (Easy spin) following the standard protocol suggested by the manufacturer. Quantity and quality of DNA extracted products were estimated by electrophoresis in a 0.8% agarose gels and visualization in a UV transilluminator device (Bio-Rad). Successfully extracted DNA was used as template in a polymerase chain reaction (PCR) to amplify a set of 11 microsatellite loci (Bspi 3.11, Bspi3.19, Bspi4.14, Bspi4.16, Bspi4.24, Bspi4.25, Bspi3.26, Bspi4.27, Bspi4.28, Bspi4.29, Bspi4.30, Trujillo et al. 2017).

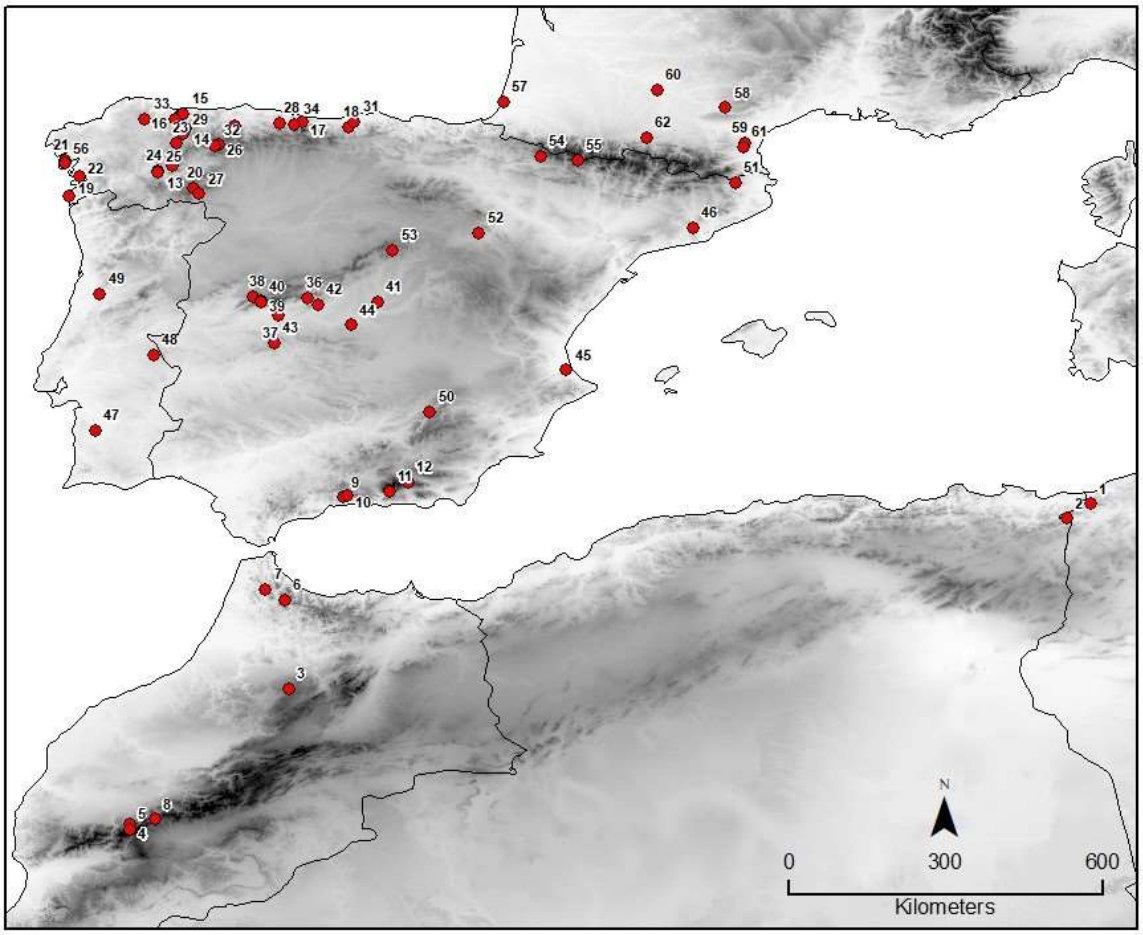

Figure 5: Red dots represent localities of sample collection for this study, and numbers, its respective code (see Table S1).

Loci were amplified in two different multiplex PCR reactions (see Table 1), containing a total volume of 10-11 µl: 5 µl of PCR Master Mix (QIAGEN), 3 µl of distilled H2O, 1 µl of primer multiplex mix, and 1-2 µl of DNA extract (~50 ng/µl). Forward primers were labelled with fluorescent tags (6-FAM, VIC, NED or PET) for visualization purposes. A negative control was always used to identify possible contaminations. PCR touchdown cycling conditions were equal in both multiplex reactions. The reaction started with an initial denaturation at 95°C for 15 min, five cycles with 95°C for 30 sec for denaturation, 1min 45 sec of annealing at 60°C (decreasing 0.5°C each cycle), 72°C for 30 sec for extension, followed by 35 cycles of 95°C 30 sec for denaturation, 58°C for 1 min 45 sec for annealing, 72°C for 30 sec for extension, and ended with a final extension of 30 min at 60°C. PCR amplification was checked by running PCR products in 2% agarose gels and visualization in an UV transilluminator device (Bio-Rad). PCR products were genotyped on an ABI3130XL capillary sequencer (Applied Biosystems) using as size standard LIZ 725 (Nimagen). Allele scoring was performed using GeneMapper version 4.0 (Applied Biosystems).

Table 1: Locus ID, repeat motif, multiplex, tail (fluorescent dye), and volume of each primer in multiplex reaction (Foward [1:10], Reverse, and Tail) for the eleven microsatellite loci developed in *Bufo spinosus* by Trujillo et al. (2017), and used in this study.

Locus ID	Repeat motif	Multiplex	Fluorescent dye	Foward [1:10] (µl)	Reverse (µl)	Fluorescent dye (µl)
Bspi4.27	GATA	M1	6-Fam	0.4	0.4	0.4
Bspi4.24	GATA	M1	Vic	0.6	0.6	0.6
Bspi4.28	CTAT	M1	Ned	0.4	0.4	0.4
Bspi3.11	CTT	M1	Ned	0.6	0.6	0.6
Bspi4.16	GATA	M1	Pet	0.6	0.6	0.6
Bspi4.25	CTAT	M2	6-Fam	0.5	0.5	0.5
Bspi4.14	GATA	M2	6-Fam	0.4	0.4	0.4
Bspi4.30	TCTA	M2	Vic	0.7	0.7	0.7
Bspi4.29	TAGA	M2	Vic	0.5	0.5	0.5
Bspi3.19	ACT	M2	Vic	0.4	0.4	0.4
Bspi3.26	AGT	M2	Ned	0.4	0.4	0.4

The mitochondrial gene cytochrome b (*cyt b*) was amplified for 88 samples using *cyt b* primers used by Recuero et al. (2012) (forward - 5' ATC TAC CTT CAC ATC GGA CGA G; reverse 5' - AGT TTR TTT TCT GTG AGT CC) to identify mitochondrial lineages, and their relationships, within *B. spinosus*. PCR reactions were performed with a total volume of 11 µl: 5 µl of PCR Master Mix (QIAGEN) 3,2 µl of distilled H2O, 0,4 µl of each primer (10 pmol/µl), and 2 µl of DNA extract (~50 ng/µl). PCR conditions followed Recuero et al. (2012): initial denaturation 92ºC for 2min., 37 cycles of 94ºC 30 sec for denaturation, 53ºC 45 sec for annealing and 72ºC 1 min 30 sec for extension, followed by a final extension for 5 min. PCR products were visualized in a 2% agarose gels and an UV transilluminator device (Bio-Rad). Sequencing of successfully amplified samples was outsourced to Genewiz company (https://www.genewiz.com).

2.3 Mitochondrial data and phylogenetic analysis

Mitochondrial DNA sequences were manually edited and aligned in BioEdit (Hall, 1999)and validated regarding their taxonomical status using the BLASTn algorithm (Zhang et al. 2000) available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The *cyt b* dataset consisted in a total of 122 sequences from 62 localities (Table S2). Haplotype quantities and its respective population were obtained in DNAsp and a mtDNA haplotype network was generated in TCS vs. 1.21 using a parsimony method and the resulting network was esthetically improved in TSC BU (Santos et al. 2016).

Mitochondrial phylogenetic relationships were assessed following a Bayesian approach implemented in Beast v2.6.2 (Bouckaert et al. 2014)The best fitting model was tested in Partition Finder 2.1.1 (Lanfear et al. 2017) under the Akaike Information Criterion (GTR+G). A lognormal relaxed clock and Coalescent constant size model was used as tree priors. Markov Chain Monte Carlo (MCMC) analysis was run using 100 million generations and discarding 25% trees after burn-in. The maximum clade credibility summary tree with posterior probabilities for each node using median values was obtained in TreeAnnotator v1.8.2 (Rambaut and Drummond, 2015), and the resulting tree was visualized and edited in Figtree v1.4.2 (Rambaut and Drummond, 2012).

2.4 Microsatellite analysis

Microchecker v2.2.3 (Van Oosterhout et al. 2004) was used to detect potential allele dropout, genotyping errors, and null alleles. For populations with 5 or more individuals, deviation from Hardy-Weinberg equilibrium (HWE) and genetic diversity indices, including observed (Ho) and expected (He) heterozygosity, were calculated in GeneAlex vs 6.51b (Peakall, Rod and Smouse, 2012). Potential linkage disequilibrium across loci across populations were computed in Genepop (Rousset, 2008) and and p-values were adjusted using the false discovery method (Benjamini, Yoav and Hochberg, 1995).

In order to prevent biased results due to many populations having only one individual, measures of allelic diversity were performed and corrected for differences in sample size by using HP-Rare (Kalinowski, 2005). We estimated genetic differentiation between localities as measured with F_{ST} fixation index in Arlequin v3.5. (Excoffier and

Lischer, 2010) This software was also used to perform Analysis of Molecular Variance (AMOVA) to estimate genetic variation in different levels (within, between and among groups). To perform this test, we hypothesized that Africa and European specimens constitutes different genetic groups for microsatellite data.

Genetic structure was analyzed in STRUCTURE (Pritchard et al. 2000), adopting the admixture model and assuming correlated allele frequencies with MCMC chain length of 200.000 interactions, 20.000 burn-in and five replicates per K-value. In this study a hierarchical analysis was performed containing three datasets: A) containing all populations; B) only European populations; C) only African populations. For dataset A, we forced K values ranging from 1 to 2 aiming to check if the potential clusters present in Africa and Europe were fully separated (for example: Africa assigned to one cluster and Europe to the other one). The results of multiple replicates were merged at Clumpak platform (Kopelman et al. 2015). Different methods were explored to infer the best K number of genetic clusters at KFinder software (Wang, 2019): the Parsimony Index (here referred as PI) (Wang, 2019), ΔK method (Evanno et al. 2005) and mean value of Ln Pr(X|K) (Pritchard, Matthew and Peter, 2000).

In order to identify and describe clusters of genetically related individuals, we performed a discriminant analysis of principal components (DAPC) (Jombart and Collins, 2015) implemented in the Adegenet R package (Jombart, 2008). DAPC is a multivariate statistical method in which variance in the sample is partitioned into a between-groups and within-group components, maximizing discrimination between groups. To perform this test, the data is first converted into a PCA (Principal Component Analysis) and then a discriminant analysis identify the clusters. Adegenet evaluates the optimal numbers of PCs (Principal Components) to retain based on calculation of α-score (which measures the proportion of observed discriminant functions and random discrimination). The optimal number of clusters (K) for the DAPC was chosen based on the lowest value provided by the Bayesian Information Criterion (BIC).

3. Results

3.1 Phylogenetic analysis

Bayesian analysis of *cyt b* mtDNA sequences identified two well supported clades (posterior probability > 0,95) (Figure 6). Clade 1 includes five European haplogroups, though not all well-supported. Clade 2 comprises two African well-supported haplogroups. We identified seven haplogroups and a total of 42 haplotypes: four and five haplotypes are distributed in Tunisia (in yellow) and Morocco (in green), respectively, while the 33 haplotypes found across Iberian and France show a moderate spatial genetic structure, with some regions showing admixture between haplogroups (Figure 7). A rare haplogroup (HG3, in pink) appear in Marin, Coruña and Grandas de Salime. The most common haplogroup was HG7, being widespread across Europe and more present in north Spain. Haplogroup 4 (HG4, in red) is mostly assigned to Mayenne and Fougerolle and the Mediterranean portion of France, and Girona, Huesca and Sardenes in Spain. Haplogroup 6 (HG6, in light blue) in Mediterranean coast of Spain and in Sistema Central.

3.2 Microsatellite analysis

From the initial microsatellite dataset, 205 samples from 62 populations were successfully genotyped. No populations revealed linkage disequilibrium and no loci revealed evidence for allele dropout, and presence of possible null alleles was shown in three populations: Valencia (locus Bspi 4.25), Montblanc (locus Bspi 4.25) and Sierra de Gredos2 (locus Bspi 4.30). Significant deviations for HWE were reported for the following populations: Beni M'Tir, Ermidas do Sado, Feija National Park, Laguna Grande de Gredos, Madrid, Montblanc, Sierra de Gredos 1, Tuchan and Valencia due to heterozygosity deficiency. However, the low number of sample size in some populations might be a possible explanation for departures from HWE. Because of deviations from HWE were not consistent across all population, all loci were kept for the analysis.

For allele richness, Pontevedra exhibited the highest value (1.91) and Sierra de Gredos 2 the lowest (1.45), and for private allele richness, Granada, and Feija National Park the highest rates (0.08) while Tuchan and Covadonga showed the lower (zero). The overall indexes for genetic diversity ranged from Na: 11 to 6.09 in Africa (Beni M'Tir, and Feija respectively) and from 7.64 to 4.18 in Europe (Sierra de Gredos 2 and Hecho

respectively); Ho: 0.76 (Covadonga) to 0.58 (Sierra de Gredos 1 and Madrid); uHe: 0.83 (Beni M'Tir) to 0.62 (Sierra de Gredos 1) (see populations in table 2). Although there is a much higher number of populations in Europe than in Africa in our dataset, general mean values of genetic diversity index were slightly higher in Africa (P-AR: 0.05; Na: 8.54; Ho: 0.67; uHe: 0.76) than in Europe (P-AR: 0.03; Na: 5.89; Ho: 0.66; uHe: 0.74), except for AR, where Europe displayed 1.98 and Africa 1.74.

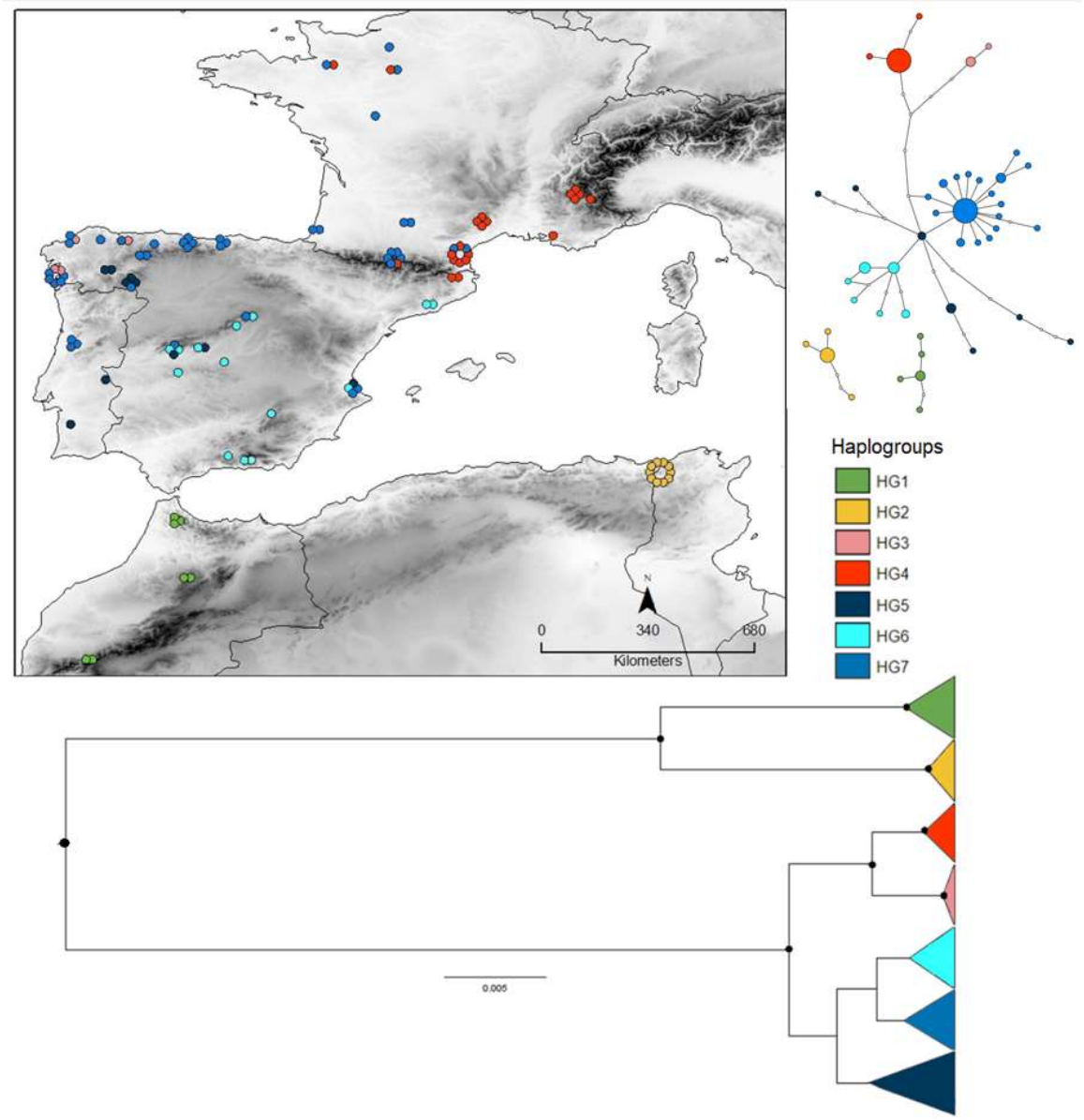

Figure 6: At top-left: representation of the seven identified *cyt b* haplogroups for *Bufo spinosus* containing samples from the present study and samples provided by Recuro et al (2012) available at Genbank. Top-right: Haplotype network inferred by TCS under the 95% criterion, showing the 42 different haplotypes color-coded by seven major haplogroups. The size of each haplotype symbol is proportional to its frequency. Bottom: Bayesian phylogenetic tree based on mtDNA *cyt b* gene. Black dotes identify the nodes with posterior probability higher than 0.95.

Table 2: Sampling information and genetic diversity indexes for each population. C: Country. Pop: Population. N: Number of samples per population. AR: allele richness. P-AR: private allele richness. Na: mean number of alleles. Ho: observed heterozygosity. uHe: expected heterozygosity. TN: Tunisia. Mo: Morocco. PT: Portugal. FR: France. SP: Spain.

C	Pop	N	AR	P-AR	Na	Ho	uHe
TN	Beni M'Tir	18	1.67	0.02	11	0.69	0.83
TN	Feija National Park	10	1.82	0.08	6.09	0.66	0.7
PT	Ermidas do Sado	7	1.79	0.06	6.64	0.69	0.8
FR	Montblanc	10	1.8	0.03	7.27	0.71	0.74
FR	Tuchan	7	4.73	0	5.91	0.64	0.76
SP	Covadonga	5	1.79	0	4.91	0.76	0.79
SP	Granada	5	1.82	0.08	4.73	0.63	0.68
SP	Hecho	5	1.71	0.07	4.18	0.64	0.66
SP	Laguna Grande de Gredos	9	1.77	0.02	6.73	0.72	0.8
SP	Madrid	9	1.76	0.03	6	0.58	0.73
SP	Ordesa	5	1.73	0.01	5.36	0.69	0.79
SP	Pontevedra	5	1.91	0.02	5.55	0.68	0.74
SP	Sierra de Gredos1	6	1.77	0.03	4.64	0.58	0.62
SP	Sierra de Gredos2	10	1.45	0.03	7.64	0.7	0.79
SP	Valencia	11	1.75	0.02	7.09	0.65	0.75

To evaluate patterns genetic differentiation in the dataset, first the AMOVA test was performed by dividing the original dataset with 62 localities based on the two main mtDNA groups, European clade (Iberia and France) and the African clade (Morocco and Tunisia). This test showed significant components of differences between groups (8.03%, F_{ST}: 0.203), between populations (12.36%, F_{SC}: 0.134), and within populations (79.61%, F_{CT}: 0.080). Second, pairwise F_{ST} was performed with 14 populations containing five or more samples each, 12 from Europe and two from Africa (table 3). According to *F* statistics proposed by Wright (1951), the overall significant values for pairwise distances between populations can be considered moderate (0.06 - 0.15) to high (0.16 – 0.25) and ranged from 0.06 (Tuchan and Valencia, Pontevedra and Valencia, Ermidas do Sado and Madrid) to 0.27 (Granada and Laguna Grande de Gredos). In terms of populations, Granada, Feija National Park and Beni M'Tir are those showing highest genetic differentiation.

Table 3: Pairwise *FST* values between localities (below the diagonal) with 5 or more samples of *Bufo spinosus*. Non-significant *p* values are represented by a star (*). 1: Ermidas do Sado; 2: Granada; 3: Hecho; 4: Madrid; 5: Montblanc; 6: Ordesa; 7: Pontevedra; 8: Tuchan; 9: Valencia; 10: Sierra de Gredos 1; 11: Laguna Grande de Gredos; 12: Sierra de Gredos 2; 13: Beni M'Tir; 14: Feija National Park. County of locallity origin: Portugal: 1; Spain: 2 – 12; Tunisia: 13 – 14.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	0.00													
2	0.18	0.00												
3	0.14	0.12	0.00											
4	0.06	0.18	0.08	0.00										
5	0.04*	0.15	0.05*	0.03*	0.00*									
6	0.12	0.20	0.07	0.11	0.05*	0.00								
7	0.07*	0.20	0.10	0.09	0.03*	0.12	0.00							
8	0.10	0.20	0.08	0.10	0.02*	0.02*	0.07*	0.00						
9	0.08	0.14	0.07	0.07	0.02*	0.08	0.06	0.06	0.00					
10	0.07	0.20	0.11	0.07	0.02*	0.16	0.05*	0.10	0.07	0.00				
11	0.11	0.27	0.23	0.13	0.10	0.25	0.14	0.20	0.15	0.02*	0.00			
12	0.11	0.26	0.19	0.15	0.07	0.21	0.15	0.17	0.12	0.12	0.19	0.00		
13	0.18	0.22	0.16	0.21	0.12	0.16	0.18	0.11	0.13	0.15	0.25	0.21	0.00	
14	0.16	0.17	0.12	0.18	0.12	0.16	0.15	0.13	0.10	0.14	0.24	0.20	0.01*	0.00

3.2.1. Genetic structure for dataset A: K=2

For dataset A in which we forced K=2 (figure 7), the genetic signal of Cluster 2 present in African populations, is also present in most of populations in the Mediterranean coast of Iberian Peninsula and France. Cluster 01 is present in most of Iberia, and except for the populations in the Mediterranean coast, little admixture is present in the populations along Iberia and Maghreb.

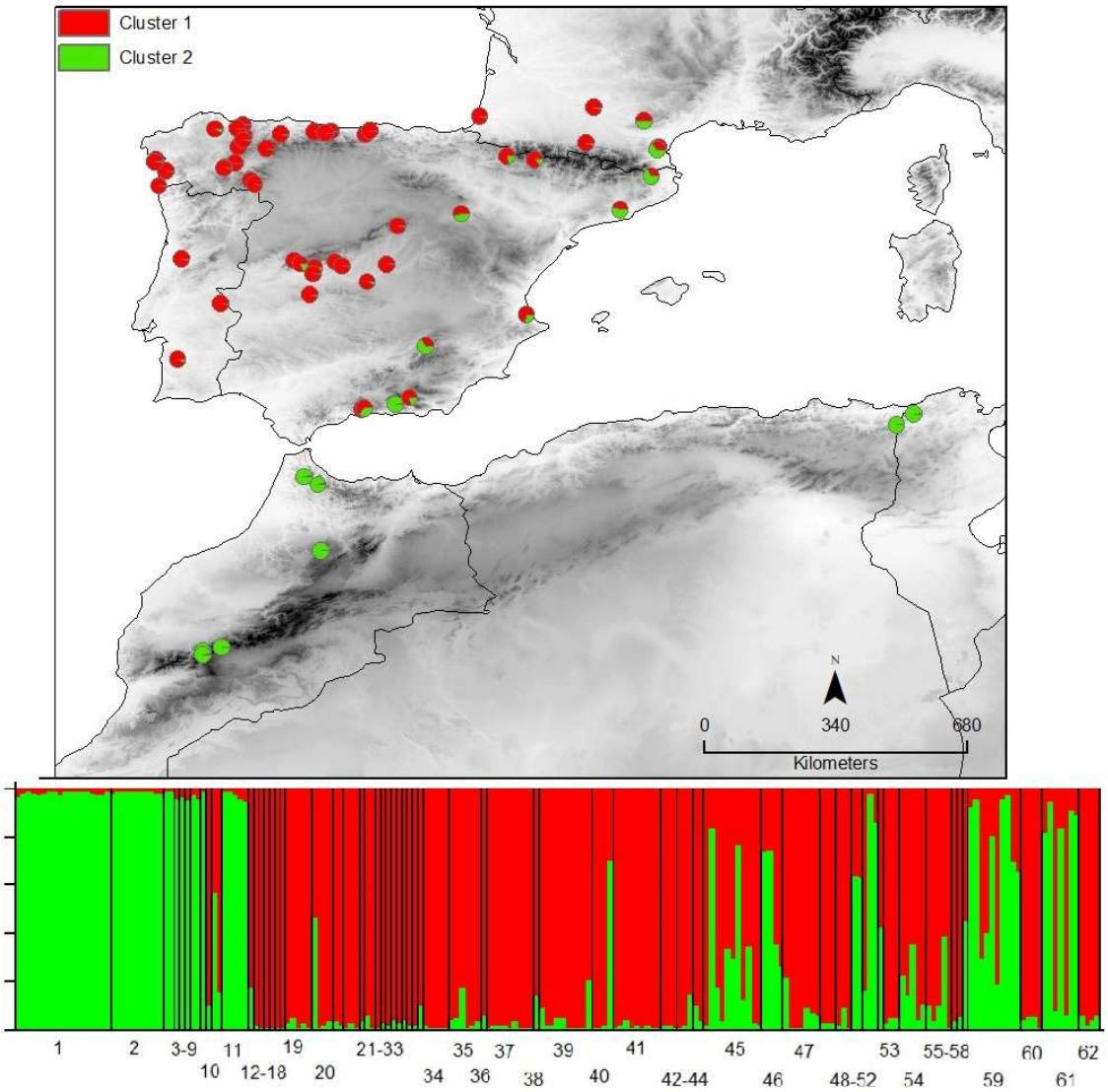

Figure 7: Above: Population Q-membership obtained in Structure and represented in pie charts for K=2. Below: individual Q-membership for K=2.

3.2.3 K values for only European samples (dataset B)

Most of the results obtained for this dataset resulted in K=4 (figure 8) as the most probable number of genetic clusters (Ln Pr(X|K) and PI methods). Under this scenario, great level of admixture can be observed along Iberia, and populations assigned to a single cluster are more concentrated in Cantabria, Asturias, and Galicia regions in Spain. Cluster 1 is widespread across Iberia but present a stronger signal along the Mediterranean coast of Iberia and France. Cluster 2 is more present in north and northwest Iberia, and southwest France. Clusters 3 and 4 are presented in major degree in central Spain and no population present exclusivity for those clusters.

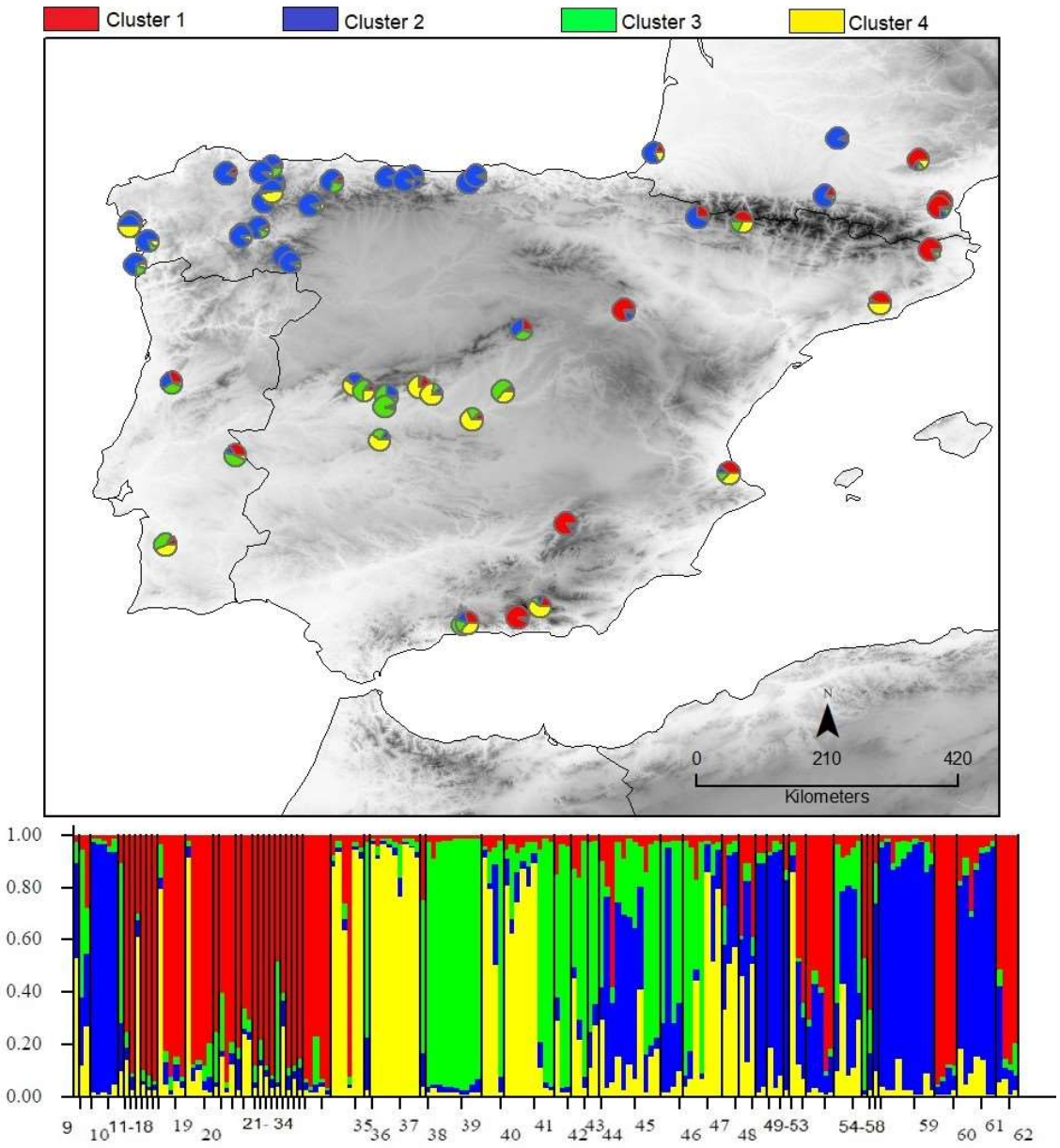

Figure 8: STRUCTURE analysis represented in pie charts at the study area for the most supported cluster membership for Evanno method (K=4). Numbers under barplots represents number of the population.

Evanno method ΔK detected K=2 clusters (figure 9) for dataset B, with widespread signal for the two genetic with signal of admixture clusters throughout the European portion of the study area. Northern Iberia and some localities in France (Iraty Lake, Léon, Beauzelle and Tuchán), Portugal (Buçaco), and Spain (Zaragoza and Jaén) are largely assigned to cluster 2. Cluster 1 is also extensively distributed, showing a strong signal in Sistema Central and along the Mediterranean coast of Iberian Peninsula.

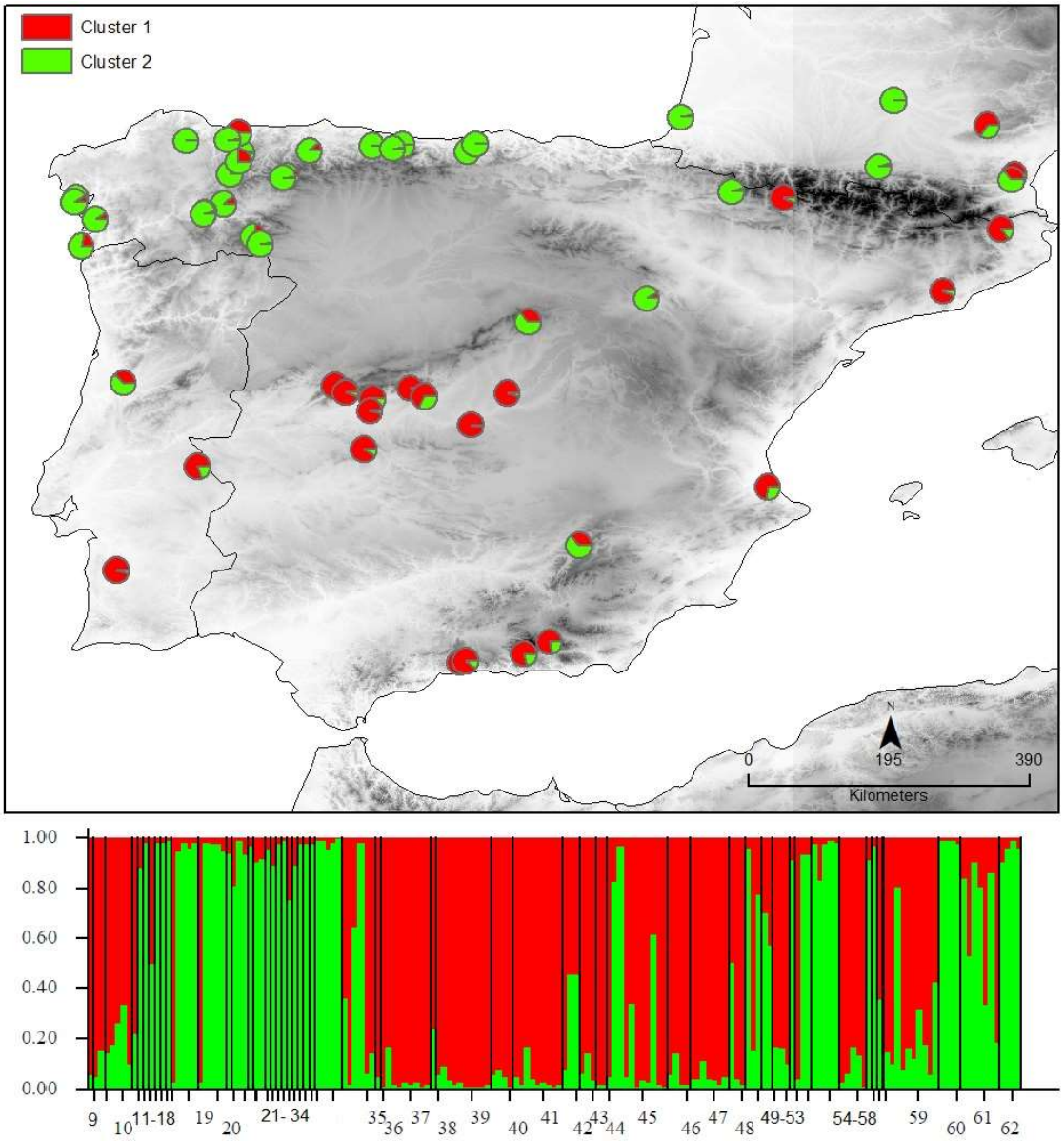

Figure 9: STRUCTURE analysis represented in pie charts at the European portion study area for the most supported cluster membership for K=2. Numbers under barplots represents number of the population.

3.2.3 K values for only African samples (dataset C)

For this dataset, ΔK recovered K=2, displaying admixture, with the two clusters equally distributed among Morocco and Tunisia (Figure 10). The other methods (Ln Pr(X|K) and PI) suggested K=1 for the dataset for Maghreb as the most probable number of genetic clusters, suggesting a single population for our sample sites in this region.

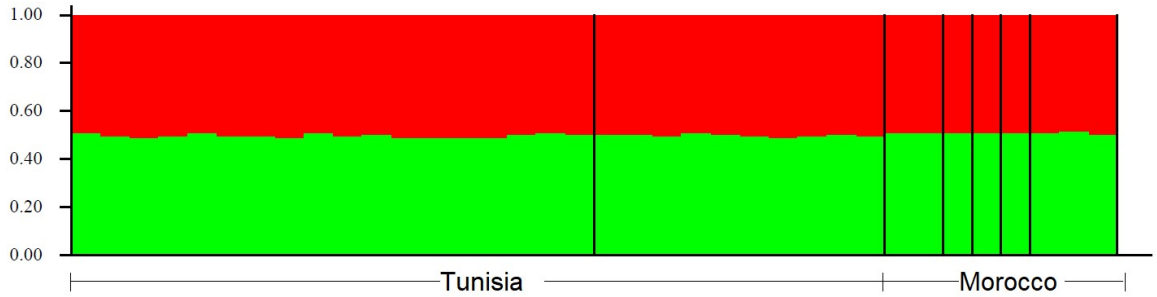

Figure10: STRUCTURE barplot of the most supported number of clusters (K=2) for Evanno's method on dataset C.

3.3. K-means for DAPC

Results of K-means clustering of Discriminant Analysis of Principal Components based on retained 100 PCs, determined K=4 (Figure 11 and 12) to have the lowest BIC value best explaining the genetic data. The scatterplot of DAPC showed a clear separation of African and European populations, with high levels of genetic admixture between the three clusters identified in Europe. Cluster 1 is present in Maghreb and do not show any signal of admixture with populations in Europe, or vice-versa. Cluster 2 is displayed in populations in Portugal, and in the Mediterranean coast of Spain and France, Zaragoza Guadalajara and Hecho, in Spain. Cluster 3 appear in populations in France Ordessa (near Pyrenees in Spain), in Meseta Central region, and in La Coruña, northwest Spain. Cluster 4 also occur in Meseta Central region and northwest Spain.

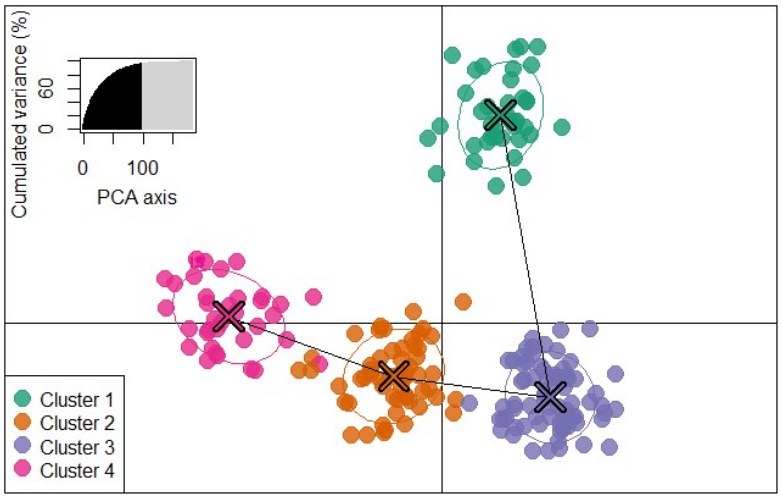

Figure 11: Scatterplot of cluster assignment based on K-mean (K=4) clustering for DAPC scores. Each dot represents a genotyped individual related to a cluster in the same color. Cluster assignment was based on K-mean clustering for DAPC scores.

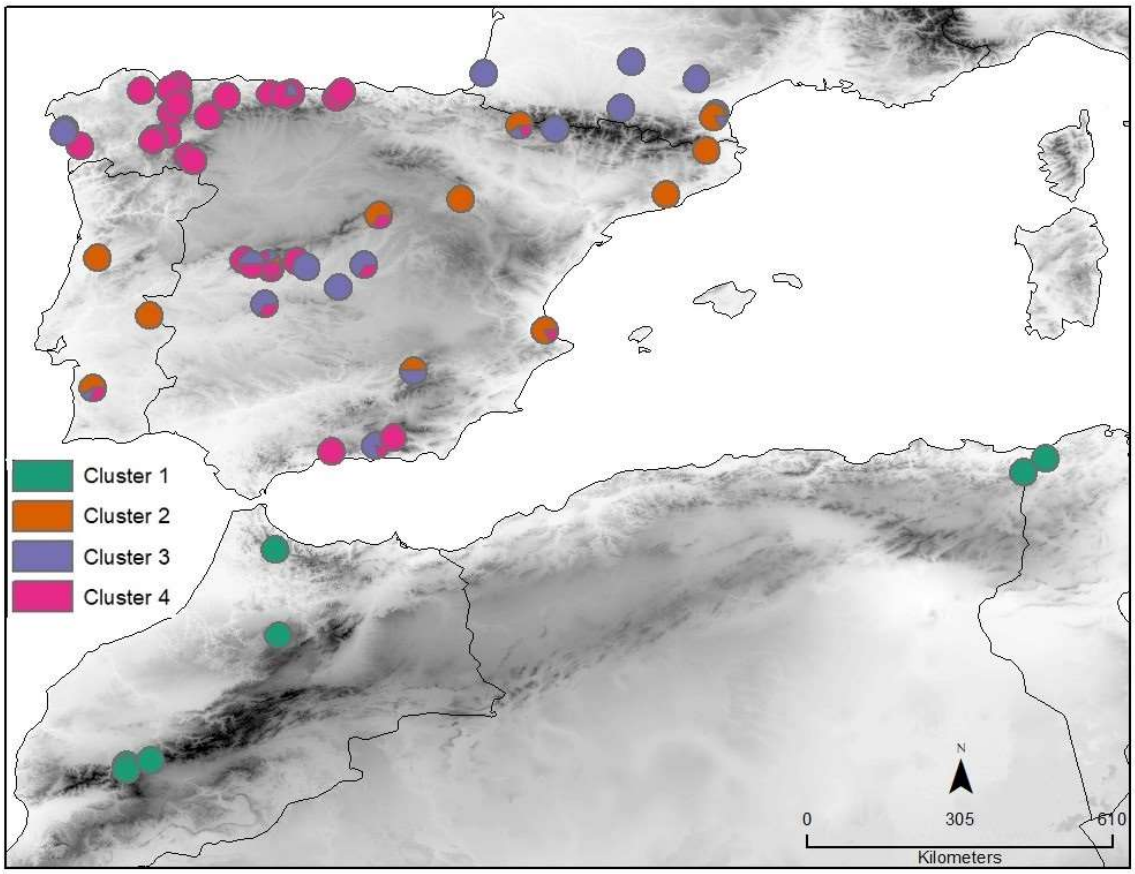

Figure 12: A: Result of DAPC genetic clustering K=4 represented in pie charts at the study area.

4. Discussion

In this study we assessed *Bufo spinosus'* evolutionary history for the first time. We obtained genetic data from microsatellite and mitochondrial markers to perform a series of genetic analyses to identify patterns of genetic structure and diversity across *B. spinosus'* range distribution. Our results revealed a discordance between mtDNA, and microsatellites results regarding samples along the Iberian Mediterranean corridor. On the one hand mtDNA results show three main independent clusters, two in North Africa, one in Morocco, one in Tunisia, and the other one in Europe. On the other hand, genetic structure revealed by microsatellite data shows genetic admixture between the two main genetic clusters along the Mediterranean corridor in Iberia.

Despite the different methods here used to detect genetic differentiation in STRUCTURE, we will focus the genetic structure discussion for datasets B and C using the ΔK Evanno's method, because it might be the most congruent in displaying K values

for genetic clusters yielding lower levels of admixture among clades. Furthermore, according to Wang (2019), is the most accurate method when population is in hierarchical structure at population genetics level**.** Nonetheless, on one hand, DAPC has shown to perform better at broader scale with long-range dispersal species (Blair et al. 2012), on the other hand, it could tend to find artificial groups where there is little population differentiation (Grumer and Leaché, 2017). Besides that, ΔK is less accurate when $K \leq 2$ according to Wang (2019). For this reason, the discussion of K=2 on dataset A, will rely on PI an Ln Pr(X|K) methods. In this study, different methods led to different levels of genetic admixture in populations of *B. spinosus.* Therefore, the results obtained in our research must be interpreted cautiously because the moderate levels of population admixture detected in some populations can represent analytical artefact.

4.1 Genetic structure between Europe and Maghreb

Natural landscape barriers may lead to major evolutionary and ecological consequences for wildlife populations. The Strait of Gibraltar is a classical barrier that enhance genetic structure within taxa (Schmitt, 2007; Husemann *et al.* 2013; Busack, 1986). To our knowledge, the first study testing Gibraltar Strait as a barrier for *Bufo spinosus* was Busack in 1986. He conducted a study including 17 taxa of amphibians (four species) and reptiles (13 species) that occur both in Iberia (Spain) and Morocco. Regarding the genetic distance of amphibians, three out of four amphibian species (*Discoglossus pictus, Bufo bufo and Pleurodeles walt*) showed higher genetic distances with allozyme data between continents than among populations sampled in each country. Our results also demonstrate that for *B. spinosus* (referred as *B. bufo* in Busack, 1986), the genetic distance between Iberia + France and Maghreb are congruent with the one found in Busack (1986).

The mtDNA pattern recovered exhibit a clear differentiation between European and Maghrebian populations, both in the phylogenetic tree and in haplotype network, as in García-Porta et al. (2012) and Recuero et al. (2012). According to the last authors, African populations of *B. spinosus* would have splitted from the Iberian lineage at the end of the Miocene, about 5.3 MY. This period coincides with the end of the MSC, associated with the flooding of Gibraltar Strait and thus, forming a barrier to gene flow for many terrestrian species that had access to North Africa through (Husemann et al. 2014). Such pattern of genetic structure is also well supported by studies performed in other taxa, such as in

reptiles (*e.g.*: *Natrix* (Kindler et al. 2013 and Kindler et al. 2018)*, Natrix maura* (Barata, Harris and Castilho, 2008), *Vipera* (Velo-Antón et al. 2012)*, Blanus* (Albert, Zardoya and García-París, 2007), and amphibians: *Discoglossus* (Zangari, Cimmaruta and Nascetti, 2006 and Vences et al. 2014)*, Alytes* (Martínez-Solano et al. 2004)*.*

By setting K=2 (Blair et al. 2012) in STRUCTURE, we tested if historical disruption in gene flow reflects a pattern of genetic differentiation consistent with the barrier effect of the Strait of Gibraltar. A most probable number of cluster K=2 was supported by Ln Pr(X|K) and PI. Our microsatellite results did not recover African populations as a differentiated genetic cluster. All the analyses tested for K= 2 recovered admixture of the two main clusters along the Iberian Mediterranean coast. Despite previous studies on *B. bufo* suggest the absence of sex-biased dispersal because 93% of females and 96% of males return to the same breeding pounds (Arntzen et al. 2017; Reading, Loman and Madsen, 1991; Smith and Green, 2005), sex biased dispersal for *B. spinosus* cannot be excluded. One scenario to explain this cyto-nuclear discordances is an expansion from Africa to Europe during the late Pleistocene, when sea level decreased dramatically, reaching only 5km wide in Strait of Gibraltar (Zazo, 1999), facilitating the exchange of fauna between continents (Husemann et al. 2014). *Bufo spinosus* might have recolonized the southeastern Iberia and expand northwards along the mediterranean corridor. Episodes of torrential rain could created small islands of soil and vegetation in open sea coming from large rivers, allowing rafting events and overseas dispersal for amphibians (Measey et al. 2007). Despite limitations established by salinity, dispersal capabilities of amphibians across the sea have been previouly evidenced for other taxa across the Strait of Gibraltar (*e.g. Pleurodeles walt* in Gutiérrez-Rodríguez, Barbosa and Martínez-Solano, 2017; *Ptychadena newtoni* in Measey et al. 2007; *Hyla meridionalis* in *Recuero* et al. 2007 and Dufresnes and Alard, 2020; *Emys orbicularis* in Velo-Antón et al. 2015; *Psammodromos algirus* in Carranza et al. 2006, *Cossidula russula* in Cosson et al. 2005). Furthermore, the brackish water do not present an impediment for *B. bufo* to swim in open water in North Baltic Sea (Seppä and Laurila, 1999), indicating that *B. spinosus* might posses a certain tolerance to salinity as well.

Nonetheless, differences in morphology regarding Iberian and African specimens have not been yet evaluated (Figure 17). Integrative analysis including morphological data,

and additional molecular markers would be more appropriate to clarify the taxonomic relationships between African and European lineages revealed by mtDNA.

Figure 17: Left: *Bufo spinosus* from an Iberian population. Right, *B. spinosus* from a Tunisian population. Pictures from Iñigo Martínez-Solano

4.2 Genetic structure of *Bufo spinosus* within Iberia and France

In the Iberia Peninsula, the mountain systems can be considered a historical barrier to gene flow for some species, playing a major role in shaping patterns of genetic structure. However, our lack of sampling for microsatellite data in northern slopes of Central System, in the southern slopes of Cantabria Mountains and along the Betic System prevent us from testing the role of Iberian mountains as permeable barriers along north-south axis for *B. spinosus*. Notwithstanding, our analysis demonstrates mountain systems in Iberia as permeable barriers for this species along east-west axis.

We detected high levels of connectivity both from microsatellite and mtDNA along southern slopes of Central System mountains. Life history traits of the species, such as dispersal rates and use of a wide variety of habitas, probably helped maintain historical and contemporary connectivity across these populations. Beyond, the Central System has been identified as a current barrier for gene flow in north-south axis for *Pelobates cultripes* (Gutiérrez-Rodríguez, Barbosa and Martínez-Solano, 2017b) and in a minor degree, to *Hyla molleri*, *Pelophylax perezi,* and *Epidalea calamita* (Sánchez-Montes et al. 2018).

The Pyrenees played a considerable role limiting species dispersal, but still, several taxa could survive the glacial periods in ice-free areas along the chain that provided glacial refugia (Charrier et al. 2014). Acting as a biogeographical barrier during

postglacial expansions, they promoted genetic structuring along the chain (*e.g. R. temporaria* (Vences et al. 2017)*, L. vivipara* (Milá et al. 2013). *Bufo spinosus* presented a concordant result between microsatellites and mtDNA displaying low levels of differentiations toward east-west and north-south axis, implying that there are passages that allow dispersal events and favors admixture in the Pyrenees. Furthermore, lower lands at eastern and western ends of the mountain range allowed passages of several animals and plants, acting as corridors of migration (Médail and Diadema, 2009; Petit et al. 1998), being a possible route of expansion toward Iberia. However, a proper sampling along Pyrenees and niche analysis would help understand if such pattern of genetic structure might come from glacial refugia within the mountains or if reflect posterior colonization from adjacent climatic refugia within Iberia and France.

Our findings detected regional connectivity the Cantabrian along an east-west axis for *B. spinosus,* probably resulted from population expansion in this direction. Similar genetic patterns, are found in *Chioglossa lusitanica* (Alexandrino et al. 2000), *Rana iberica* (Teixeira et al. 2018b)*,* and *Vipera seoanei* (Martínez-Freiría, Velo-Antón and Brito, 2015). Contrarily, Cantabrian mountains has shown to be an efficient barrier for *Lissotriton helveticus* (Recuero and García-París, 2011)*, Salamandra salamandra* (García-París et al. 2003)*, Lacerta vivipara* (Milá et al. 2010) and *R. temporaria* (Vences et al. 2017)*,* displaying different genetic groups along longitudinal axis.

The Betic Strait was a wide marine channel that was progressively closed in late Miocene, forming the area today occupied by the Guadalquivir River basin. During the Pliocene, uplifts of the Iberia Peninsula formed deep fluvial systems, leading to vicariant events within this region (García-París, Alcobendas and Alberch, 1998) promoting deep genetic structure in many taxa (*e.g. V. latastei* (Velo-Antón et al. 2012), *Discoglossus* (García-París and Jockush, 1999; Vences et al. 2014) and S. *salamandra* (García-París, Alcobendas and Alberch, 1998; Antunes et al. 2018). On the opposite, for our data microsatellite (K=2 on dataset B) and haplotypic diversity for *B. spinosus* suggest south Iberia as a permeable barrier, displaying no genetic structure along east-west axis. Still, there is a large gap of sampling along this region, and a dataset supplied with more localities would endure genetic structure of *B. spinosus* within south-east Iberia.

Our analysis of mtDNA data identified a subtle geographic structure of genetic variation throughout the European range of *B. spinosus.* The haplogroups HG4 and HG7 displaying a star-like shape, presenting high frequencies, and being geographically disperse through east France and in central and north Iberia (HG4), and south France, Girona and Sardenes, in Spain (HG7), are indicative of ancestral population and population expansion. Thus, these low genetic heterogeneity in France, might be due to loss of alleles during post-glacial expansion toward Iberia. Furthermore, the genetic diversity encountered within Iberia suggest that populations did not encountered a considerable quantity of fragmentation during the Pleistocene glaciations, maintaining great haplotypic diversity and little geographic fragmentation (Garcia-Porta et al. 2012). Since the species have shown a generalist behavior before and even during the LGM, tolerating a wide climatic and ecological condition ever since (Bisbal-Chinesta and Blain, 2018), its wide ecological breadth probably helps to maintain high levels of regional connectivity in mountains systems within Iberia.

4.3 Genetic structure of *Bufo spinosus* within Maghreb

In the Maghreb, geographic barriers such as the Riffan corridor, Atlas mountains, Mouluya River basin and Soumman Valley, as well as the climatic cycles in the Quaternary (Médail and Diadema, 2009; Beddek et al. 2018) shaped species range and their genetic structure. Several east-west splits along North Africa have been identified in phylogeographic studies, revealed by distinct mitochondrial haplogroups (e.g. *P. saharicus* in Lansari et al. 2015; *Salamandra algira* in Dinis et al. 2019), and cryptic speciation for other amphibians (e.g. *Hyla meridionalis* and *P. saharicus* in Beddek et al. 2018, *S. algira* in Ben Hassine et al. 2016) (Beddek et al. 2018; Salvi et al. 2018; Dufresnes et al. 2019). We obtained contrasting results regarding African populations. On the one hand, microsatellites did not find evidence of genetic structure within the Maghreb, which might be a reflex of heterozygosity deficiency that could have led to allele fixation in these populations. On the other hand, the mitochondrial *cyt b* gene revealed the occurrence of two independent haplogroups showing a clear split between Moroccan and Tunisian populations, in concordance with previous studies (Garcia-Porta et al. 2012; Recuero et al. 2012). In Tunisia, *B. spinosus* is considered a rare species, being restrict to humid-subhumid forests in the extreme north-western region of the country (Ben Hassine and Nouira, 2012). Likely, these areas served as refugia during past climatic oscillations and still maintain, to these days, a suitable condition for *B. spinosus* persistence. Allopatric differentiation in Maghreb were led by isolation of populations into refugia during climactic

fluctuations (*e.g.* Beddek et al. 2018; Dufresnes et al. 2019) (Huseman et al. 2014). Within Morocco, Atlas Mountains are a major barrier splitting in east-west lineages (*e.g. Mauremys leprosa* in Veríssimo et al. 2016), serving as refugia and areas of diversification (Freitas et al. 2018). However, lack of sampling in eastern slopes of Atlas Mountains in Morocco and in Algeria impede further assessments of what vicariant process might have drove divergency between lineages of *B. spinosus* in Maghreb. Moreover, isolation in glacial refugia in Atlas Mountains could explain the east-west pattern found in *B. spinosus* mtDNA and some other taxa (*A. erithrurus* in Fonseca et al. 2009; *Podarcis* in Pinho, Ferrand and Harris, 2006; *Pleurodeles poireti* in Veith et al. 2004).

Nevertheless, these results should be interpreted with caution because cyto-nuclear discordances can often lead to false evolutionary and taxonomic conclusions (such as mirage of cryptic species in Dufresnes et al. 2020). Factors such as allele homoplasy and ancestral polymorphism may blur phylogeographic patterns. *Bufo spinosus'* cyto-nuclear discordance can result from the longer persistence of ancestral nuclear polymorphism comparing to ancestral mitochondrial polymorphisms, derived from the uniparental inheritance of mitochondrial genome. Because of the stochastic nature of coalescent process in which markers can retain its own genetic signature may led to incomplete lineage sorting (Towes and Brelsford, 2012). Besides, more data would help to clarify allele sharing of microsatellite genetic signature within Morocco and Tunisia. Despite this, lack of comprehensive sampling (both in number and extension) impeded a thorough assessment of patterns of genetic structure and range delimitation for each haplogroup and would clarify which vicariant process led to divergency in lineages from Maghreb. Furthermore, we do not discard the presence of new cryptic lineages across North Africa, particularly south of Atlas Mountains in Morocco, and in Algeria, where new lineages of other generalist species were recently discovered (*e.g.,* Veríssimo et al. 2016; Martínez-Freiría et al. 2017).

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6. Supplementary material

Table S1: List of samples per sites and its respective countries for microsatellite analysis. N: number of samples.

Country	Site	Code	N	Country	Site	Code	N
Tunisia	Beni M'Tir	1	18	Spain	Madrid	41	9
Tunisia	Feija National Park	2	10	Spain	Marin	22	3
Morocco	Ifrane	3	2	Spain	Mijares-Casavieja	36	1
Morocco	Imlil	5	1	Spain	Navia de Suarna	23	1
Morocco	Moulay	7	2	Spain	Ordesa	55	5
Morocco	Oukaimeden	4	1	Spain	Pelahustán	42	3
Morocco	Talassemtane	6	1	Spain	Pontevedra	19	5
Morocco	Tizi n´Tichka	8	1	Spain	Puerto de la Ragua	12	1
Portugal	Buçaco	49	3	Spain	Quiroga	24	2
Portugal	Ermidas do Sado	47	7	Spain	Quiroga cam	25	1
Portugal	Portalegre	48	3	Spain	Rio de Porcos	28	1
Spain	A Seara	13	1	Spain	Rio Tendi	29	1
Spain	Barcelona	46	4	Spain	Sanabria	27	1
Spain	Bres	16	1	Spain	Sierra de Gredos1	35	6
Spain	Cáceres	43	3	Spain	Sierra de Gredos2	39	10
Spain	Caldevilla	17	1	Spain	SW Proaza	30	1
Spain	Canillas de Aceituno	10	2	Spain	Toledo	44	2
Spain	Cantabria	18	1	Spain	Torrelavega	31	1
Spain	Castropol	15	1	Spain	Urbanización Nueva Analucía	9	1
Spain	Covadonga	34	5	Spain	Valencia	45	11
Spain	Curotiña	21	1	Spain	Vilariño del Sil	32	1
Spain	Girona	51	3	Spain	Villablino	26	1
Spain	Granada	11	5	Spain	Xistral	33	1
Spain	Grandas de Salime	14	1	Spain	Zamora	20	5
Spain	Guadalajara	53	3	Spain	Zaragoza	52	1
Spain	Hecho	54	5	France	Beauzelle	57	1
Spain	Jaén	50	2	France	Iraty Lake	62	4
Spain	La Coruña	56	1	France	Léon	60	4
Spain	Laguna de la Nava	40	4	France	Mont Canigou	58	1
Spain	Laguna del Trampal	38	1	France	Montblanc	59	10
Spain	Laguna Grande de Gredos	37	9	France	Tuchan	61	7

Table S2: List of samples per sites and its respective countries for mitochondrial DNA analysis. N: number of samples.

Country	Locality	N	Access number	
Tunisia	Beni M'Tir	6	JN647441	JN647442
Tunisia	Feija National Park	5	JN647443	JN647444
Morocco	Ifrane	3	JN647429	JN647430
Morocco	Imil	1	x	
Morocco	Oukaimeden	1	x	
Morocco	Moulay	2	x	
Morocco	Talassemtane	1	x	
Morocco	Tizi	1	x	
Portugal	Bucaco	2	x	
Portugal	Ermidas	5	x	
Portugal	Portalegre	3	JN647431	
France	Beauzelle	2	JN647420	
France	Bergerie Hôpital	1	JN647448	
France	Fougerolles de Plessis	1	JN647327	
France	French Pyrenees (near Arguenos)	1	JN647435	
France	In between Vautorte and St Denis de Gastines, dept. Mayenne	1	JN647446	
France	Iraty	1	x	
France	Juigny	1	JN647426	
France	La Manouesse	1	JN647428	
France	Montblanc	2	x	
France	Moulin ouest	1	JN647447	
France	Mount Canigou	1	JN647421	
France	Saint Bonnet en Champsaur	4	JN647457	JN647459
			JN647458	JN647460
France	San Esteve ses Rovires	1	JN647453	
France	Sorbs (Ville Vieille)	1	JN647449	
France	Sotch de Caylus	1	JN647450	
France	St. Mars sur la Futane	1	JN647328	
France	St Pierre des Nids near Pre-en-Pail, dept. Mayenne	1	JN647445	
Spain	A Coruña	1	JN647433	
Spain	A Pobra do Caramiñal	1	JN647422	
Spain	Asturias	6	x	
Spain	Avila	8	x	
Spain	Barcelona	1	x	
Spain	Bres	1	x	
Spain	Caceres	2	x	
Spain	Cantabria	1	x	
Spain	35 km NE Capileira	1	JN647425	
Spain	Curotina	2	x	
Spain	Embrun	1	JN647427	

Spain	Fanlo a Escalona	1	JN647438
Spain	Girona	1	x
Spain	Granada	3	x
Spain	Guadalajara	1	x
Spain	Hecho	1	JN647437
Spain	Huesca	2	x
Spain	Jaen	1	x
Spain	Lago Enol. Covadonga	1	JN647454
Spain	Laguna de los Peces (Parque de Sanabria). San Martín de los Gallegos	1	JN647434
Spain	Laguna Grande de Gredos	1	JN647424
Spain	Laújar	1	JN647439
Spain	Leon	2	x
Spain	Majaelrayo a Cantalojas	1	JN647436
Spain	Malaga	3	x
Spain	Marin	2	x
Spain	Navia	1	x
Spain	Pelahustán. Carretera de Cenicientos	1	JN647452
Spain	Plá dels Corrals (Simat de la Valldigna)	1	JN647432
Spain	Pontevedra	3	JN647455
Spain	Quiroga	2	x
Spain	Río Madera. Orcera	1	JN647456
Spain	Rio Tendi	1	x
Spain	Sadernes	1	JN647423
Spain	Sanabria	1	x
Spain	SwProaza	1	x
Spain	Toledo	2	x
Spain	Torrelavega	1	x
Spain	Touchan	1	x
Spain	Valdemanco	1	JN647451
Spain	Valencia	1	x
Spain	Vilarino	1	x
Spain	Villablina	1	x
Spain	Xistral	1	x
Spain	Zamora	1	x