University of Algarve
Faculty of Marine and environmental sciences

Universidade do Algarve
Faculdade de Ciências do Mar e do Ambiente

Biogeography of the Fire Salamander (*Salamandra salamandra*, Linnaeus 1758) in Portugal

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The contents of this report are exclusive responsibility of the Author, and they weren't used to obtain any other academic degree besides this one, in this university or other.

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Á minha família, principalmente os meus pais e ao meu irmão, por serem o pilar que me suporta

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Abstract

Several studies show that the Betic and Messinian crisis may be linked to origin of several Iberian endemic species, while the Pleistocene ice ages cycles are responsible for the fragmentation of species into subspecies and lineages divergence. The present study of the relationships between *Salamandra salamandra* subspecies (*S. s. gallaica* and *S. s. crespoi*) that inhabit the southwestern area of the Iberian Peninsula could contribute to a better understanding of the real impact of these events on the biota. Cytochrome b mitochondrial DNA fragments of 197 individuals from 12 locations were analyzed and a noticeable division was found. Several key nucleotide positions were found as being responsible for this clear division, matching the know distribution of Portuguese salamander subspecies encountered using several analysis (Nested Clade Phylogenetic Analysis, haplotype network and genetic distances), where the center-north populations were grouped into Clade I (*S. s. gallaica* distribution) and south populations were grouped in Clade II (*S. s. crespoi* distribution). The calculated neutrality indexes show that every sampled population is relatively stable and that different types of selection appear to be happening in Alcoutim, Serra da Estrela, Sesimbra and Sintra. Monchique in Clade II and Sintra in Clade I both showed high levels of diversity which associated with its geographical characteristics, support the hypotheses of them being past refugia for these subspecies. Douro and Tejo rivers together with the effects of the Pleistocene glaciations played an important role in the highly structured geography differentiation of *S. s. gallaica* populations, whereas in the case of the *S. s. crespoi* subspecies they seem to have had no effect, as this one presents a more continuous distribution.

Resumo

Vários estudos ligam a origem de várias espécies endémicas da Península Ibérica com a crise Bética e Messiniana, enquanto as idades do gelo do Pleistoceno encontram-se relacionadas com a fragmentação de espécies em subespécies e divergência entre linhagens. O presente estudo das relações entre subespécies de *Salamandra salamandra* (*S. s. gallaica* e *S. s. crespoi*) que habitam a área sudoeste da Península Ibérica, pode contribuir para um melhor entendimento do verdadeiro impacto que estes eventos têm na Biota. Fragmentos de DNA mitocondrial do citocromo *b* de 197 indivíduos pertencentes a 12 localizações foram analisados, encontrando-se uma divisão notória nos dados. Várias posições nucleotídicas chaves foram identificadas como sendo responsáveis por esta clara divisão, coincidindo esta com a distribuição conhecida de cada subespécie portuguesa de salamandra, em que as populações com distribuição centro-norte agrupam-se no Clado I (distribuição da *S. s. gallaica*) e as do sul no Clado II (distribuição da *S. s. crespoi*). Estes resultados foram apoiados por várias análises (NCPA, rede de haplotipos e distancias genéticas). Os índices de Neutralidade calculados mostram que cada população aparenta ser relativamente estável e que diversos tipos de selecção parecem estar a actuar em algumas localidades (Sintra, Serra da Estrela, Sesimbra e Alcoutim). Tanto Monchique no Clado II como Sintra no Clado I mostram grande diversidade genética, o que associado às características ambientais desses locais, permite suportar a teoria de que essas zonas possam ter sido refúgios passados. Os rios Tejo e Douro juntamente com os efeitos das glaciações do Pleistoceno desempenharam um papel fundamental na estruturação e diferenciação geográfica encontrada nas populações da espécie *S. s. gallaica*, enquanto que no caso da subespécie *S. s. crespoi* estas aparentam não ter tido efeito, já que esta subespécie apresenta uma distribuição mais continua.

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I. General Introduction

Phylogeography is concerned with the principles and processes governing the geographical distributions of genealogical lineages, especially those at the intraspecific level (Avise 1998).

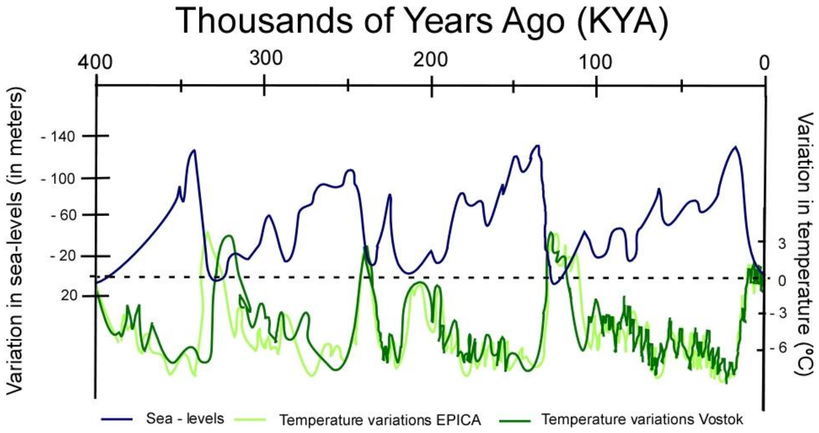

Figure 1 - Variation in sea-level (Waelbroeck et al. 2002), and temperature in the last 500 KY (Petit et al. 1999, EPICA 2004).

Global temperatures have changed dramatically from the beginning of the Tertiary period, some 65.5 million years ago (MYA). The most recent extraordinary global oscillations in temperatures produced the last ice ages marking the beginning of the Pleistocene period (1.8 MYA to 10 thousand years ago). Throughout the ages cyclical features, the Croll-Milankovitch cycles that last around 100 thousand years (Muller and MacDonald 1997) have induced glaciations on the planet, promoting expansion of great ice sheets from the pole that have covered most of the northern hemisphere (Hewitt 1996, Thompson and Russell 2005). The last glacial maximum was 18 thousand years ago (KYA) ending around 12 KYA, marking the beginning of the Holocene which is the present geological period (Committee 2007). Those cold dry periods were spaced by shorter interglacial ages of warmer climate, like the present one, lasting approximately 10 KY each (EPICA 2004, Committee 2007). Warmer temperatures induced enlarged deglaciations that lead a significant increase in sea-level (Figure 1) and consequently large marine transgressions. There are a number of recorded instances where these transgressions have prevented gene flow between amphibian populations. One example is found in Baja California peninsula, where endemisms are a consequence of a series of marine transgressions during the late Neogene (5.5–1

MYA) (Riddle et al. 2000). Another example is the separation of the Betic-Rif mountain belt from the Iberian mainland during the Betic crises that prevented the exchange of terrestrial fauna between southern Europe and Northern Africa (Fromhage et al. 2004).

Recent findings using Artic ice cores (Figure 1), sediment and pollen records show the last warm inter-glacial period occurred 135 – 115 MYA (Petit et al. 1999, EPICA 2004). During this warmer period, strong temperature variations of approximately 7-10 degree Celsius were observed in very short time periods of just a few decades (Hemming 2004). There were obvious consequences of this fact, namely the shifts in species distribution (Hewitt 2004a). The more recent event of dramatic temperature decrease took place approximately 10 KYA and became knew as the "Younger Dryas". It affected oceanic currents at a global level, increasing the size of the polar ice sheets and disrupted the climate. For instance, the tundra reached the region that is presently the south of France, around 47° of longitude. Nevertheless, this event had a small duration in geological terms considering that in the last 6 KY the oceanic currents acquired the present day configuration as well as the geographical distribution of the vegetation biotas (Hewitt 1999).

One of the most relevant effects created by the sharp decrease in temperature and precipitation was the expansion of the ices sheets, which affected both marine and terrestrial habitats alike, forcing the organisms to migrate to lower latitudes (Fedorov and Goropashnaya 1999, Hewitt 2004b; Garzón, Dios and Ollero 2007) while simultaneously establishing barriers to dispersion. In fact, there are a number of published instances (Olalde et al. 2002, Bohme 2003, Deffontaine et al. 2005, Bossuyt et al. 2006, Burbrink et al. 2008) where Biotas at a global scale were affected, leading most of the fauna and flora of these regions to be compressed in area towards the equator. Then, as this cold period ended and territories became available, a post glacial expansion occurred and species migrated from southern refugia to these newly accessible territories, accompanying the rise in global temperatures (Hewitt 2000, Thompson and Russell 2005). As species migrated to occupy new areas, they have encountered empty niches, or niches already occupied by recent colonizers. In the first case where there was no competition for resources a rapid population expansion occurred, which in turn lead to a decline of the genetic diversity in these newly re-colonized areas (Hewitt 2004b). It was observed a decrease in the genetic variability compared to the source population and an increase in homozygosity. In the second instance, where there was competition for the niche, the species either co-existed or one of them was forced into extinction on that location (Steinfartz et al. 2000, Hewitt 2004b). This kind of processes together with the cyclical events of contraction and expansion realized by many species during the ice ages and interglacial periods lead to the accumulation of genetic differences of many lineages frequently ending in speciation. Of particular relevance in terms of species and genetics, is the process of contraction into highly diversified southern refugia that enhanced some species patchy distribution. Thus, the genetic structure of populations was modified by the colonization dynamics and their strong spatial structure is a consequence of that specific past process (Hewitt 2000, 2004b). The population genetic structure of organisms and their geographical distribution are an appropriate tool to propose possible scenarios regarding colonization routes and demographic oscillations of geographical expansions. However, the analysis of these expansions is difficult because each population of each species is affected differently by the topography, and has diverse response capabilities. Comparative phylogeography allows the identification of common events that have affected transversally a number of different species (Hewitt 1996, Provan and Bennett 2008).

II. Main geological events in the Iberian Peninsula

Recent studies have found the existence of several refugia in Europe, located in the southern Iberia, Italic and Baltic Peninsulas (Hewitt 2000, 2004a). All these regions share an irregular and complex topography dominated by mountain areas. The unique geography of Iberia was formed during the last 23 MY, in the Oligocene and Pliocene Periods, while the present structure of the hydrological regime was established at the end of the Quaternary Period (Garcia-Paris et al. 1998, Gomez and Lunt 2007). One paleogeographical event that marked this region was the Betic Crisis that started ca. 15-16 MYA when a Betic sea strait opened, isolating the Betic region from the rest of

Iberia (Duggen et al. 2003). Then, approximately 12-10 MYA a land bridge formed between these two regions connecting them for a brief period in time, due to crustal narrowing of the eastern Betic Sea strait. Later, ca. 8 MYA the Betic sea strait reopened, and during the next 2 MY progressive fragmentation marked this isolated region. This isolation ended during the Messinian crisis (Miocene-Pliocene transition), when the Iberian Peninsula became connected with the North African continent from 5.3 to 5.9 MYA resulting in the closure of the strait of Gibraltar and the drying out of the Mediterranean Sea (Krijgsman et al. 1999, Fromhage et al. 2004). After the end of this crisis, climatic oscillations and the decrease in sea level promoted the emergence of many small islands near the strait, allowing many species to disperse between Europe and North Africa (Carranza et al. 2006).

During the last glacial maximum the Pyrenees were covered in ice preventing the free flow of animals and plants (Davis et al. 2003, Gomez and Lunt 2007). The existence of many endemic species of fauna and flora suggest vicariant events promoted by this geographical isolation caused by the climate change in a topographic diverse area (Thompson and Russell 2005, Gomez and Lunt 2007). Altitudinal range shifts of many species responding to major climatic changes also had dramatic effects, such as the increase in divergent allopatric lineages, some of which are unique to this refugia locations (Provan and Bennett 2008). It is probable that the major extant European species appeared before the ice cycles of the Quaternary Period, thus implying that to fully understand the real impact of these cycles in the biota, one needs to look not at the species level, but at a lower sub-species level. At this level, the relatively recent effects of contraction and expansion in the distribution of the biota will be more evident (Hewitt 1996, Steinfartz et al. 2000).

The diversity of habitats and various types of climate created in part by its irregular topography, enabled the Iberia peninsula to become an important refugium for a number of different species. For example, the Scots pine *Pinus sylvestris* has very restricted habitat conditions, only present in humid altitudes between 1000 and 2000 meters. Due to this stringent habitat conditions, several species and subspecies were formed, two of which in the Pyrenees and other two in the rest of Iberia, reflecting several refugia inside this peninsula (Soranzo N et al. 2000). Another example is the snake *Natrix maura* that has several lineages present throughout Western Europe, and all of these lineages are present in Iberia, showing there the highest diversity as well as the most ancient haplotypes (Guicking D et al. 2002, Barata et al. 2008).

Iberia is under the effect of two different climate types, the North Atlantic on the northwest part and the Mediterranean on the southeast part. This promotes and increases the natural tendency of many species to have patchy distributions, favoring the divergence and the development of new features as well as promoting the increase of genetic divergence (Garcia-Paris et al. 1998, Gomez and Lunt 2007).

The Iberian Peninsula is therefore a privileged geographic area to work in phylogeographic investigations (Steinfartz et al. 2000, Hewitt 2001, Gomez and Lunt 2007), providing important information to assist conservation efforts.

III. Amphibian phylogeography of the Iberian Peninsula

The temperatures and precipitation declined during all ice ages, turning most of Europe into a steppe-tundra land and deciduous forests that once were common throughout Europe became confined to some secluded locations in the Mediterranean Peninsulas (Deffontaine et al. 2005). Due to convergence of a number of characteristics such as irregular topography and climatic diversity, the Iberian Peninsula became a diversified habitat for amphibians. Presently, Iberia is the most species-rich region in Europe (Martínez-Solano and García-París 2005), with eight endemic amphibian species among twenty-eight native ones (Gomez and Lunt 2007).

Discoglossus* and *Alytes

There are two endemic toad genus in this area: *Discoglossus* and *Alytes* (Godinho R et al. 1999). The genus Alytes is composed of five species, four of them existing in the Iberian Peninsula inhabiting temperate forests and prairies with small marshes (Figure 2). *A. cisternasii* is endemic to the southeastern region of the peninsula*,* while *A. dickhilleni* inhabits the south region. *A. muletensis* is endemic to the Balearic islands and the species *A. obstetricians* is more vastly distributed, being also present in Western Europe (Gomez and Lunt 2007).

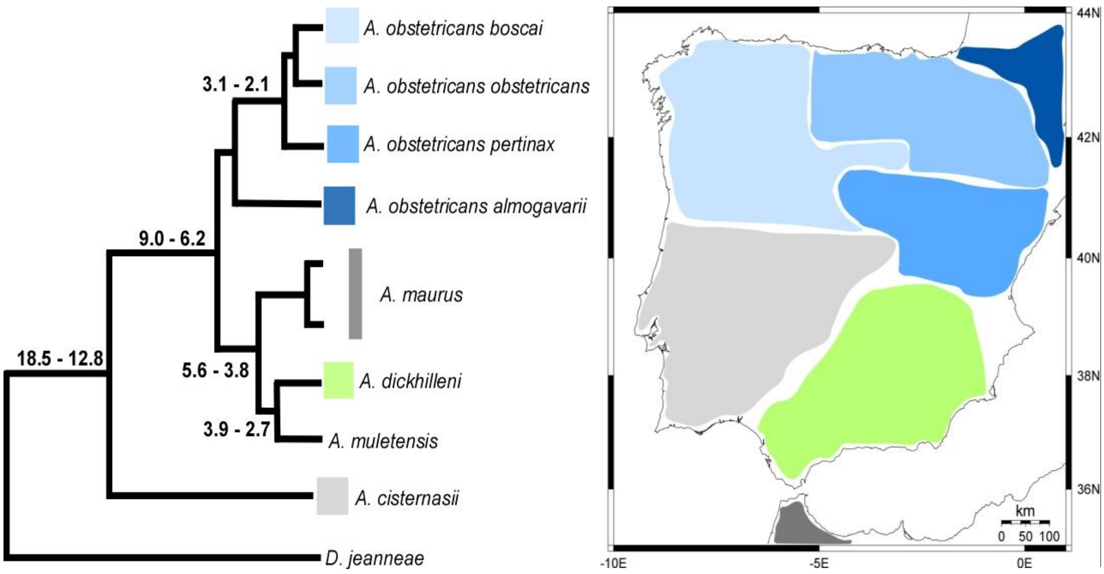

Figure 2 - Representation of main phylogenetic clades of *Alytes* spp. present in Iberia (on the left, with numbers representing MY) and their geographic distribution (on the right), based on protein data by Martínez-Solano et al. (2004). Only clades with high bootstrap support (>70%) are represented.

Recent studies (Martínez-Solano et al. 2004) proposed a biogeographic scenario to explain the current distribution of the genus in Iberia (Figure 2). The formation of large salt lakes throughout Iberia, 18.5 - 12.8 MYA (Monzón et al. 1989) prompted the fragmentation of the ancestor of the extant species into two lineages: *A. cisternasii* and the common ancestral of all other Iberian *Alytes* species. This last group spread thorough Iberia, occupying also the Betic massif (ca. 14 MYA). Later, upon the formation of the Betic strait, the population inhabiting the massif became isolated from Iberia (9 – 6.2 MYA). Due to the reopening of the Strait of Gibraltar (ca. 5 MYA) (Duggen et al. 2003), the isolated southern population originated the current species *A. maurus* in North Africa and *A. muletensis* in the Balearic islands. The same ancestral of A*. muletensis* also differentiated into the present southern Iberian species *A. dickhilleni*. Later (3.1 - 2.1), *A. obstetricians* suffered a fragmentation in Iberia where it originated three sub-species, *Alytes o. obstretricans*, *A. o. boscai* and *A. o. pertinax* (Figure 2). This fragmentation into subspecies is probably linked with the formation of main fluvial drainages areas in the Iberian Peninsula or perhaps is a consequence of the Pleistocene ice ages. However, recent indications of multiple refuges of this species during ice ages give more support to this latter hypothesis (Gomez and Lunt 2007).

The *Discoglossus* genus has a more recent divergence history with two endemic Iberian species, *D. galganoi* and *D. jeannean*. This latter species prefers basic limestone and gypsum substrate inhabiting the central-east Iberian region, while *D. galganoi* prefers granitic or shale substrate that exist in west-central Iberian area. Both species display different levels of population structure, with *D. galganoi* displaying a more geographically structured genetic diversity (Gomez and Lunt 2007) divided into two groups, a northern group and a central-south group that separated around 1.1–0.4 MYA (figure 3). The central-southern group is further divided into a central and a southernmost subgroup, whose separation dates back to 0.2 MYA. Recent hypotheses associate these divergences with the Pleistocene Ice ages (Martinez-Solano 2004).

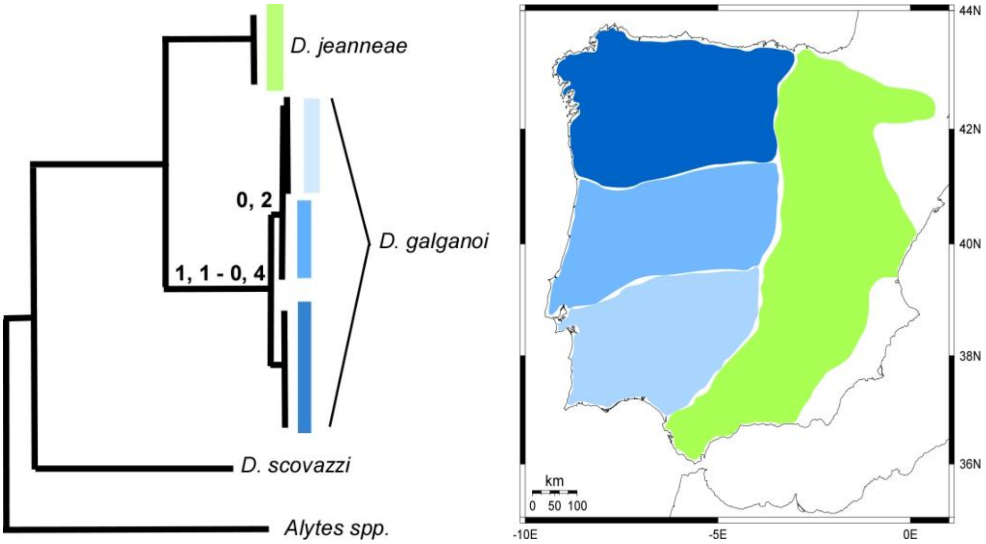

Figure 3 - Representation of main phylogenetic clades of *Discoglossus* spp. present in Iberia (on the left, with numbers representing MY) and their geographic distribution (on the right), based on data by Martinez-Solano (2004). Only clades with high bootstrap support (>70%) are represented.

Lissotriton

The genus *Lissotriton*, formerly known as *Triturus* (Garcia-Paris M et al. 2004, Steinfartz et al. 2007), presents four species inhabiting the deciduous forests of the Iberian Peninsula. *L. helveticus* inhabits the north area around the Pyrenees, while the western-central region of Iberia is inhabited by endemic *L. boscai*. The marbled species *L. marmoratus* exists in the north of Iberia and west France, and *L. pymaeus* is distributed in the south of the peninsula (Figure 5). The more basal split in Lissotriton, happened 44 - 48 MYA, between the marble species (*L. marmoratus*, *L. pymaeus*) and all other Iberian species. Later, the common ancestral of *L. helveticus* (more basal species) and *L. boscai* diverged ca. 20-22 MYA, during the Miocene, while the three other species lineages separated much more recently. Both the marbled newts diverged around 5 MYA during the time of the Messinian crisis and the formation of main fluvial drainages areas in Iberia (Doadrio 1988, Martinez-Solano et al. 2006, Steinfartz et al. 2007).

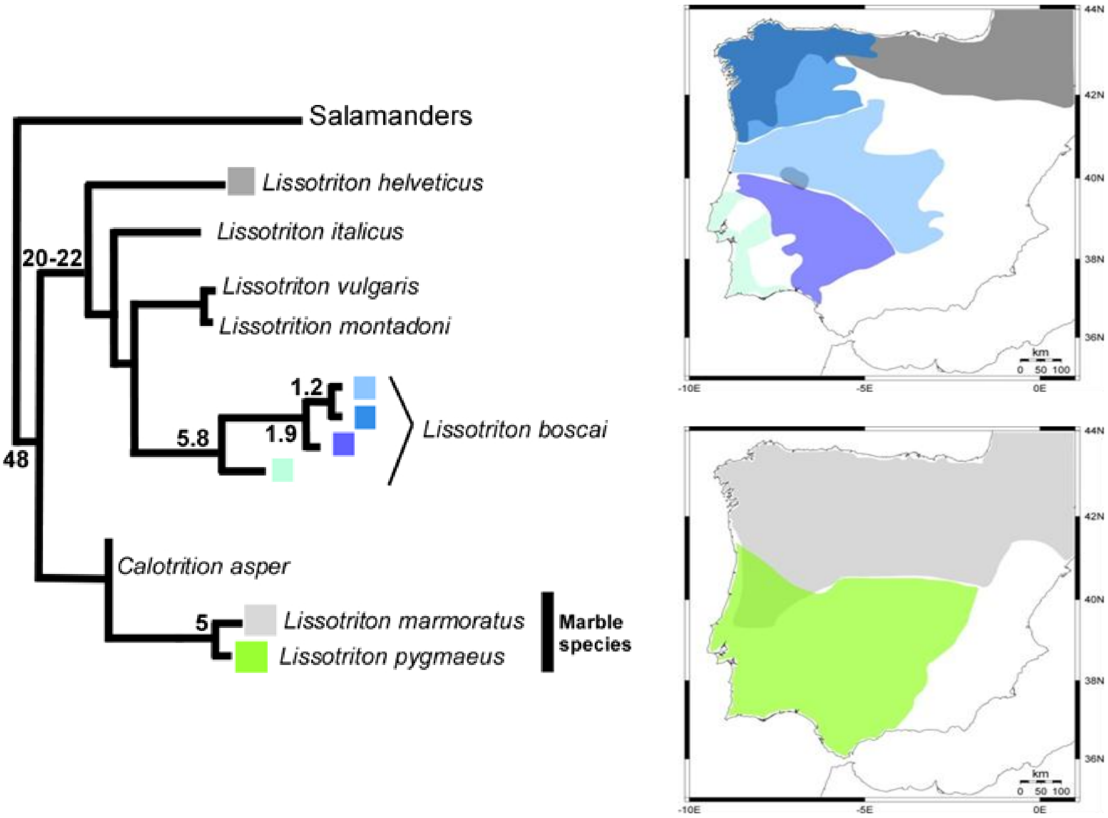

Figure 4 - Representation of main phylogenetic clades of *Lissotriton spp.* present in Iberia (on the left, with numbers representing MY) and their geographic distribution (top right are the marbled species and bottom right are *L. helveticus* and *L. boscai*.), based on data by Martinez-Solano *et al.* (2006), and Steinfartz *et al.* (2007). Only clades with high bootstrap support (>70%) are represented.

L. boscai shows a very strong geographic genetic structure associated with two distinct lineages separated during the Messinian crisis (5.8 MYA). One lineage inhabits the southwestern area of Iberia while the other occupies the rest of the Iberian Peninsula, and shows a distinct division into three clades (Figure 5) that are a consequence of the Pleistocene ice ages according to recent hypotheses (Martinez-Solano et al. 2006). The first clade inhabits the central-south area of Portugal having diverged approximately 1.9 MYA, while the other two clades diverged more recently around 1.3 MYA (Martinez-Solano et al. 2006).

Pleurodeles

The *Pleurodeles* genus has two species present in Iberia: *P. walt* and *P. poireti* (Figure 6). *P. walt* occupies most of Iberia and Moroccan area in North Africa and shows a characteristic genetic structure divided into two groups, a southeastern group that inhabits the south region of Iberia and North African region, and a Northwestern group that occupies the rest of the Iberian Peninsula. *P. poireti* is only present in North Africa, but also has two main lineages (Figure 6).

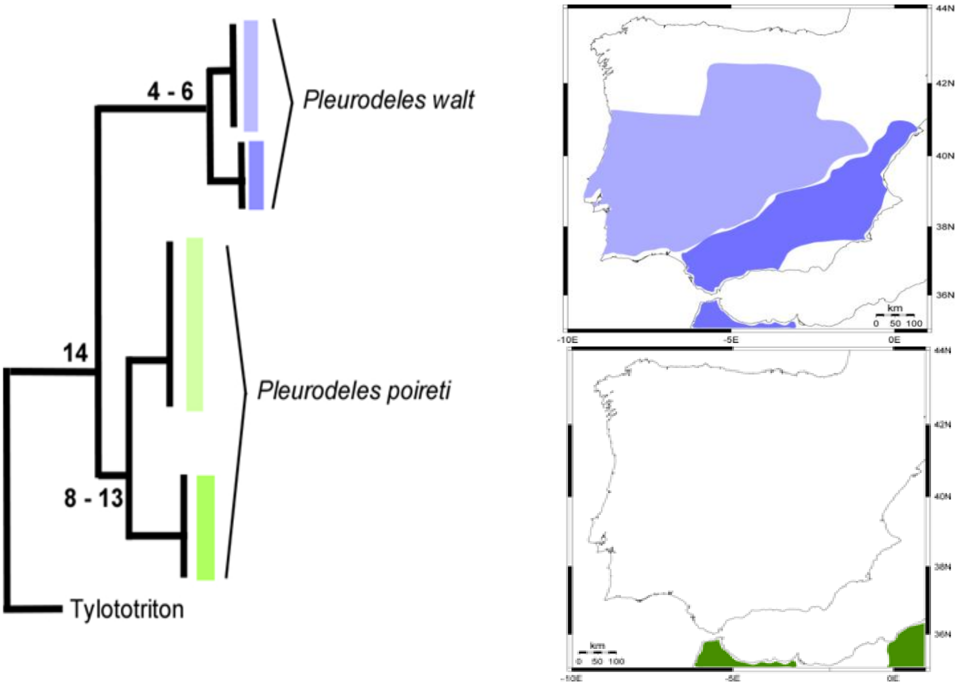

Figure 5 - Representation of the main phylogenetic clades of *Pleurodeles spp.* present in Iberia (on the left, with the numbers representing MY and considering the divergence between *P. walt* and *P. poireti* a consequence of the Betic crisis) and their geographic distribution (on the right), based on data by Veith *et al.* (2004). Only clades with high bootstrap support (>80%) are represented.

Recent hypotheses using the divergence between *P. walt* and *P. poireti* as a consequence of the Betic crisis (ca. 14 MYA ago), point to a less precise separation of the two lineages of *P. poireti* (8-13 MYA). While, the divergence of the two *P. walt* groups is more recent, ca. 4 to 6 MYA and is associated with the Messinian crisis event (Veith et al. 2004).

Pelodytes and *Pelobates*

Pelobates has one representative in Iberia, *Pelobates cultripes*, while *Pelodytes* has two, *Pelodytes Ibericus* and *Pelodytes punctatus*, but all occupy the southern Iberia region (Figure 7). If the disjunction between *P. cultripes* and *P. varaldii* is calibrated using the Betic crisis (ca. 15 MYA), then the split between *P. Ibericus* and *P. punctatus* is placed in a large time frame, between 1-7 MYA. But a recent discovery that *P. Ibericus* is divided into two groups has to be taken into account since its relationship to the other species of this genus is yet to be determined (Veith et al. 2006).

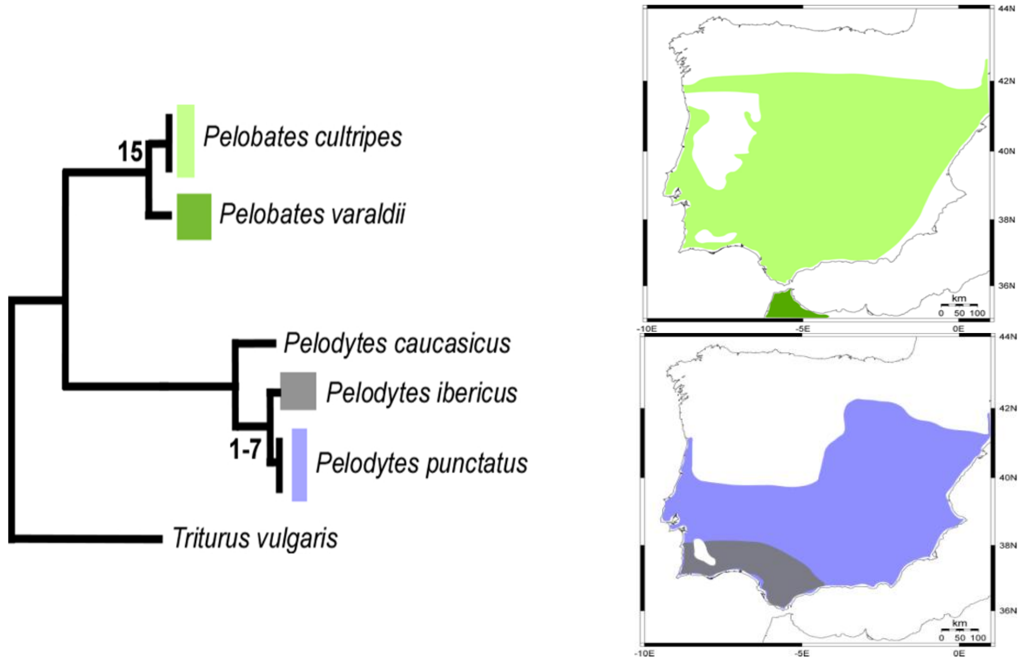

Figure 6 - Representation of main phylogenetic clades of *Pelobates spp.* and *Pelodytes spp.* present in Iberia (on the left, with numbers representing MY) and their geographic distribution (on the right), based on data by Veith *et al.* (2006). Only clades with some bootstrap support (>63%) are represented.

Rana iberica

Rana iberica is another Iberian endemism that inhabits the central-northwestern zone of Iberia, having a high haplotype diversity associated with a strong geographic structure.

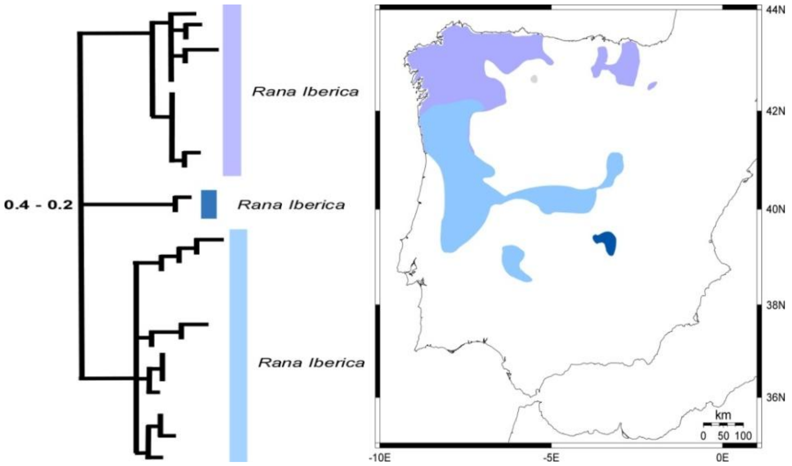

Figure 7 - Representation of main phylogenetic clades of *Rana Iberica* present in Iberia (on the left, with numbers representing MY) and their geographic distribution (on the right), based on data by Teixeira (2007). Only clades with high bootstrap support (>70%) are represented.

This species shows three distinct mitochondrial lineages, all product of a recent divergence (0.4 - 0.2 MYA). One lineage occupies the northwestern area of Iberia, another the central Iberia and the last lineage occupies a secluded zone in Sierra de Guadalupe (Figure 4). Both the Northern and central lineages show distinct signs of recent population expansion, exhibiting a star-shaped haplotype network and a significant neutrality tests (Teixeira 2007).

Salamandridae

The *Salamandridae* family has several Iberian endemic species and subspecies, in which two genus are more relevant, *Salamandra* and *Chioglossa*. We will focus in detail in a section further ahead on the *Salamandra* genus, and here we will refer only to the sole representative of the genus *Chioglossa*, *Chioglossa lusitanica*. This species inhabits streams of moist forest situated in the northwest region of the peninsula (Almeida et al. 2001).

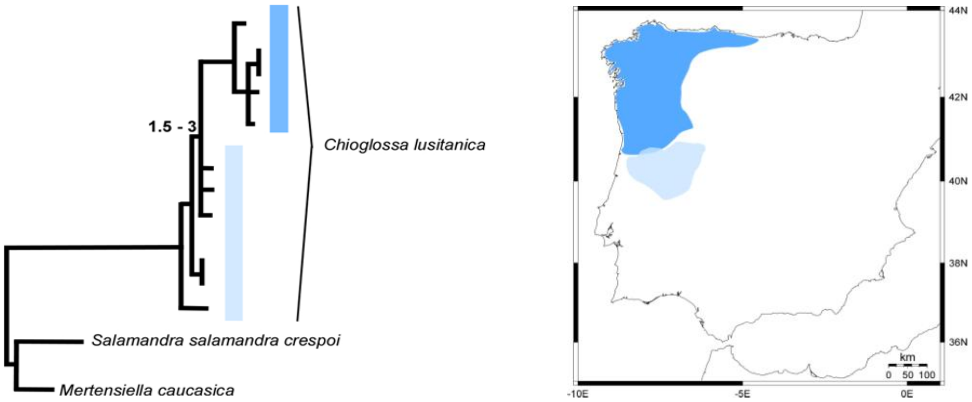

Figure 8 - Representation of main phylogenetic clades of *Chioglossa lusitanica* present in Iberia (on the left, with numbers representing MY) and their geographic distribution (on the right), based on data by Alexandrino *et al.* (2007), and Sequeira *et al.* (2005). Only clades with some bootstrap support (>60%) are represented.

Recent studies found a genetic subdivision into two groups (Figure 8) according to geographical location. These two groups diverged during the early Pleistocene at 1.5 – 3 MYA, probably due to the Pleistocene ice ages. Small events of secondary contact between the two groups may have occurred since limited spatial introgression is observed. It was also determined that each of these groups had a different center of origin, or glacial refuge, each on an opposite side of the Mondego River. It was also determined that both the Mondego and the Douro rivers functioned as barriers to gene flow (Alexandrino et al. 2000, Sequeira et al. 2005, Alexandrino et al. 2007).

III a. Brief conclusion of the Iberian amphibian phylogeography

Most of the Iberian endemic amphibians show signs of multiple divergence and long term vicariance, exhibiting in many cases a split into northern and southern clades. Several species emerged during the Betic and Messinian crisis, while the Pleistocene ice ages of the last 100 thousand years seem to be responsible for the fragmentation of species into subspecies and lineages divergence (Hewitt 2000, Gomez and Lunt 2007). Many of the species that emerged from these divisions had several hypothesized refugiums. The main ones are on the base of the Pyrenees, the Betic regions, Serra da Estrela and the Central Iberia (Figure 9). This locations acted as centers of dispersion for many species presently widespread throughout Europe (Hewitt 1999, Gomez and Lunt 2007).

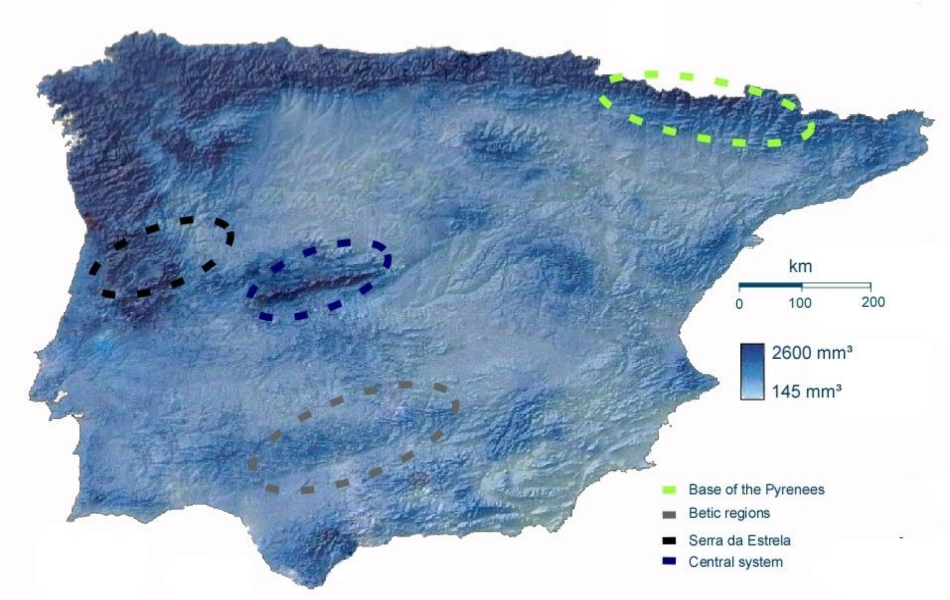

Figure 9 – The base map shows the distribution of annual precipitation in a blue scale on top of the Iberian topographic regime and is based on Teixeira (2007). The circles highlight the zones where numerous studies place the existence of Iberian refugiums for several European amphibians.

These locations all share an irregular topography coupled with high precipitation values, which appear to be key environmental characteristics for amphibians (Gomez and Lunt 2007, Teixeira 2007). Forecasts for the precipitation values in Iberia during last glacial maximum indicate that during this period, besides the decrease in temperature there was also a significant decrease in precipitation, with the present lowest precipitation values matching the highest forecasted for that ice age. Significant is the fact that during that cold period the Iberian recorded refugia locations displayed the higher precipitation values (Garzón, Dios and Ollero 2007, Teixeira 2007).

It is also noteworthy that amphibians tend to display strong genetic variation between populations and genetic structure patterns reflecting ancient rather than recent processes (Martínez-Solano et al. 2004, Veith et al. 2004, Martinez-Solano et al. 2006, Veith et al. 2006, Recuero et al. 2007). This phenomenon coupled with their characteristic philopatric behavior and low vagility, make this group very suitable as model organisms to study the effects of geological events on the genetic structuring of populations (Martínez-Solano and García-París 2005).

IV. General description of *Salamandra salamandra*

The species *Salamandra salamandra*, Linnaeus 1758, commonly named as Fire Salamander in Europe and as "Salamandra-de-Pintas-Amarelas" in Portugal belongs to the True Salamanders Clade (Order Caudata; Family Salamandridae) (Almeida et al. 2001, Steinfartz et al. 2007).

It is a long-lived urodelo (tailed) amphibian and iteroparous (producing offspring in successive cycles or seasons) species, widely distributed across central and west Europe. The *S. salamandra* is characterized for having a big large flat head with dark eyes and a slender build, with dark smooth and glossy skin. Its morphological relevant features are its size that can reach 21 centimeters, and the partially distinct basic patterns formed by its yellow spots that can be characteristic of certain regions (Malkmus 2004, Rebelo Personal Communication 2008). Like most other members of the Salamandridae family, the *S. salamandra* characterizes itself for showing a dual life cycle, with a water dependant larval period and an adult period with mix dependence between the aquatic and terrestrial environment (Rebelo and Leclair 2003, Schmidt et al. 2007, Vences and Köhler 2008). The brood can be biannual or annual, being mainly biannual in sub-species that live in mountain areas. However, it is evident that this aspect of the life cycle is variable and presumably a method of response used by this species to adapt to the habitat unpredictability (Rebelo and Leclair 2003). Matting is usually nocturnal and the fertilization internal, happening outside the aquatic environment because the individuals are poor swimmers. The larval period extends from one to six months and at this stage larvae are carnivorous, feeding mainly on small aquatic invertebrates while the adult form feeds primarily on macro invertebrates that dwell on habitat floor (Almeida et al. 2001, Rebelo and Leclair 2003, Rebelo Personal Communication 2008). The larval state of the species has a stream-dwelling nature with a large head, prominent external gills and a low dorsal crest, showing a grayish our brownish coloration with dark spots upon hatch. During their development these dark spots became wider and more distinct, while white spots emerge as well. Then, during the metamorphosis process this white spots acquire its yellow color, giving this species its characteristic appearance (Garcia-Paris et al. 1998, Almeida et al. 2001, Malkmus 2004).

The Iberian Peninsula represents the west limit of distribution for the *S. salamandra* (Figure 10), and in this location this species can be found in various types of environments that range from wet-mesic forest, to arid scrubland and dry steppe (Griffiths 1996, Rebelo and Leclair 2003, Malkmus 2004). The abundance of this species shifts due to local conditions, and the geographic distribution is patchy due to their limited dispersal abilities and environment requirements (Garcia-Paris et al. 2003). Nevertheless, this species expresses a great adaptability existing in several biomes, and apparently is only restricted by the conditions of humidity that it needs to complete its life-cycle. It exists more abundantly in mountain areas with annual precipitation above 800-900 mm of water per cubic meter, and is present in all of Portugal vertical dimension. It also has a preference for mountain areas with small foothills and stumpy herbaceous vegetation (Griffiths 1996, Rebelo and Leclair 2003, Malkmus 2004). The northern wet Atlantic climate with increased precipitation and humidity (Figure 9) could help explain the abundance of this species in the northern area of the region. It also would explain the presence on that area of many other amphibian species, some of them endemic and phylogenetically close to *S. salamandra*, like the taxa *Chioglossa lusitanica* (Godinho R et al. 1999, Rebelo and Leclair 2003). In Portugal, *S. salamandra* usually starts its period of activity with the arrival of the first autumn rains in the start of October, extending it until the beginning of the summer in April/May. This species is therefore active during the autumn and winter months and inactive during the hot summer period. It also shows crepuscular and nocturnal behavior, sometimes doing small migrations on rainy days and nights, but being in general territorial (Almeida et al. 2001, Rebelo Personal Communication 2008). Most of these features are common with other species of *Salamandra* found on other locations, like Central Europe and Israel (Rebelo and Leclair 2003).

According to the *IUCN - Red List of Threatened Species* and the Berna Convention, the *S. salamandra* is classified as "Least Concern" meaning that it is not a threatened species. This is due to the wide area of distribution, high abundance in some locations, great tolerance in terms of habitat and for not having a specific impending threat upon them (http://www.redlist.org/). The major threats that this species faces are the fragmentation of the habitat, and its huge susceptibility to pollutants like most other amphibians (Griffiths 1996).

V. The *Salamandra salamandra* complex in the Iberian Peninsula

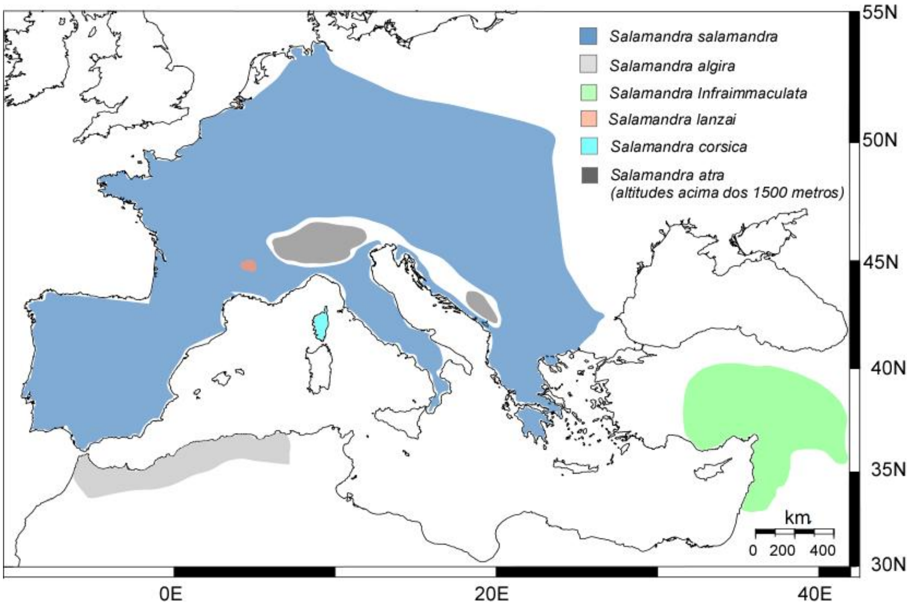

Figure 10 – Map with the current recorded distributions of *Salamandra* species in Europe (based in Steinfartz *et al.* (2000) and North Africa.

Recent studies show that the genus *Salamandra* has arisen from the divergence of the same ancestral lineage of the genus *Mertensiella* around 25 MYA, and that the European genetic entity of *Salamandra* diverged from its African sister 8 MYA when the Betic strait reopened. In Europe (Figure 10), this entity suffered a radiation originating the four European species of *Salamandra* and its many sub-species (Griffiths 1996, Godinho R et al. 1999, Steinfartz et al. 2000, Garcia-Paris et al. 2003).

All species of *Salamandra* show a different divergence history, but all started to diverge before the glacial cycles of the Pleistocene and after these cycles all started to expand their reach from southern glacial retreats, thus re-colonizing Europe (Garcia-Paris et al. 1998, Steinfartz et al. 2000, Garcia-Paris et al. 2003).

Of the sixteen recorded sub-species existing in the Mediterranean area, nine are present on the Iberian Peninsula, but the range of distribution of each sub-species is still an unresolved matter, due to his high polymorphism both in terms of morphology and genetics, leading to the creation of the so called *Salamandra* complex and the classification of this species as polytypic (Steinfartz et al. 2000, Garcia-Paris et al. 2003). This separation is even more unclear because cases of individuals with hybrid characteristics were recorded (Garcia-Paris et al. 1998, Steinfartz et al. 2000, Rebelo and Leclair 2003, Rebelo Personal Communication 2008). Recent studies in the Iberian Peninsula concerning the diversity of sub-species of *S. salamandra* determined that the southern sub-species *S. s. longirostris,* was the most basal sub-species (Garcia-Paris et al. 2003, Steinfartz et al. 2000).

The two sub-species of Fire Salamander present in Portugal diverge mainly in size and color pattern. The *S. s. gallaica* individuals are generally smaller, and normally display irregular big yellow spots which can sometimes have small touches of red in them, while *S. s. crespoi* has a regular pattern of small yellowish spots (Figure 12). Nevertheless, these distinctions are at the extreme of a range, and are only clear in individuals from isolated populations. Therefore, this morphological distinction of the two subspecies is often difficult to distinguish (Almeida et al. 2001, Malkmus 2004, Rebelo Personal Communication 2008).

Figure 11 – On the left is *Salamandra salamandra gallaica* from Serra da Estrela, and on the right is *Salamandra salamandra crespoi* from Monchique (Malkmus 2004)

Relevant for the present work are the findings of Steinfartz *et al*., (2000) and Garcia-Paris *et al*., (2003) concerning the relationships between *S. s. gallaica* and *S. s. crespoi*. In Figure 11 we have represented the *S. salamandra* clade recognized by Garcia-Paris *et al*., (2003) and all significant clades involving subspecies of salamander in continental southwest Europe and Africa identified by Steinfartz *et al*., (2000). According to the findings of this two studies *S. s. crespoi* and *S. s. gallaica*, with the exception of *S. s. gallaica* of G5 (Figure 11-I), are not grouping together, and thus do not share the same most recent common ancestor. This *S. s. gallaica* specimen of group G5 (Figure 11-I, *S. s. gallaica*[3]*)* was collected in Santiago do Cacém, a locality that lies clearly within the geographical distribution area described for *S. s. crespoi* and was most likely misidentified.

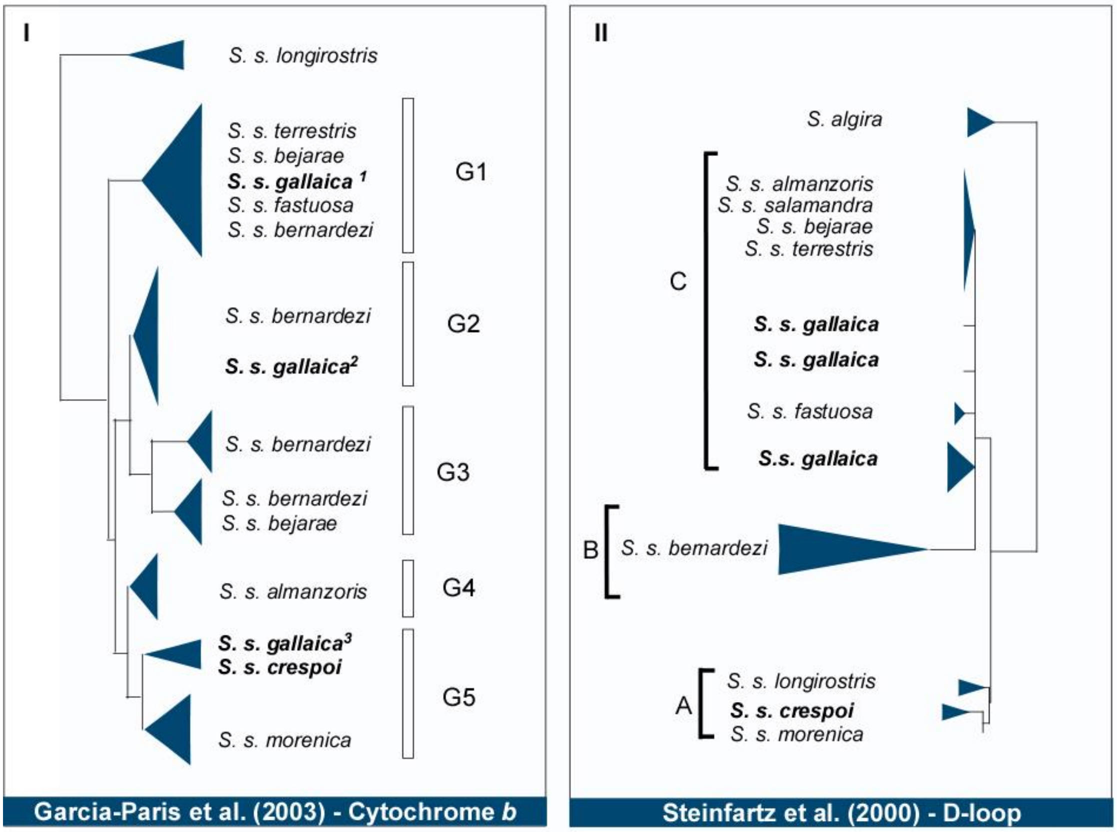

Figure 12 – I: Relationships between haplotypes of *Salamandra salamandra* based on a neighbor-joining analysis of a fragment of the mitochondrial DNA cytochrome *b* gene (modified from Figure 2 of Garcia-Paris *et al.* (2003)). The five groups represented, G1–G5, are based on bootstrap values higher than 57%. [1] Individuals from Lugo, Pontevedra and Zamora (North of Spain); [2] individuals from Lugo (North of Spain); and [3] individuals from Santiago do Cacém.
Figure 11 – II: Bootstrap consensus tree (>70%) of mitochondrial D-loop sequences based on a neighbor-joining analysis (modified from Figure 2 of Steinfartz *et al.* (2000)). A number of non-relevant taxa to this subject were excluded in both figures.

S. s. crespoi occurs in the clade G5 that groups southern Iberian populations (*S. s. morenica, S. s. crespoi* and *S. s. longirostris*). Garcia-Paris *et al.* (1998) proposed that this clade (clade G5) has arisen from a Pleistocene refugia area in the South of Spain. The closer genetic proximity between *S. s. morenica* and *S. s. crespoi* opposed to *S. s. longirostris* depicts the allopatric effects of the Betic crisis. According to Steinfartz et al. (2000) the southern sub-species *S. s. longirostris, S. s. morenica* and *S. s. crespoi* were among the first to diverge forming clade A, while the *S. s. gallaica* clade C includes subspecies from Central, Western and Eastern Europe and it is largely unresolved. Thus, the presence of both *S. s. gallaica* and *S. s. crespoi* in the Western Iberia Peninsula area is not due to a divergence from a single recent common ancestor, rather to different cladogenetic events among the *Salamandra* clade. According to some studies, the transition between this two subspecies (*S. s. gallaica* and *S. s. crespoi*), has been reported to occur in the southwest area of Portugal near the Torgal Creek (lower Alentejo) and on an unclear area around Mértola – Alcoutim region in the southeastern region of Portugal (Almeida et al. 2001, Rebelo and Leclair 2003, Malkmus 2004).

Alexandrino, J., E. Froufe, J. W. Arntzen, and N. Ferrand. 2000. Genetic subdivision, glacial refugia and postglacial recolonization in the golden-striped salamander, Chioglossa lusitanica (Amphibia: Urodela). Molecular Ecology **9**:771–781.

Alexandrino, J., J. Teixeira, J. Arntzen, and N. Ferrand. 2007. Historical biogeography and conservation of the golden-striped salamander (Chioglossa lusitanica) in northwestern Iberia: integrating ecological, phenotypic and phylogeographic data. Pages 189-205 Phylogeography of southern European refugia.

Almeida, N. F., P. F. Almeida, H. Gonçalves, F. Sequeira, J. Teixeira, and F. F. Almeida. 2001. Anfíbios e Répteis de Portugal. Guias FAPAS. Fundo para Protecção da Vida Selvagem, Porto.

Avise, J. C. 1998. The history and purview of phylogeography: a personal reflection. Molecular Ecology **7**:371-379.

Barata, M., D. J. Harris, and R. Castilho. 2008. Comparative phylogeography of northwest African Natrix maura (Serpentes: Colubridae) inferred from mtDNA sequences. African Zoology **43**:1-7.

Bohme, M. 2003. The Miocene Climatic Optimum: evidence from ectothermic vertebrates of Central Europe. Palaeogeography Palaeoclimatology Palaeoecology **195**:389-401.

Bossuyt, F., R. M. Brown, D. M. Hillis, D. C. Cannatella, and M. C. Milinkovitch. 2006. Phylogeny and Biogeography of a Cosmopolitan Frog Radiation: Late Cretaceous Diversification Resulted in Continent-Scale Endemism in the Family Ranidae. Systematic Biology **55**:579 - 594.

Burbrink, F. T., F. Fontanella, R. A. Pyron, T. J. Guiher, and C. Jimenez. 2008. Phylogeography across a continent: The evolutionary and demographic history of the North American racer (Serpentes : Colubridae : Coluber constrictor). Molecular Phylogenetics and Evolution **47**:274-288.

Carranza, S., E. N. Arnold, and J. M. Pleguezuelos. 2006. Phylogeny, biogeography, and evolution of two Mediterranean snakes, Malpolon monspessulanus and Hemorrhois hippocrepis (Squamata, Colubridae), using mtDNA sequences. Molecular Phylogenetics and Evolution **40**:532-546.

Committee, U. S. G. S. G. N. 2007. Divisions of geologic time—Major chronostratigraphic and geochronologic units: U.S. Geological Survey Fact Sheet

Davis, B. A. S., S. Brewer, A. C. Stevenson, and J. Guiot. 2003. The temperature of Europe during the Holocene reconstructed from pollen data. Quaternary Science Reviews **22**:1701-1716.

Deffontaine, V., R. Libois, P. Kotlik, R. Sommer, C. Nieberding, E. Paradis, J. B. Searle, and J. Michaux. 2005. Beyond the Mediterranean peninsulas: evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (Clethrionomys glareolus). Molecular Ecology **14**:1727-1739.

Doadrio, I. 1988. Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. Bonner Zoologische Beitrage **39**:113–128.

Duggen, S., K. Hoernle, P. van den Bogaard, L. Rupke, and J. Phipps Morgan. 2003. Deep roots of the Messinian salinity crisis. Nature **422**:602-606.

EPICA. 2004. Eight glacial cycles from an Antarctic ice core. Nature **429**:623-628.

Fedorov, V. B. and A. V. Goropashnaya. 1999. The Importance of Ice Ages in Diversification of Arctic Collared Lemmings (*Dicrostonyx*): Evidence from the Mitochondrial Cytochrome *B* Region. Hereditas **130**:301-307.

Fromhage, L., M. Vences, and M. Veith. 2004. Testing alternative vicariance scenarios in Western Mediterranean discoglossid frogs. Molecular Phylogenetics and Evolution **31**:308-322.

Garcia-Paris M, Montori A, and H. P. 2004. Amphibia. Lissamphibia. Fauna Iberica. Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Cientificas, Madrid.

Garcia-Paris, M., M. Alcobendas, and P. Alberch. 1998. Influence of the Guadalquivir River Basin on Mitochondrial DNA Evolution of Salamandra salamandra (Caudata: Salamandridae) from Southern Spain. Copeia **1998**:173-176.

Garcia-Paris, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zones in Iberian populations of fire Salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution **57**:129-143.

Garzón, M.B., Dios, R.S., Ollero, H.S. (2007): Predictive modelling of tree species distributions on the Iberian Peninsula during the Last Glacial Maximum and Mid-Holocene. Ecography, **30**: 120-134.

Godinho R, Teixeira J, Rebelo R, Segurado P, Loureiro A, Álvares F, Gomes N, Cardoso P, Camilo-Alves C, and B. JC. 1999. Atlas of the continental Portuguese herpetofauna: an assemblage of published and new data. Revista Española de Herpetología **13**:61–82.

Gomez, A. and D. H. Lunt. 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula.

Griffiths, R. A. 1996. Newts and Salamanders of Europe. First edition. Blackwell Publishing.

Guicking D, Joger U, and W. M. 2002. Molecular phylogeography of the Viperine snake (*Natrix maura*) and the Dice snake. Biota **3**:47-57.

Hemming, S. R. 2004. Heinrich events: Massive late Pleistocene detritus layers of the North Atlantic and their global climate imprint. Reviews of Geophysics **42**:RG1005.

Hewitt, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society **58**:247-276.

Hewitt, G. M. 1999. Post-glacial re-colonization of European biota. Biological Journal of the Linnean Society **68**:87-112.

Hewitt, G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature **405**:907-913.

Hewitt, G. M. 2001. Speciation, hybrid zones and phylogeography - or seeing genes in space and time. Molecular Ecology **10**:537-549.

Hewitt, G. M. 2004a. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **359**:183-195.

Hewitt, G. M. 2004b. The structure of biodiversity - insights from molecular phylogeography. Frontiers in Zoology **1**:4.

Krijgsman, W., F. J. Hilgen, I. Raffi, F. J. Sierro, and D. S. Wilson. 1999. Chronology, causes and progression of the Messinian salinity crisis. Nature **400**:652-655.

Malkmus, R. 2004. Amphibians and Reptiles of Portugal, Madeira and the Azores-Archipelago. Koeltz Sceintific Books.

Martinez-Solano, I. 2004. Phylogeography of Iberian Discoglossus (Anura : Discoglossidae). Journal of Zoological Systematics and Evolutionary Research **42**:298-305.

Martínez-Solano, I. and M. García-París. 2005. The impact of historical and recent factors on genetic variability in a mountain frog: the case of <i>Rana iberica</i> (Anura: Ranidae). Animal Conservation **8**:431-441.

Martínez-Solano, I., H. A. Gonçalves, J. W. Arntzen, and M. García-Paris. 2004. Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: Alytes). Journal of Biogeography **31**:603-618.

Martinez-Solano, I., J. Teixeira, D. Buckley, and M. Garcia-Paris. 2006. Mitochondrial DNA phylogeography of Lissotriton boscai (Caudata, Salamandridae): evidence for old, multiple refugia in an Iberian endemic. Molecular Ecology **15**:3375-3388.

Monzón, P. A., A. L. Cabrera, and A. E. Roca. 1989. Contexto estructural y paleogeográfico de los sistemas lacustres cenozoicos de España. Acta geológica hispánica **24**:3-4.

Muller, R. A. and G. J. MacDonald. 1997. Glacial Cycles and Astronomical Forcing. Science **277**:215-218.

Olalde, M., A. Herrán, S. Espinel, and P. G. Goicoechea. 2002. White oaks phylogeography in the Iberian Peninsula. Forest Ecology and Management **156**:89-102.

Petit, J. R., J. Jouzel, D. Raynaud, N. I. Barkov, J. M. Barnola, I. Basile, M. Bender, J. Chappellaz, M. Davis, G. Delaygue, M. Delmotte, V. M. Kotlyakov, M. Legrand, V. Y. Lipenkov, C. Lorius, L. Pepin, C. Ritz, E. Saltzman, and M. Stievenard. 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature **399**:429-436.

Provan, J. and K. D. Bennett. 2008. Phylogeographic insights into cryptic glacial refugia. Trends in Ecology & Evolution **23**:564-571.

Rebelo, R. Personal Communication 2008. Morphology and coloration in the specie *Salamandra salamandra* that inhabits Portugal. Faro.

Rebelo, R. and M. H. Leclair. 2003. Differences in size at birth and brood size among Portuguese populations of the fire salamander, Salamandra salamandra. Herpetological journal **vol. 13**:179 -187.

Recuero, R., A. Iraola, X. Rubio, A. Machordom, and M. García-París. 2007. Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. Journal of Biogeography **34**:1207-1219.

Riddle, B. R., D. J. Hafner, L. F. Alexander, and J. R. Jaeger. 2000. Cryptic vicariance in the historical assembly of a Baja California Peninsular Desert biota. Proceedings of the National Academy of Sciences of the United States of America **97**:14438-14443.

Schmidt, B., M. Schaub, and S. Steinfartz. 2007. Apparent survival of the salamander Salamandra salamandra is low because of high migratory activity. Frontiers in Zoology **4**:19.

Sequeira, F., J. Alexandrino, S. Rocha, J. W. Arntzen, and N. Ferrand. 2005. Genetic exchange across a hybrid zone within the Iberian endemic golden-striped salamander, Chioglossa lusitanica. Molecular Ecology **14**:245-254.

Soranzo N, Alia R, Provan J, and P. W. 2000. Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insights into the postglacial history of European *Pinus sylvestris* populations. Molecular Ecology **9**:1205-1211.

Steinfartz, S., M. Veith, and D. Tautz. 2000. Mitochondrial sequence analysis of Salamandra taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of Salamandra salamandra. Molecular Ecology **9**:397-410.

Steinfartz, S., S. Vicario, J. W. Arntzen, and A. Caccone. 2007. A Bayesian Approach on Molecules and Behavior: Reconsidering Phylogenetic and Evolutionary Patterns of the Salamandridae with Emphasis on Triturus Newts. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution **308**:139–162.

Teixeira, J. 2007. Biogeografia, evolução e conservação da herpatofauna endémica do noroestes da Península Iberica. Porto University, Porto.

Thompson, M. and A. Russell. 2005. Glacial Retreat and its Influence on Migration of Mitochondrial Genes in the Long-toed Salamander (*Ambystoma macrodactylum*) in Western North America. Pages 205-246 Migration of Organisms.

Veith, M., L. Fromhage, J. Kosuch, and M. Vences. 2006. Historical biogeography of Western Palaearctic pelobatid and pelodytid frogs: a molecular phylogenetic perspective. Contributions to Zoology **75**:109-120.

Veith, M., C. Mayer, B. Samraoui, D. D. Barroso, and S. Bogaerts. 2004. From Europe to Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed salamanders (Genus Pleurodeles). Journal of Biogeography **31**:159–171.

Vences, M. and J. Köhler. 2008. Global diversity of amphibians (Amphibia) in freshwater. Hydrobiologia **595**:569-580.

Waelbroeck, C., L. Labeyrie, E. Michel, J. C. Duplessy, J. F. McManus, K. Lambeck, E. Balbon, and M. Labracherie. 2002. Sea-level and deep water temperature changes derived from benthic foraminifera isotopic records. Quaternary Science Reviews **21**:295-305.

Thesis Objectives

To examine the processes driving patterns of genetic differentiation and persistence of the two *Salamandra salamandra* subspecies in Portugal, four specific issues will be addressed based on the phylogeographic analysis of the cytochrome *b* mitocondrial gene fragment: a) spatial distribution of the genetic diversity exhibited by both subspecies; b) assessment of genetic differentiation between the two subspecies; c) establishment of the geographical distribution and limits of the subspecies, with recognition of possible contact zones and d) identification of possible demographic events that may have contributed to the maintenance of the two forms in Portugal.

Amphibia-Reptilia XX (2009): XX-XXX

Phylogeography of *Salamandra salamandra*, L. in Portugal: It's not all black and yellow

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It has been shown that the Betic and Messinian crisis may be associated to the origin of several Iberian endemic species, while the Pleistocene ice ages cycles are responsible for the fragmentation of species into subspecies and lineages divergence. The present study explores the genetic relationships between 197 *Salamandra salamandra* individuals from the two putative subspecies inhabiting the Iberian Peninsula southwestern area, *S. s. gallaica* and *S. s. crespoi*, based on a cytochrome b mitochondrial DNA fragment. A total of 12 locations were sampled, with a mean average of 16 individuals per sample. The existence of two groups of populations is clearly supported by 17 diagnostic nucleotide positions. The center-north populations match the known distribution of *S. s. gallaica* and southern populations correspond to *S. s. crespoi* distribution. Haplotype and nucleotide diversities ranged from 0.000 to 0.012 and from 0.000 to 0.833, respectively. High levels of diversity were encountered in Monchique (h=0.83 and π=0.012), and Sintra (h=0.81 and π=0.010) associated with its environmental characteristics, supporting the hypotheses of being past refugia for these subspecies. The calculated neutrality indexes show that all populations display a stable historical demographically (no significant Fs or R_2 tests). Nested clade analysis did not produce any evidence for the existence of major barriers to past gene flow within *S. s. crespoi*, whereas suggests the influence of Tejo-Sado Rivers upon the genetic structure of *S. s. gallaica*. Also a late Pliocene/early Pleistocene origin of the split between S. *s. crespoi* and *S. s. gallaica* is determined. This information is important regarding amphibian conservation plans for Iberia.

The Iberian Peninsula due to its irregular topography, diversity of habitats and various types of climate was one of the most important refugia in Europe (Davis et al. 2003, Gomez and Lunt 2007). The existence of several endemic species of fauna and flora suggest the occurrence of vicariant events promoted by climate change that induced geographical isolation. However, it is noteworthy that most of the extant European species appeared before the last ice cycle of the Quaternary Period (Hewitt 1996, Steinfartz et al. 2000).

Numerous geological events shaped the Iberian Peninsula, some of which had direct impact on the biota of many amphibians. The formation of salt lakes inside Iberia ca. 15.3 – 13.5 MYA, the formation of Neo-Pyrenees 7-10 MYA, the Betic (15 MYA) and Messinian crisis (5.9-5.3 MYA), and the formation of fluvial drainages areas inside Iberia (ca. 5 MYA) seem to be responsible for the divergence encountered in a number of genus in the western Mediterranean (e. g. *Alytes*, *Lissotriton*, *Discoglossidae* and *Pleurodeles* genuses) (Martinez-Solano 2004, Martínez-Solano et al. 2004, Veith et al. 2004, Arntzen et al. 2007, Steinfartz et al. 2007). The Pleistocene ice ages of the last 100 thousand years seem to be responsible, in turn, for the fragmentation of species into subspecies and lineages divergence (Hewitt 2000, Gomez and Lunt 2007) seen in *A. obstretricans and L. boscai* (Martinez-Solano 2004, Martínez-Solano et al. 2004). All 8 Iberian subspecies of *Salamandra salamandra*, Linnaeus 1758, began to diverge before the glacier cycles of the Pleistocene (Garcia-Paris et al. 2003, Steinfartz et al. 2000). These factors enhanced the different degrees of genetic divergence and high polymorphism found within the complex of species and subspecies belonging to the genus *Salamandra*, making the determination of the geographical distribution limits of each entity difficult. (Garcia-Paris et al. 1998, Steinfartz et al. 2000, Garcia-Paris et al. 2003, Rebelo and Leclair 2003).

The habitat of the *S. salamandra* like most amphibians is highly dependent on the amount of precipitation, being more abundant in mountain areas with annual precipitation above 800 mm/m^3 (Griffiths 1996, Malkmus 2004).

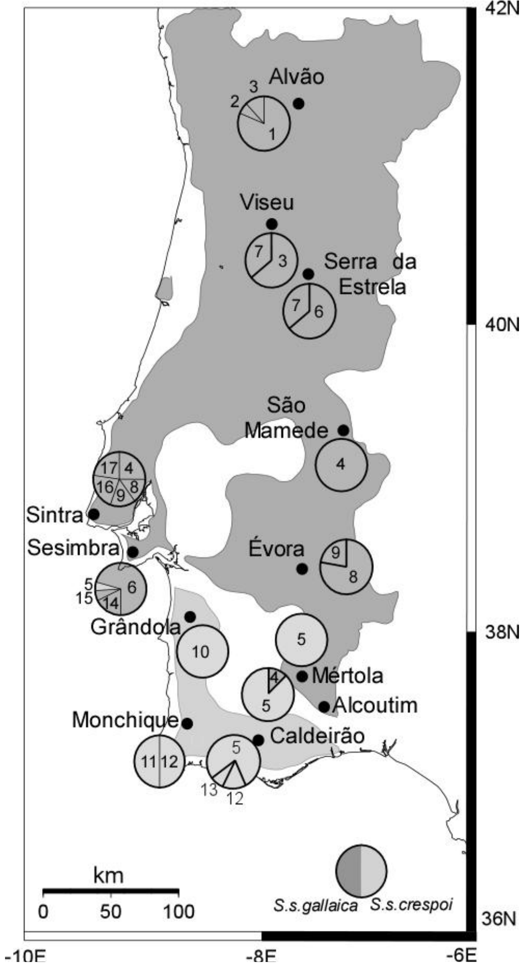

Figure 1 - Sampling locations and haplotypes frequency (pie slice size) found on those locations. The 1 to 17 represent the different haplotypes encountered, and the different background shading indicates the know distribution (Almeida et al. 2001) of the two subspecies of salamander existing in Portugal.

S. s. gallaica and *S. s. crespoi* divergence is based on the size. *S. s. gallaica* is usually smaller displaying irregular big yellow spots with ocasional small touches of red, whereas *S. s. crespoi* has a regular pattern of small yellowish spots. However, these distinctions are not always notorious as this sub-species can sometimes display mixed characteristics (Rebelo and Leclair 2003, Malkmus 2004, Rebelo Personal Communication 2008).

In this study, processes driving patterns of genetic differentiation and persistence of the two *Salamandra salamandra* subspecies in Portugal were examined, recurring to: i) spatial distribution of the genetic diversity exhibited by the both subspecies; ii) assessment of genetic differentiation between the two subspecies; iii) establishment of the geographical distribution and limits of the subspecies, with recognition of possible contact zones and iv) identification of possible demographic events that may have contributed to the maintenance of this two forms.

The results combined will be a major contribution to develop biogeographic scenarios that explain the genetic structure found within this species in Portugal, contributing to a better understanding of the real impact of geological events and Pleistocene Ice Ages in this group. Additionally, important information for amphibian conservation and management will be provided (Hewitt 1996, Steinfartz et al. 2000).

Material and Methods

For this study we aimed for the most inclusive geographical distribution of the species *S. salamandra* in Portugal. Selection of localities (Figure 1) included some potential contact zones hypothesized by Caetano (2003). We sampled a total of 197 individuals from 12 locations (Table 1). DNA was extracted from tail tips of adults and larvae using a slightly modified version of the NaCl method present in Sambrook *et al.* (2001). The cytochrome *b* region of the DNA was amplified using primers MVZ 15, MVZ 16 and cyt-b2 (Moritz et al. 1992). PCR reactions occurred in a total volume of 25 µl, containing 0.4 µM of each primer, 750 µM of dNTP's, 5 µL of 5x GoTaq Flexi buffer (pH 8.5, 1.5 mM of $MgCl_2$), 0.2 units of GoTaq DNA polymerase (Promega) and 1 µL of extracted DNA. The PCR profile consisted of a 3 minutes denaturation period at 95ºC, followed by 40 cycles of denaturation, annealing and elongation (45 seconds at 95ºC, 45 seconds at 48ºC, 1 minute at 72 ºC, respectively), and a final 10 minute step at 72ºC. Samples were purified by ethanol-sodium acetate precipitation and sequenced on an ABI Prism® 3130XL Genetic Analyzer equipped with 16 capillaries. Sequences where aligned and trimmed using GENEIOUS version 3.6.1 (Drummond et al., 2006).

Estimation of all genetic parameters (Table 1) was performed using DnaSP version (Rozas J et al. 2003), SAMOVA (Dupanloup I et al. 2002) and Arlequin version 3.000 (Excoffier et al. 2005). Diversity indexes (k, *h;* π and their standard deviation), as well as parsimony informative sites were calculated for each location. The evolution model and associated parameters for use in Arlequin estimates were determined by Modeltest 3.06 (Posada 2008).

Geographic population structure was assessed using a spatial analysis of molecular variance SAMOVA 1.0 (Dupanloup I et al. 2002). The method implemented in SAMOVA employs a simulated annealing procedure and uses allele frequency data along with geographic coordinates of the sampled populations to identify population groups maximizing genetic differentiation. We determined the most likely number of *K* groups by repeatedly running SAMOVA with variable numbers of groups and by choosing the number resulting in a maximum *Fct* value (Dupanloup I et al. 2002). The configuration with the largest *Fct* value among the 100 tested was retained as the best grouping of populations. Sequences were collapsed into haplotypes using the program COLLAPSE (Posada 2004), and a maximum parsimony network was built with TCS program (Clement M et al. 2000). Tamura-Nei genetic distances were calculated using program MEGA (Tamura K et al. 2007).

A Nested Clade Phylogeographic Analysis (NCPA) was also performed using ANeCA version 1.2 (Figure 2), which is a fully automated implementation of the method (Panchal 2007). Despite recent controversy (Petit 2008), the NCPA is still one of the most used analytical tools in biogeography

(Templeton 2008) and its results can be considered with caution. The sequences were collapsed into haplotypes and a haplotype network was constructed using maximum parsimony. Next, using a nested statistical design, the haplotypes were divided by the number of mutations that separated each of them. The haplotypes that diverged only by one mutation were grouped in the one-step clades, then each of these clades were analyzed and the ones that differed only by one mutation were nested creating two-step clades. This process continued, creating higher levels of hierarchy until all the clades were grouped into a single clade, the Total Cladogram clade (Templeton 1998).

Two different tests for geographical association were conducted: a categorical test using the χ^2 distribution in which clades showing genetic and/or geographical variation were tested against their geographical location, and a statistical analysis of clade distances. This last analysis is composed of two different measures. Dc measures the spread of individuals within clades, and Dn reflects the spread on individuals from one clade with respect to other clades of the same nesting level (Templeton et al. 1995, Martinez-Solano 2004). These tests were performed by GeoDis (Posada et al. 2000) and the evolutionary patterns were sorted using the modified version of the inference key present in ANeCA (Panchal 2007). Inferences on population historical demography (data not showed) were made both by mismatch analysis and by using the neutrality indexes (Tajima D, R^2, Fu Fs, F* and D*), calculated in DnaSP (Rozas J et al. 2003). Mismatch pairwise differences distribution were made assuming a constant size population and a growth-decline expansion in the population models. When populations experience recent exponential growth in size, they exhibit a unimodal distribution because most individuals will be genetically highly similar, i.e., all coalescent events occur near the root of the tree. In opposition, stable populations that did not experience significant changes in its size should have a multimodal distribution (Mes 2003, Fritz et al. 2006). A number of neutrality tests have been used as indicators of past demography. The Fs test is a one-sided test that uses information from the haplotype network and reflects an excess of low frequency variants, meaning that when this test gives significant negative values a noteworthy amount of rare haplotypes have been detected indicating possible past expansion or background selection (Fu 1997, Ramos-Onsins and Rozas 2002). Tajima's D test correlates the nucleotide diversity detected in a two-on-two nucleotide paring with its segregating sites, effectively detecting deviations from selective neutrality, because in those conditions the value of this test is zero (Ramos-Onsins and Rozas 2002, Mes 2003). So, significant negative values of this test can indicate an excess of rare mutations which can be a sign of population expansion or selection, and positive values point out to possible population subdivision, constant selection for some haplotypes, or possible population bottleneck (Kreitman 2000, Fay and Wu 2005).

Table 1 - Table with the sampled locations, their corresponding coordinates, size in base pairs of the DNAmt fragment used (the 362 base pairs data are grey shaded) and matching number of individuals collected in each location (N). It also shows the corresponding diversity indexes to each location, containing the number of detected haplotypes (H), polymorphic sites (PS), parsimony informative sites (PIS), average number of nucleotide differences (K), haplotype diversity (*h*), its corresponding standard deviation (SD), nucleotide diversity (π) and its standard deviation (SD). The haplotypes are also grouped into the Clades determined by the haplotype network. The light grey shade indicates 362 base pair fragment data.

Locality	Geographical Coordinates	Sampling Period	Length bp	N	Diversity indexes							
					H	PS	PIS	K	*h*	SD	π	SD
Alvão	41°23'N; 7°48'W	2003 - 2004	690	18	3	2	1	0,32	0,31	0,13	0,0005	0,0002
Viseu	40°40'N; 7°54'W	2008	690	3	2	7	0	4,67	0,67	0,31	0,0068	0,0032
Serra da Estrela	40°24'N; 7°31'W	2003 - 2004	690	20	2	9	9	4,31	0,48	0,07	0,0063	0,0009
São Mamede	39°19'N; 7°18'W	2003 - 2004	690	19	1	0	0	0,00	0,00	0,00	0,0000	0,0000
Sintra	38°47'N; 9°26'W	2003 - 2008	690	36	5	16	16	7,00	0,81	0,02	0,0101	0,0005
Sesimbra	38°27'N; 9°01'W	2003 - 2004	690	20	4	29	1	3,20	0,49	0,12	0,0046	0,0034
Évora	38°25'N; 7°40'W	2003 - 2004	690	11	2	1	1	0,33	0,33	0,15	0,0005	0,0002
Alcoutim	37°33'N; 7°28'W	2003 - 2004	690	11	2	29	0	5,27	0,18	0,14	0,0076	0,0060
Caldeirão	37°20'N, 7°58'W	2003 - 2004	690	25	3	3	3	0,79	0,35	0,11	0,0012	0,0004
Grândola	38°06'N; 8° 37'W	2003 - 2004	690	1	1	0	0	-	-	-	-	-
Mértola	37°37'N; 7°40'W	2003 - 2004	690	2	1	0	0	-	-	-	-	-
Monchique	37°19'N; 8°33'W	2003 - 2004	690	2	2	1	0	-	-	-	-	-
Grândola	38°06'N; 8° 37'W	2003 - 2004	362	15	3	2	1	0,61	0,51	0,12	0,0017	0,0005
Mértola	37°37'N; 7°40'W	2003 - 2004	362	15	2	11	11	3,77	0,34	0,13	0,0104	0,0039
Monchique	37°19'N; 8°33'W	2003 - 2004	362	4	3	9	0	4,50	0,83	0,22	0,0124	0,0058
Clade 1			690	127	12	24	22	6,12	0,87	0,01	0,0089	0,0004
Clade 2			690	41	5	3	3	0,74	0,35	0,09	0,0011	0,0003
Amostra Total			690	168	17	43	41	13,42	0,89	0,01	0,0195	0,0009
Clade 2			362	66	6	4	3	0,89	0,55	0,07	0,0025	0,0003
Amostra Total			362	197	13	16	15	4,92	0,87	0,01	0,0136	0,0004

Table 2 – Table with the different haplotypes detected and corresponding variable positions identified in the 690 base pairs fragment, along with the number of individuals which bear each haplotypes and locations where they were encountered. The haplotypes are also divided into the Clades determined by the haplotype network. The light grey shaded positions highlight the diagnostic nucleotide positions that differentiate the *S. s. gallaica* subspecies (clade I) from the *S. s. crespoi* subspecies (clade II).

Clade	Haplotypes	Nucleotide variable positions of the Cytochrome *b* fragment																																										Populations
		6	24	54	114	145	168	175	183	186	190	226	228	234	309	339	381	390	402	405	412	418	438	453	456	459	471	509	513	531	537	538	574	579	582	594	624	636	640	660	661	663	672	681	
I	**4**	C	C	C	C	A	C	A	C	T	G	A	A	C	C	T	T	C	T	C	G	C	C	C	A	C	T	C	G	C	T	C	G	C	C	T	C	T	A	G	G	A	G	C	Alcoutim; Sintra; São Mamede
	8	-	-	-	-	-	-	G	-	-	-	C	-	T	-	C	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	T	-	T	T	-	-	-	G	-	-	-	-	-	Evora; Sintra
	9	-	-	-	-	-	-	G	-	-	-	C	-	T	-	C	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	T	A	T	T	-	-	-	G	-	-	-	-	-	Evora; Sintra
	16	-	-	-	-	-	-	G	-	-	-	C	-	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	T	-	-	-	A	G	A	-	Sintra
	14	-	-	-	-	-	-	G	-	-	A	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Sesimbra
	6	-	-	-	-	-	-	G	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Serra da Estrela; Sesimbra
	15	-	-	-	-	C	-	G	-	-	A	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Sesimbra
	17	-	-	-	-	-	-	G	-	-	-	C	-	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	-	-	-	-	A	G	A	-	Sintra
	1	-	T	-	-	-	-	G	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	Alvão
	2	-	T	-	-	-	-	G	-	-	-	C	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	Alvão
	3	-	-	-	-	-	-	G	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	Alvão; Viseu
	7	-	-	-	-	-	-	G	-	-	-	C	-	T	T	C	-	T	-	T	-	-	-	-	-	-	-	-	-	-	-	-	T	T	-	C	-	-	-	-	-	G	G	-	Serra da Estrela; Viseu
II	**5**	T	-	A	T	-	G	G	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	-	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	Alcoutim; Mertola; Caldeirão; Sesimbra
	12	T	-	A	T	-	-	-	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	T	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	Monchique; Caldeirão
	13	T	-	A	T	-	A	G	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	-	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	Caldeirão
	10	T	-	A	T	-	-	G	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	-	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	Grândola
	11	T	-	A	T	-	-	G	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	T	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	Monchique

The R_2 test has been shown to be the most powerful test to examine population expansions in small samples (Ramos-Onsins and Rozas 2002). Fu F* and D* tests are calculated by using the number of unique haplotypes inside the sample, indicating the same type of process as Tajima's D test but using slightly different parameters, and having slightly different abilities to detect each processes. For example, Fu's F* test has a better ability to detect background selection (Simonsen et al. 1995, Fritz et al. 2006). Their statistical significance was tested using 1000 random generated samples under the null hypotheses of selective neutrality and constant size population using coalescent algorithms (Hudson 1990), thus obtaining *P*-values in proportion with the number of random samples that were smaller or equal to the observed values.

Results

From the 197 individuals, 29 did not amplify the 690 base pairs (bp) cytochrome b fragment. A smaller 362 bp cytochrome b fragment was successfully amplified from these individuals and will be used occasionally, as it contains relevant information on 3 locations (Grândola, Monchique and Mértola) poorly represented in the 690 bp dataset (Table 1). There were no insertions or deletions detected in the alignment and no stop codons were found when the alignment was translated into amino acids. The total dataset revealed 45 substitutions (37 transitions and 8 transversions) of which 43 are polymorphic sites. The nucleotide composition has a strong bias towards high T's (32.34%) and low G's (13.53%), having somewhat similar quantities of C's (25.03%) and A's (29.10%).

A total of 17 haplotypes were identified (GenBank accession numbers XXXXXXXX - XXXXXXXX), 5 represented by more than 15 individuals, and 4 haplotypes are represented by a single individual. The three haplotypes best represented with 27, 29 and 33 individuals have a widespread distribution in the centre and south of Portugal, and the unique haplotypes encountered were in Grândola, Sesimbra and Sintra (Table 2). The haplotype diversity per location (*h*) ranged from 0 to 0.83 with an average of 0.35. Sintra is the location with the highest number of haplotypes (*h* = 0.81) and São Mamede the location with the lowest, having a single haplotype (Table 1).

The haplotype network from the 95% statistical parsimony analysis (Figure 2) resulted in two independent networks separated by 23 mutations. One of the networks comprises haplotypes present in northern-central region (dark grey haplotypes forming clade I) and the other network includes haplotypes from southern locations of Portugal (light grey haplotypes forming clade II). This result has a further support as SAMOVA, conducted repeatedly by increasing the number of groups (*K*) reached a maximum F_{CT} value of 0.771 (P value<0.01) with *K* = 2 (results not shown), grouping the exact same regions. Some of the variable positions of the alignment (Table 2) in a total of 17, appeared to be essentially non-random (diagnostic), allowing prompt allocation of each haplotype to one of the two clades. Differentiation among the locations of each group was high with an F_{ST} value of 0.886 (P-value <0.001).

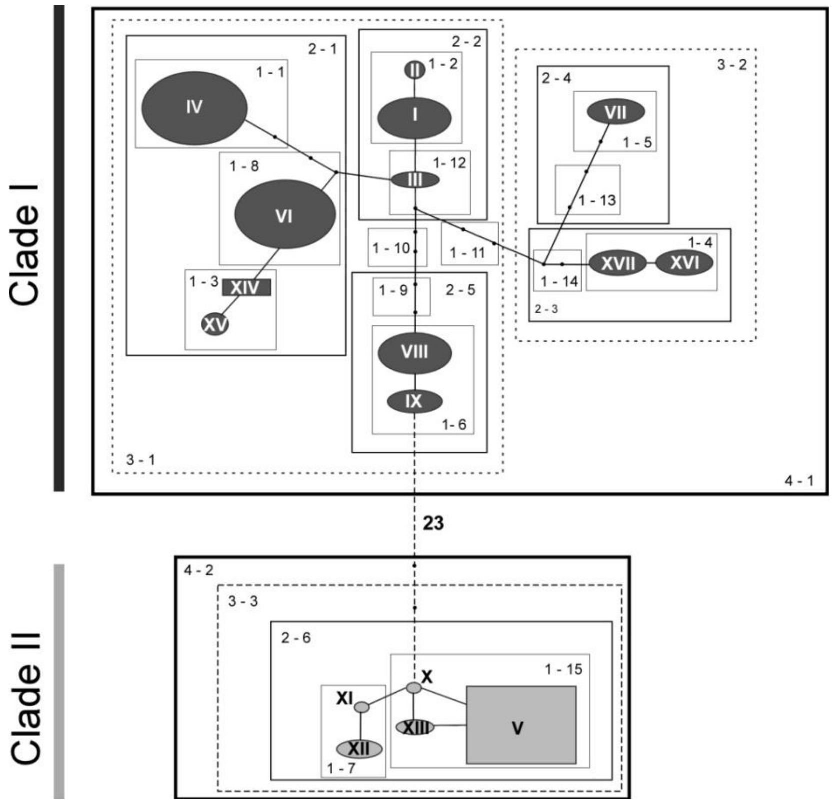

Figure 2 – Network and nested design of *S. salamandra* obtained for the cytochrome *b* haplotypes (each haplotype has a dimension corresponding to their frequency) using the 690 base pairs fragment. Two distinct networks were detected when using 95% maximum-parsimony, separated by 23 mutations. Several clades were identified and each hierarchical level is differentiated by different dashing. The most relevant clades detected are: the 4-1 clade that groups the haplotypes belonging to *S. s. gallaica* (marked by the dark grey), and the 4-2 clade that groups the *S. s. crespoi* haplotypes (marked by light grey).

Table 3 – Corrected Tamura-Nei genetic distances between sampled locations (below diagonal), and associated standard error (above diagonal). In light grey shade are the inter-clade distances and on white are the intra-clade ones.

	Alvao	Estrela	Evora	Sesimbra	Sintra	Mamede	Viseu	Alcoutim	Mertola	Monchique	Caldeirao
Alvao		0,002	0,004	0,003	0,002	0,003	0,002	0,007	0,007	0,007	0,007
Estrela	0,007		0,004	0,003	0,002	0,003	0,002	0,007	0,007	0,007	0,007
Evora	0,010	0,013		0,003	0,004	0,005	0,004	0,007	0,007	0,007	0,007
Sintra	0,010	0,012	0,011		0,003	0,003	0,002	0,006	0,007	0,007	0,007
Sesimbra	0,006	0,007	0,014	0,013		0,003	0,002	0,007	0,007	0,007	0,007
Mamede	0,007	0,009	0,015	0,012	0,008		0,003	0,007	0,008	0,008	0,008
Viseu	0,005	0,007	0,011	0,010	0,008	0,009		0,006	0,007	0,007	0,007
Alcoutim	0,036	0,038	0,035	0,036	0,036	0,040	0,036		0,001	0,002	0,001
Mertola	0,039	0,041	0,037	0,039	0,039	0,044	0,039	0,004		0,002	0,000
Monchique	0,040	0,041	0,038	0,039	0,040	0,043	0,039	0,007	0,004		0,002
Caldeirao	0,039	0,041	0,038	0,039	0,039	0,044	0,039	0,005	0,001	0,003	

When both datasets are compared we observe the fusion of a number of haplotypes (1+2, 3+6, 8+9, 7+16+17) present in the 690 bp dataset into less but more frequent haplotypes when considering the 362 bp dataset. Two new haplotypes have arisen only due to the inclusion of individuals from Grândola. Using only the 690bp dataset and excluding the locations with less than 5 individuals (Grândola, Mértola, Monchique, Viseu, Monchique), most locations have high haplotype diversities (0.31≤*h*≤0.51), in sharp contrast with São Mamede and Alcoutim that display very low haplotype diversities (*h* < 0.20). Sintra has high haplotype diversity (*h* = 0.81) (Table 1). The corrected intra-clade Tamura–Nei genetic distances between haplotypes (results not shown) were always lower (0.001-0.016) than the inter-clade distances (0.035-0.045, with an average of 0.04 ± 0.01 S.E.). Considering the sampling locations (Table 3), Clade I shows a higher genetic distances (0.005-0.040) and Clade II shows lower distances (0.001-0.007), while the locations have higher values.

The NCPA can assist in determining which historical processes could eventually be responsible for the current geographical distribution of haplotypes (Figure 2). The null hypotheses of no association between geographic distribution and haplotype distribution was rejected for clades 1-6, 1-7, 1-15, 2-1, 2-2, 2-6, 3-1, 3-2, 4-1 and the Total Cladogram. At the one-step clade level, no further inferences could be made. At the two-step level when the Northern haplotypes 1, 2 and 3 in the vicinity of each other are grouped in clade 2-2, the pattern of restricted gene flow with isolation by distance could be inferred. This pattern was also found in clade 2-1 that joins together individuals from Sesimbra (haplotypes 6, 14 and 15), Sintra, São Mamede and one individual from Alcoutim (these three locations share haplotype 4). At the next hierarchical level, long-distance colonization and/or past fragmentation patterns can probably explain the geographic distribution of haplotypes grouped in clade 3-1. In clade 3-2, allopatric fragmentation of populations was probably responsible for the genetic distribution of the three equally frequent haplotypes dispersed throughout the irregular terrain that linked the two exclusive Sintra haplotypes (16 and 17), with Viseu and Serra da Estrela haplotype (7). The Total Cladogram shows a clear association between genetic and geographical distribution but no pattern of demographic events could be identified.

The neutrality indexes, Tajima's D, Fu's F* and D* statistic tests all revealed significant similar departure from neutral selection, with a significant contribution for positive values in individuals from Serra da Estrela and Sintra, and negative for individuals from Alcoutim and Sesimbra. The only exception is Mértola that has a significant high value of Fu's D* (1.44) when the 15 individuals with the 362 bp are analyzed. The total sample also shows a positive significant Fu's D* (mean of 1.67) and F* tests (mean of 2.23). Fs and R_2 tests did not show any significant departure from neutral selection, having in general positive high values (mean of 3.94). When considering the two separate clades, the values obtained are not statistically significant. Alcoutim, Serra da Estrela, Sesimbra and Sintra display a bimodal mismatch distribution while the remaining locations displayed a unimodal distribution (results not shown). Locations with less than 5 individuals were not included in the analysis.

Discussion

Most of the Iberian endemic amphibians exhibit a common pattern of a northern and southern geographical split (Paulo et al. 2001, Martinez-Solano 2004, Veith et al. 2004, Martinez-Solano et al. 2006, Veith et al. 2006). Our results agree with this observed pattern, with two deeply divergent clades having a geographical distribution split corresponding to the Northern-Central region and to the Southern region of Portugal. Several key nucleotide positions (Table 2) are responsible for this clear divergence between the two clades and the geographic distribution of haplotypes of each clade generally matches the range distribution known for the two different subspecies of *S. salamandra* that exist in Portugal (Almeida et al. 2001, Malkmus 2004). There is a marked low intermixing between these two lineages (Table 2), with almost every location having individuals from one clade only. Clade I haplotypes have a geographical distribution compatible with the known range of *S. s. gallaica* and Clade II to *S. s. crespoi*. Similar results of low intermixing between lineages were also found in the species *L. boscai* (Martinez-Solano et al. 2006). There are only two locations, Sesimbra and Alcoutim, at the central-eastern and southern-western edges of the distribution of the two subspecies (Almeida et al. 2001), where individuals with haplotypes of the two clades can be found in sympatry (Table 2). The observation of a joint presence of haplotypes that are characteristic of each subspecies is not due to secondary contact, as the two subspecies are not derived from the same most recent common ancestor (Garcia-Paris et al. 2003, Steinfartz et al. 2000), but is a sympatry arisen from the geographical range expansion of both subspecies. Also of interest is the redefinition of the described geographical distribution of the two subspecies. In fact most references to date do not mention explicitly the presence of *S. s. crespoi* in the Sesimbra region and in both Alcoutim and Mertola.

In all locations there are signs of demographic stability indicated by the multimodal pattern of the mismatch distribution, and the non significant Fs and R_2 neutrality indexes. The neutrality tests (Tajima D, Fu and Li D* and F*) seem to hint the possibility of selection acting in some locations (Sintra, Serra da Estrela, Alcoutim e Sesimbra); however, significant negative values were obtained in localities where haplotypes from both clades are found (Alcoutim and Sesimbra). Nevertheless other studies have indicated eventual size-linked selection acting on *S. salamandra* as latitude decreases (Rebelo Personal Communication 2008), and therefore one cannot discard that possibility in locations where both subspecies are found.

As for other species, the southwestern area of Portugal (Monchique) has already been identified as a important refugium for several

Iberian herpetofauna, namely *L. boscai* (Martinez-Solano et al. 2006), *A. cisternasii* (Teixeira 2007) and *Lacerta schreiberi* (Paulo et al. 2001), due to its environmental and geographic characteristics. Although the evidence is scarce, that could also be the case for *S. s. crespoi*, as the two individuals sampled displayed different haplotypes (Table 1) (or if one considers the 362 data-set, 4 individuals correspond to 3 haplotypes), which renders a relatively high haplotype diversity for this location. For the same reason but with stronger support, Sintra and to a lesser extend Viseu and Serra da Estrela, could have played the same role for *S. s. gallaica*. This kind of refugia locations share the same characteristics of other amphibians refugia, maintaining some level of forest throughout long time periods, and having mountainous irregular terrain with high precipitation levels (Gomez and Lunt 2007, Teixeira 2007). Also the contraction of the distribution of this species into these locations can be attributed to the superior precipitation levels forecasted for those areas, during the cold dry Pleistocene glaciations, as the precipitation values are a key component in amphibian's habitat determination (Griffiths 1996, Malkmus 2004, Garzón, Dios and Ollero 2007).

Nested clade distance analysis of mtDNA sequence variation revealed a high level of phylogeographic structure in both salamander subspecies. The rejection of the null hypothesis of random distribution of haplotypes was common at the higher-level nesting's, reflecting deep lineage divergence. The genetic distances and NCPA (Figure 2 and Table 3) support the claim that there is no barrier to gene flow for *S. s. crespoi* (Clade II), while the same analysis shows that *S. s. gallaica* (Clade I) has a signature of population structure. NCPA divides this Clade into several subclades (Figure 2 and Table 3) with distinct demographic processes as some locations show almost no signs of differentiation (like Viseu and Alvão, and Sesimbra and São Mamede), while others (i.e. Serra da Estrela and Evora) display higher values of genetic distance. The closest *S. s. gallaica* haplotypes (Figure 2) that are grouped into two level clades in the NCPA (2-1 and 2-2) show signs of restricted gene flow with isolation by distance. In clade 2-2 despite the small geographic range of the haplotype distribution, the restricted gene flow can be explained by the existence of the Douro River crossing its geographic range. This river has been recognized as a barrier to gene flow in amphibians, namely in the species *Chioglossa lusitanica* (Alexandrino et al. 2000). Clade 2-1 also has a river with same recognized effect crossing its range (the Tejo-Sado Rivers). Particularly the Tejo river has been also recognized responsible for separating different lineages of *Discoglossus galganoi* (Martínez-Solano et al. 2004). This fact, together with the more wide range that characterizes the irregular terrain of Clade 2-1 can explain the restricted gene flow inferred for that clade. The broader scale division (clade 3-1) encountered inside this salamander subspecies (*S. s. gallaica*) is inferred as due to past population fragmentation as to the Pleistocene ice ages (Hewitt 2000). This is supported by other amphibian studies that found similar results all linked to the same event (Martinez-Solano 2004, Martínez-Solano et al. 2004, Martinez-Solano et al. 2006).

The age of the common ancestor to the two clades including *S. s. longirostris*, *S. s. morenica* and *S. s. crespoi* and the remaining *S. salamandra* Iberian subspecies is estimated between 2 to 4 MYA, based on a calibration clock for D-loop of 0.8%/MY (Steinfartz *et al.* 2007). Cytochrome b sequences in the present study give a slightly longer estimated divergence time of 5.2 MY if all iberian *S. salamandra* subspecies are included (genetic distance of 2.9%, assuming a substitution rate of 0.77% (Tan and Wake 1995) per million years). Present findings support the hypothesis of a late Pliocene/early Pleistocene vicariance event in Iberia Peninsula, such as the formation of extensive fluvial drainage areas (Martinez-Solano 2004; Steinfartz *et al.* 2007), that prompt the divergence of the common Iberian ancestral of *S. salamandra*. Similar results were found for *Lissotriton boscai*, as this species showed a similar lineage divergence time and geographic distribution (Martinez-Solano 2004; Steinfartz *et al.* 2007). The present distribution of this two subspecies is possibly the result of several different past cladogeneic events. One plausible evolutionary scenario for the present day geographical and genetic differentiation may be hypothesized as follows: 1) the formation of fluvial drainage areas throughout Iberia in the Pliocene resulted in the subspecies divergence; 2) later, both subspecies may have expanded their area of distribution colonizing central Iberian territory; 3) upon the effects of the last dry glacial cycles and interglacial periods, both have retracted the distribution to a number of refugia, such as Sintra and Monchique, and 4) during the cycles renewed expansions occurred. The maintenance of the geographical and genetic differentiation may also be reinforced by the existence of two large freshwater basins (Douro, Tejo-Sado) and the occurrence of marine transgressions around southwestern coast of Portugal that covered a great part of the alentejo area during the Neogene period, together with a more recent desertification in the southern part of the country (Cachão and Silva 2000).

Genetic intra-specific variation is currently a generalized key parameter when determining conservation priorities (Moritz 2002). Therefore the strong genetic structure exhibited by *S. salamandra* subspecies in Portugal, should be taken into account when devising conservation plans to protect herpetofauna. The diagnostic variable positions showed here are an expedite way to discriminate the two subspecies of *Salamandra* and can be used for monitoring those purposes.

References

Alexandrino, J., E. Froufe, J. W. Arntzen, and N. Ferrand. 2000. Genetic subdivision, glacial refugia and postglacial recolonization in the golden-striped salamander, Chioglossa lusitanica (Amphibia: Urodela). Molecular Ecology **9**:771–781.

Almeida, N. F., P. F. Almeida, H. Gonçalves, F. Sequeira, J. Teixeira, and F. F. Almeida. 2001. Anfíbios e Répteis de Portugal. Guias FAPAS. Fundo para Protecção da Vida Selvagem, Porto.

Arntzen, J. W., G. E. Themudo, and B. Wielstra. 2007. The phylogeny of crested newts (Triturus cristatus superspecies): nuclear and mitochondrial genetic characters suggest a hard polytomy, in line with the paleogeography of the centre of origin. Contributions to Zoology **76**:261-278.

Caetano, S. 2003. Filogeografia de *Salamandra salamandra* (caudata:Salamandridae) no Sul de Portugal.

Cachão, M., Silva, C.M. (2000): The three main marine depositional cycles of the Neogene of Portugal. Ciências da Terra (UNL), **14**: 303-312.

Clement M, P. D, and C. K. 2000. TCS: a computer program to estimate gene genealogies. **10**:1657-1660

Davis, B. A. S., S. Brewer, A. C. Stevenson, and J. Guiot. 2003. The temperature of Europe during the Holocene reconstructed from pollen data. Quaternary Science Reviews **22**:1701-1716.

Doadrio, I. 1988. Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. Bonner Zoologische Beitrage **39**:113–128.

Drummond AJ, Kearse M, Heled J, Moir R, Thierer T, Ashton B, Wilson A, and S.-H. S. 2006. GENEIOUS.

Dupanloup I, Schneider S, and Excoffier L. 2002. A simulated annealing approach to define the genetic structure of populations. Molecular Ecology **11**:2571-2581.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online **1**:47-50.

Fay, J. C. and C. I. Wu. 2005. Detecting hitchhiking from patterns of DNA polymorphism. Pages 65-77 Selective Sweep.

Fritz, U., M. Barata, S. D. Busack, G. Fritzsch, and R. Castilho. 2006. Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, Mauremys leprosa (Reptilia, Testudines, Geoemydidae). Zoologica Scripta **35**:97-108.

Fu, Y. X. 1997. Statistical Tests of Neutrality of Mutations Against Population Growth, Hitchhiking and Background Selection. Genetics **147**:915-925.

Garcia-Paris, M., M. Alcobendas, and P. Alberch. 1998. Influence of the Guadalquivir River Basin on Mitochondrial DNA Evolution of Salamandra salamandra (Caudata: Salamandridae) from Southern Spain. Copeia **1998**:173-176.

Garcia-Paris, M., M. Alcobendas, D. Buckley, and D. B. Wake. 2003. Dispersal of viviparity across contact zones in Iberian populations of fire Salamanders (*Salamandra*) inferred from discordance of genetic and morphological traits. Evolution **57**:129-143.

Garzón, M.B., Dios, R.S., Ollero, H.S. (2007): Predictive modelling of tree species distributions on the Iberian Peninsula during the Last Glacial Maximum and Mid-Holocene. Ecography, **30**: 120-134.

Gomez, A. and D. H. Lunt. 2007. Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula.

Griffiths, R. A. 1996. Newts and Salamanders of Europe. First edition. Blackwell Publishing.

Hewitt, G. M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. Biological Journal of the Linnean Society **58**:247-276.

Hewitt, G. M. 2000. The genetic legacy of the Quaternary ice ages. Nature **405**:907-913.

Hudson, R. 1990. Gene genealogies and the coalescent process. Pages 1-44 Oxford Surveys in Evolutionary Biology. Oxford University Press, Oxford.

Kreitman, M. 2000. Methods to detect selection in populations with applications to the human. Annual Review of Genomics and Human Genetics **1**:539-559.

Malkmus, R. 2004. Amphibians and Reptiles of Portugal, Madeira and the Azores-Archipelago. Koeltz Sceintific Books.

Martinez-Solano, I. 2004. Phylogeography of Iberian Discoglossus (Anura : Discoglossidae). Journal of Zoological

Systematics and Evolutionary Research **42**:298-305.

Martínez-Solano, I., H. A. Gonçalves, J. W. Arntzen, and M. García-Paris. 2004. Phylogenetic relationships and biogeography of midwife toads (Discoglossidae: Alytes). Journal of Biogeography **31**:603-618.

Martinez-Solano, I., J. Teixeira, D. Buckley, and M. Garcia-Paris. 2006. Mitochondrial DNA phylogeography of Lissotriton boscai (Caudata, Salamandridae): evidence for old, multiple refugia in an Iberian endemic. Molecular Ecology **15**:3375-3388.

Mes, T. H. M. 2003. Demographic expansion of parasitic nematodes of livestock based on mitochondrial DNA regions that conflict with the infinite-sites model. Molecular Ecology **12**:1555-1566.

Moritz, C. 2002. Strategies to Protect Biological Diversity and the Evolutionary Processes That Sustain It. Systematic Biology **51**:238 -254.

Moritz, C., C. J. Schneider, and D. B. Wake. 1992. Evolutionary Relationships Within the Ensatina eschscholtzii Complex Confirm the Ring Species Interpretation. Systematic Biology **41**:273-291.

Panchal, M. 2007. The automation of Nested Clade Phylogeographic Analysis. Bioinformatics **23**:509-510.

Paulo, O. S., C. Dias, M. W. Bruford, W. C. Jordan, and R. A. Nichols. 2001. The persistence of Pliocene populations through the Pleistocene climatic cycles: evidence from the phylogeography of an Iberian lizard. Proceedings of the Royal Society B: Biological Sciences **268**:1625-1630.

Petit, R. J. 2008. On the falsifiability of the nested clade phylogeographic analysis method. Molecular Ecology **17**:1404-1404.

Posada, D. 2004. COLLAPSE. Free Software Foundation Incorporation Boston

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution **25**:1253-1256.

Posada, D., K. A. Crandall, and A. R. Templeton. 2000. GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. Molecular Ecology **9**:487-488.

Ramos-Onsins, S. E. and J. Rozas. 2002. Statistical Properties of New Neutrality Tests Against Population Growth. Mol Biol Evol **19**:2092-2100.

Rebelo, R. Personal Communication 2008. Morphology and coloration in the specie *Salamandra salamandra* that inhabits Portugal. Faro.

Rebelo, R. and M. H. Leclair. 2003. Differences in size at birth and brood size among Portuguese populations of the fire salamander, Salamandra salamandra. Herpetological journal **vol. 13**:179 -187.

Rozas J, Sánchez-DelBarrio JC, Messegyer X, and Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics **19**:2496-2497.

Sambrook, J., P. MacCallum, and D. Russell. 2001. Molecular Cloning: A Laboratory Manual. Third Edition edition. Cold Spring Harbor Laboratory Press, Nerw York.

Simonsen, K. L., G. A. Churchill, and C. F. Aquadro. 1995. Properties of Statistical Tests of Neutrality for DNA Polymorphism Data. Genetics **141**:413-429.

Steinfartz, S., M. Veith, and D. Tautz. 2000. Mitochondrial sequence analysis of Salamandra taxa suggests old splits of major lineages and postglacial recolonizations of Central Europe from distinct source populations of Salamandra salamandra. Molecular Ecology **9**:397-410.

Steinfartz, S., S. Vicario, J. W. Arntzen, and A. Caccone. 2007. A Bayesian Approach on Molecules and Behavior: Reconsidering Phylogenetic and Evolutionary Patterns of the Salamandridae with Emphasis on Triturus Newts. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution **308**:139–162.

Tamura K, Dudley J, Nei M, and Kumar S. 2007. Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24**:1596-1599.

Tan, A. and D. Wake. 1995. MtDNA Phylogeography of the California Newt, *Taricha torosa*. Molecular Phylogenetics and Evolution **4**:383-394.

Teixeira, J. 2007. Biogeografia, evolução e conservação da herpatofauna endémica do noroestes da Península Iberica. Porto University, Porto.

Templeton, A. R. 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. Molecular Ecology **7**:381-397.

Templeton, A. R. 2008. Nested clade analysis: an extensively validated method for strong phylogeographic inference. Molecular Ecology **17**:1877-1880.

Templeton, A. R., E. Routman, and C. A. Phillips. 1995. Separating Population Structure from Population History: A Cladistic Analysis of the Geographical Distribution of Mitochondrial DNA Haplotypes in the Tiger Salamander, Ambystoma tigrinum. Genetics **140**:767-782.

Veith, M., L. Fromhage, J. Kosuch, and M. Vences. 2006. Historical biogeography of

Western Palaearctic pelobatid and pelodytid frogs: a molecular phylogenetic perspective. Contributions to Zoology **75**:109-120.

Veith, M., C. Mayer, B. Samraoui, D. D. Barroso, and S. Bogaerts. 2004. From Europe to Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed salamanders (Genus Pleurodeles). Journal of Biogeography **31**:159–171.

Concluding Remarks

- Our results conform to the common pattern of a northern and southern geographical split in most Iberian amphibians, resulting in two well separated groups.

- The two identified groups can be easily separated on the basis of any of the 17 diagnostic positions.

- Each group matches the geographical distribution of the two subspecies of *S. salamandra* existing in Portugal: Clade I with a northern-central range similar to *S. s. gallaica*, and Clade II with a southern distribution matching *S. s. crespoi*.

- In most localities only one subspecies is present, except in Alcoutim and Sesimbra, where the genetic variants characteristic of these two species were for the first time identified in sympatry.

- Alcoutim and Mértola were inhabited mostly by *S. s. crespoi* (Alcoutim had only one *S. s. gallaica* individual) while the bibliography indicates these areas should only have the species *S. s. gallaica*.

- All localities display historical demographic stability.

- There is anecdotal evidence for Monchique and more robust evidence for Sintra and Serra da Estrela - Viseu area that support the hypotheses of them being refugia areas (Monchique for *S. s. crespoi*, and Sintra for *S. s. gallaica*).

- There is high level of phylogeographic structure found in the two salamander subspecies, especially in *S. s. gallaica*, likely marked by the effects of the Tejo, Douro and Sado Rivers acting as barriers.

- Our results indicate a late Pliocene/early Pleistocene (3.8 – 5.2 MYA) origin of the split between S. *s. crespoi* and *S. s. gallaica*.