UNIVERSIDADE DE LISBOA

Faculdade de Ciências

Departamento de Biologia Animal

MESTRADO EM BIOLOGIA EVOLUTIVA E DO DESENVOLVIMENTO

Assessing the speciation continuum in *Timon lepidus*

Telma Guedes Laurentino

Dissertação orientada por:

Professor Doutor Octávio S. Paulo

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I dedicate this thesis to all passionate knowledge-seekers before me, currently investigating the natural world, and to those yet to come.

Abstract

Understanding speciation requires the integration of ecology, evolution and the role of history in shaping the diversification or decline of lineages. To gain understanding on how variation is generated and maintained within and between natural populations, we must understand both how variation in phenotype may affect the fitness of individuals in their local environment, and how natural selection is shaping the genome of those organisms.

Next generation sequencing together with new analytical approaches have fundamentally changed the scope of studies of non-model organisms and thus, the available tools to answer long-standing questions underwent remarkable evolution. We are now, more than ever before, equipped to establishing missing links between phenotype, genotype and environment, which will provide a detailed picture of the adaptive evolutionary process.

Studies of the genomics of speciation along the speciation continuum are emerging in several non-model organisms, mainly where speciation is driven by ecology and divergent selection. The present study was the first applying RAD-Seq to natural populations of *Timon lepidus*, which allowed the analysis of thousands of polymorphic molecular markers simultaneously, across this lizard's genome.

The objective was to assess the putative incipient process of speciation between two subspecies, and further understand how populations adaptively diverge in heterogeneous environments.

The SNP data generated allowed us to address different scopes of *T. lepidus* evolutionary history, allowing the assessment of the population genomics of this species considering differently acting evolutionary forces.

The main pattern of divergence between populations reflects local adaptation rather than the expected incipient speciation pattern accordant with taxonomy, and further evidence of local adaptation and repeated ecological evolution are provided both by genomic and environmental information of this species.

Phenotype assessment proved to be inconclusive regarding the taxonomic arrangement of populations and additional research should uncover this patterns.

Therefore, the current taxonomy should be reviewed in the light of the speciation continuum, taking into account the pattern of local adaptation expressed by these populations.

Keywords: Speciation, subspecies, local adaptation, evolutionary history, RADseq, *Timon lepidus*

Resumo

A biodiversidade sempre fascinou o homem e, como tal, compreender o processo pelo qual surgem as espécies é uma problemática que acompanha os biólogos evolutivos desde a génese desta ciência.

Especiação é definido como o processo genético de diferenciação entre populações divergentes, de organismos sexuados, que culmina na distribuição descontínua de fenótipos e genótipos, mesmo entre populações com a proximidade geográfica. É o balanço entre este processo e a taxa de extinção que dá origem á biodiversidade.

A teoria de especiação, assim como o conceito de espécie, evoluíram bastante desde a publicação do *magnum opus* de Charles Darwin: "On the origin of species by means of natural selection, or the preservation of favoured races in the strungle for life", isto é: "Da origem das espécies por meio da seleção natural, ou a preservação de raças favorecidas na luta pela sobrevivência". Darwin é considerado o pai da biologia evolutiva e foi o primeiro a estabelecer o elo de ligação entre o processo adaptativo e o processo de origem de novas espécies, no entanto, passados 150 anos este continua a ser um tópico central da biologia evolutiva.

Entender o processo de especiação requer a integração de conhecimentos de ecologia, evolução e do papel da história evolutiva na modelação da diversificação de linhagens evolutivas. Para compreender como a variação é gerada e mantida dentro e entre populações naturais, é necessário compreender simultaneamente como a variação no fenótipo afeta a *fitness* dos indivíduos no seu ambiente local, e como a seleção natural modela o genoma desses mesmos organismos. Apesar da grande quantidade de investigação que tem vindo a ser realizada na área da especiação, principalmente nos últimos 20 anos, questões como a base genética e molecular da evolução adaptativa continuam ainda por desvendar. Permanece também obscuro como é que a seleção natural num número limitado de genes pode culminar em divergência *genome-wide*. Assim sendo, estabelecer os elos de ligação ente fenótipo, genótipo e ambiente poderá providenciar uma panorâmica detalhada do processo evolutivo.

As tecnologias de sequenciação de nova geração e a evolução da performance computacional, juntamente com o desenvolvimento das abordagens analíticas em bioinformática tiveram um impacto fundamental na capacidade de estudo de organismos não-modelo. Assim, as ferramentas que nos capacitam de investigar questões biológicas de longa data sofreram uma evolução extraordinária, fazendo com que estejamos hoje, mais do que nunca, equipados para compreender o processo de adaptação, assim como o processo de especiação. Estudos tendo como alvo o contínuo de divergência genética gerado ao longo do processo de especiação, emergem agora em diversos sistemas de organismos, especialmente naqueles onde a especiação é

impulsionada por heterogeneidade ambiental e consequentemente seleção ecológica disruptiva.

O presente estudo é o primeiro a aplicar sequenciação de nova geração ao lagarto ibérico *Timon lepidus*, de nome comum Sardão. Esta espécie apresenta distribuição mediterrânica, e é encontrada no sudoeste europeu com ampla distribuição na Península Ibérica. Três subespécies ibéricas continentais estão descritas, com base em morfologia e evidências moleculares e genéticas. As três subespécies apresentam distribuição parapátrica. *Timon lepidus lepidus* distribui-se pela maioria da área ocupada pela espécie, estando associada a zonas de clima tipicamente mediterrânico. Esta é substituída por *Timon lepidus ibericus* na extremidade noroeste da península, onde o clima é temperado oceânico, e na extremidade sudeste pela subespécie *Timon lepidus nevadensis*, que está fortemente associada a um bioclima desértico oceânico. Assim, gera-se um gradiente ecológico de noroeste para sudeste ao longo do qual variáveis bioclimáticas, como temperatura e precipitação, se modificam. Este lagarto classifica-se como um heliotérmico, querendo isto dizer que está dependente da exposição ao sol para termorregular e atingir níveis fisiológicos ótimos. Esta característica, associada ao facto de que se regista divergência morfológica e genética entre as três subespécies, sugere fortemente que as condições ecológicas contrastantes ao longa da área de distribuição podem conduzir à ocorrência de adaptação local, potencialmente promovendo o processo de especiação.

Estudos de filogeografia e história demográfica nesta espécie apontam *Timon lepidus nevadensis* como a espécie mais divergente, com o evento cladogenetico estimado há 9.4 milhões de anos. Esses estudos, realizados com marcadores mitocondriais e nucleares, detectam também estruturação genética e subdivisões geográficas entre populações de *Timon lepidus*, desvendando assim uma história evolutiva profundamente influenciada pelas oscilações climáticas causadas pelas idades do gelo, durante o período Pleistoceno. Seis linhagens mitocondriais com distribuição discreta são identificadas. *Timon lepidus nevadensis*, a subespécie mais divergente, apresenta uma linhagem única, exclusivamente associada a sua área de distribuição. No entanto, o mesmo padrão não se encontra para *Timon lepidus ibericus* que partilha linhagem mitocondrial com populações *Timon lepidus lepidus*. Esta linhagem mitocondrial designa-se por L3, e denota o facto de estas subespécies poderem partilhar eventos de história evolutiva. Assim sendo, as populações de *Timon lepidus lepidus* podem partilhar, ou não, história evolutiva com populações de *Timon lepidus ibericus*.

Tendo em vista a complexidade de forças evolutivas que interagem na modelação da estruturação populacional e padrões taxonómicos, é principal objetivo da presente dissertação avaliar o processo de especiação incipiente putativo entre *Timon lepidus lepidus* e *Timon lepidus ibericus*. Assim, pretende-se compreender como populações de ambas as subespécies divergem e se adaptam a ambientes heterogéneos.

A fim de abordar esta questão, três populações do clade L3: duas atribuídas a *Timon lepidus ibericus* (Galiza e Gerês) e uma atribuída a *Timon lepidus lepidus* (Montezinho) foram analisadas conjuntamente com uma população de um clade mitocondrial diferente, atribuída a *Timon lepidus lepidus* (Serra da Estrela). Aos indivíduos amostrados destas populações foi aplicada a técnica de RAD-Seq para obtenção de informação genómica, sob a forma de milhares de marcadores polimórficos espalhados pelo genoma, designados *Single Nucleotide Polymorphisms* (SNPs). A matriz gerada com esta informação permite a análise genómica das populações, de forma a compreender os níveis de estruturação genómica e como estas divergem.

Os dados de SNPs permitiram a construção de duas matrizes contendo diferentes panoramas da história evolutiva do *Timon lepidus*: uma incluindo todas as populações, permitindo assim o efeito da filogeografia diferencial das populações, e outra comtemplando apenas populações com uma história evolutiva comum, permitindo assim considerar os eventos demográficos e as diferentes forças evolutivas que atuam na produção do padrão de divergência destas populações.

Resultados concordantes com estudos anteriores mostram que Galiza e Serra da Estrela são fortemente marcadas por demografia e história evolutiva, respetivamente, que as diferenciam das restantes populações.

O principal padrão encontrado reflete adaptação local das populações. Este resultado é inesperado tendo em conta a taxonomia atual atribuída às populações em estudo. Evidencias adicionais de adaptação local e adaptação ecológica paralela são também fornecidas pela relação entre a informação genómica e ambiental.

A informação fenotípica, no entanto, provou ser inconclusiva tendo em conta a atribuição taxonómica das populações, não contribuindo para a compreensão do padrão geral de divergência genómica das mesmas. Assim, é sugerido que a taxonomia atual seja revista à luz do conceito génico de especiação, e tendo em conta o padrão de adaptação local que foi descoberto na presente dissertação.

A análise de taxons em vários estágios do contínuo de especiação permite perceber padrões gerais quando combinado com a história natural do sistema em estudo, e o presente estudo denota, uma vez mais, a complexidade de interações entre forças evolutivas envolvida tanto no processo de especiação como no de adaptação local.

Palavras-chave: especiação, adaptação local, evolução, *Timon lepidus*, sequenciação de nova geração, subespécie.

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1. INTRODUCTION

In 1859, Charles Darwin publishes one of the most enlightening essays in the history of biology: *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life,* a seminal work for evolutionary biology where, for the first time, a coherent body of observations that solidified the concept of biological evolution is summarized into a completely materialistic scientific theory.

Darwin's thesis can be broken into five theories I) Evolution *per se*, i.e. the alteration of biological characters over time, II) Gradualism, meaning that those characters would change in a small-stepped cumulative process, IV) Natural selection, a theory co-authored by Wallace (1858), which includes the concepts of individual variability, differential reproductive success and consequent increase of the frequency of individuals who better respond to environmental demands, the V) Multiplication of species, or population speciation, based on the principle that changes in the proportions of individuals within a population would lead to formation of new species , and finally III) Common descent, a theory according to which all species descend from a common ancestor and thus, life could be portrayed as one great family tree.

Even though Darwin was by no means the first to postulate a history of evolution, his vision differed greatly from the ideas of his predecessors (Futuyma, 1997) as he concluded that the link between species, past and present, was similar to a genealogic tree and therefore irregular, devoid of tendency or privileged direction (Darwin, 1837).

Later, but not without previous inflamed debate, Darwin's ideas were combined with Mendel's genetics, through mathematic models and statistics, and population genetics was born with the evolutionary synthesis (reviewed in Futuyma, 1997). A much popular phrase then takes form in the words of Dobzhansky (1973) "nothing in biology makes sense except in the light of evolution".

Nowadays, evolutionary biology is still focused on the understanding of two major aspects of nature: organism adaptation to the environment, denominated microevolution, and speciation or the origin of the discontinuous distribution of phenotypes, and genotypes, in sexually reproducing organisms into units that we call species (Butlin *et al.* 2012). Biodiversity can then be seen as the pattern resulting from the balance between extinction and speciation, the branching of the tree-of-life.

1.1 Species: one of the most controversial conceptualization in biology

Species is one of the fundamental units of biology and the concept is used ubiquitously. However, the debate over the definition of species goes back to Aristotle and Theophrastus (Mayr, 1986).

As one cannot deal with the origin of species without trying to define it, Charles Darwin also dealt with this matter and in a letter to Hooker (December 24, 1856) he wrote: "I have just been comparing definitions of species... It is really laughable to see what different ideas are prominent in various naturalists' minds when they speak of 'species'; in some, resemblance is everything and descent of little weight, in some, resemblance seems to go for nothing, (…) in some, sterility an unfailing test, with others it is not worth a farthing. It all comes, I believe, from trying to define the undefinable" (L.L.D II: 88).

Later, in the 20th century, with the uprising of the modern synthesis, one of the most popular species concepts arises: the biological species concept (Mayr, 1942). Mayr's *biological,* or *reproductive isolation, species concept is* based on "groups of interbreeding natural populations that are reproductively isolated from others such groups" so that they diverge, leading to populations that, when in secondary contact, no longer tend to interbreed. This would result in distinct gene pools over evolutionary time scales.

The basic reasonableness and operational advantages of Mayr's criterion struck immediate and wide favorable response, and his principle has been applied with considerable enthusiasm (Wilson & Brown, 1953; Coyne & Orr 2004).

However, the controversy is still very much alive nowadays (e.g. Sangster, 2014; Zimmermann & Radespiel, 2014; Dvořák et al 2015), giving rise to a huge body of literature on how species can, may or should be delimited, or even if the concept makes any sense regarding asexual organisms (Fontaneto & Barraclough, 2015).

Scientific efforts are being made towards a unified species concept (De Queiroz, 2007; Hausdorf, 2011). However, a great amount of species definitions still exist, and a generally applicable conception is yet to come (Coyne & Orr, 2004). The core problem is that various properties acquired by diverging lineages throughout time are viewed as necessary properties of species: species criterion (Fig. 1, SC), and each one of these criteria was considered necessary cutoffs under each alternative concept (De Queiroz, 2007). This set of proprieties, such as reciprocal monophyletic, ecological distinction, reproductive isolation, etc. forms a grey zone within which the alternative species conceptions conflict (Fig. 1).

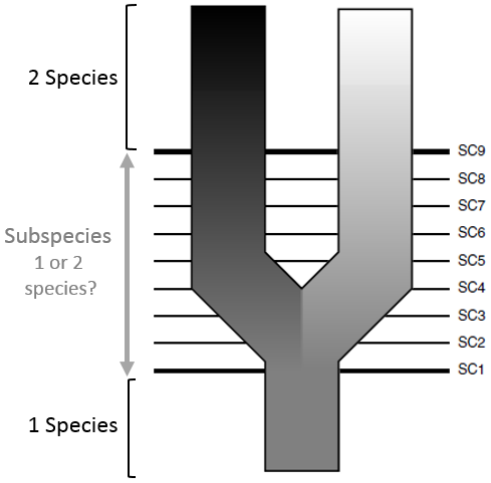

Figure 1. Speciation process and Species criterion (SC). SC 1 to 9 represent various criterion that diverging lineages acquire along divergence time. The entire set of SC forms a grey zone within which alternative species concepts conflict. Adapted from (De Queiroz, 2007).

On the extremes of the grey continuum, however, all species definitions agree unanimously about the number of species: before the acquisition of the first propriety (SC1) there is a single species, and after the acquisition of the last (SC9) there are two distinct evolving lineages (De Queiroz, 2007).

The grey zone thus comprises what we call a subspecies. Along with his analysis of the main concept, Mayr (1942) conceived subspecies as genetically distinct, geographically separate populations belonging to the same species, therefore interbreeding freely at the zones of contact. Subspecies taxonomy is biologically meaningful since they are unique evolutionary lineages, and the divergence between subspecies is not qualitatively different from that between species, and can actually be quantitatively evaluated when using multidata approaches (Sackett et al., 2014).

A species concept not only defines what a species is, but also aids the understanding of the tempo and mode of speciation. Thus, research projects focusing on the conditions and factors involved in speciation depend on the species concept, as many other biological studies depend on the delimitation of species (Hausdorf, 2011), establishing an intimate link between how we define a species and how it is formed by speciation.

When referring to species in the present thesis, which focus on a sexually reproducing diploid organism, the biological species concept is adopted, thus equating speciation to the evolution of reproductive isolating mechanisms, which essentially prevent gene exchange between newly arising taxa. However, as stated by Turelli *et al.* (2001), the use of this definition does not imply reproductive isolation as necessary for

morphological, ecological or genetic divergence, nor thus it exclude other aspects of divergence between sympatric or allopatric taxa.

1.2 SPECIATION: A HOT TOPIC FORM MORE THAN 150 YEARS

Speciation is described as the genetic process of differentiation between diverging populations, of sexually reproducing organisms, which results in a discontinuous distribution of phenotypes and genotypes, even with geographical proximity. The balance between this process and extinction gives rise to biodiversity and, consequently, this has been a central topic of evolutionary science.

An enormous amount of research on speciation has been conducted since the publication of Darwin's magum opus, especially during the past 20 plus years (R. Butlin et al., 2012; Coyne & Orr, 2004). However, despite many important advances, we are still fairly ignorant of which mechanisms are involved in the origin of species (Turelli *et al.* 2001; Butlin *et al.* 2012; Seehausen *et al.* 2014). Research on speciation is currently under auspicious development, as it takes advantage of the new possibilities of the post-genomic era, and the main contemporary questions regard the component mechanisms and evolutionary forces that lead to reproductive isolation, the connection of the process itself with patterns of biodiversity, and the understanding of the genomic signatures of the speciation process (R. K. Butlin & Ritchie, 2009; R. Butlin et al., 2012).

Some of the traditional organizing principles, such as the classification of speciation mechanisms exclusively by geographical arrangement into: allopatric, parapatric or sympatric (Fig. 2), no longer provide a satisfactory conceptual framework (R. Butlin et al., 2012; Schulter, 2001). Classification of speciation models nowadays focuses on driving mechanisms of reproductive isolation (Schulter, 2001; Via, 2001), and concepts, such as the speciation islands, are currently under heated debate (Pennisi, 2014).

Speciation was once generally thought to require vicariant phenomena, i.e. physical barriers that prevented gene flow between populations, thus leading to reproductive isolation (Fig. 2). Due to the complete ceasing of gene flow, reproductive compatibility between geographically isolated populations is lost, and speciation becomes inevitable in allopatry. Given enough time, both pre- and postzygotic isolating mechanisms would arise as inevitable by-products of genetic divergence caused by isolation itself, adaptation to alternative environments, or both (Schulter, 2001; Turelli et al., 2001).

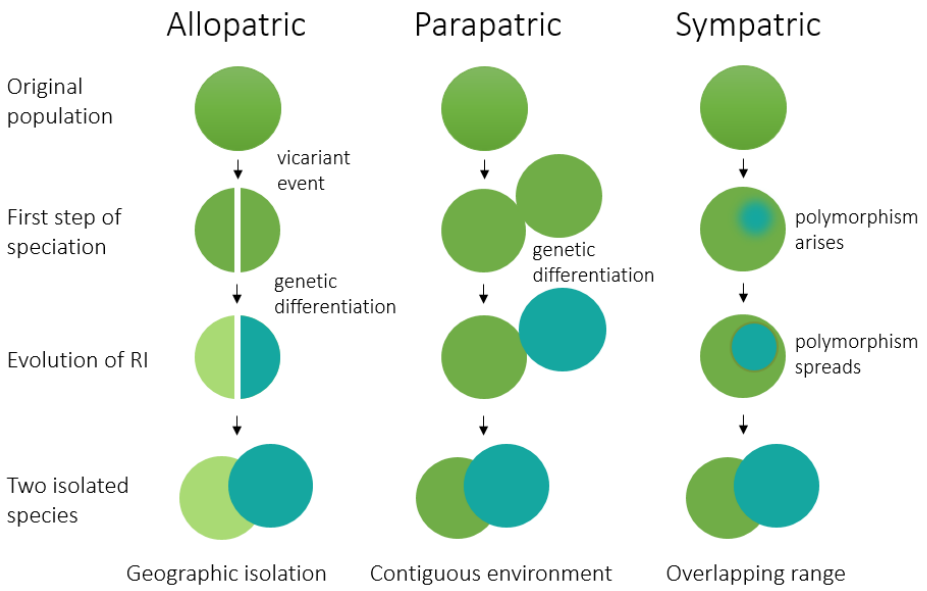

Figure 2. Modes of speciation regarding geographical arrangement of populations. Allopatric speciation requires geographic isolation, thus totally inhibiting gene flow, while both parapatric and sympatric speciation can occur in the presence of gene flow.

Yet, species do form without a physical barrier, and population genetics has shown that mechanisms that can produce divergence among allopatric populations can also cause divergence in parapatry (Fig. 2) and sympatry (Fig. 2).

Parapatric speciation occurs between populations occupying contiguous habitats, and the speed of the process will be impacted by the strength of the evolutionary forces generating divergence. If these are strong, causing local adaptation or maintaining alternative adaptive peaks, then divergence of traits leading to reproductive isolation can occur over small spatial scales. Even if alleles favorable in all the species range spread across hybrid zones, such introgression might not prevent parapatric divergence, because selection can dominate gene flow over quite small scales (Turelli et al., 2001).

Another type of speciation-with-gene-flow, and a more controversial one, is sympatric speciation, which occurs between populations sharing overlapping habitats, and is driven by disruptive or divergent selection (Turelli et al., 2001; Via, 2001). Reproductive isolation can readily evolve in sympatry, either through divergent selection directly on habitat choice or as a pleiotropic effect of disruptive selection on other traits (Rice and Salt, 1990). Also, linkage between loci under disruptive selection and those causing assortative mating would facilitate speciation by limiting the disruptive effects of recombination (Via, 2001).

However, despite being reliant on the same evolutionary forces, the dynamics of genomic divergence in the presence of gene flow can differ greatly from allopatric divergence (Feder, Egan, & Nosil, 2012). When gene flow accompanies the speciation process, antagonism is generated between divergent selection, and migration and gene flow. The first builds up favorable combinations of locally adapted genes, while the second break down those combinations and tend to homogenize populations. Hence, features of genome structure that reduce recombination between populations (e.g. chromosomic inversions) can enhance the efficiency of divergent selection, by creating and maintaining linkage disequilibrium (LD). By contrast, there is no antagonism between selection and recombination during speciation in allopatry, since geographic barriers to migration totally inhibit gene flow (R. Butlin et al., 2012; Coyne & Orr, 2004; Feder et al., 2012).

A seminal contribution for the understanding of speciation with gene-flow is the model created by Wu (2001; Fig. 3), which pictures the genome behaving like a porous membrane, which allows for recombination in some, but not all, genomic regions: populations will diverge at few loci that are locally advantageous, whereas divergence in non-adaptive regions of the genome will be prevented by homogenizing gene flow. This dynamic will result in a continuum of speciation, which can culminate in a cladogenetic event, or produce incipient species.

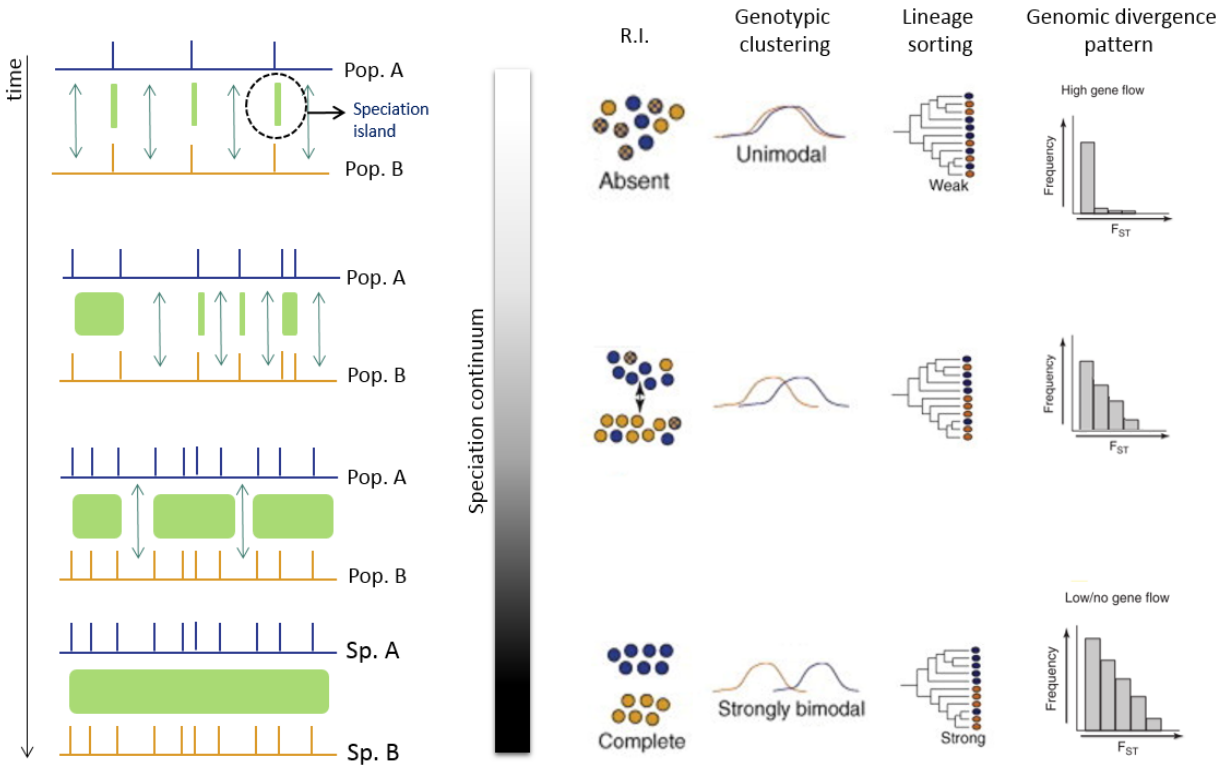

Figure 3. The speciation continuum. **Blue** represents population A and **Yellow** represents population B. **Vertical bars** represent locus under selection. Gene flow between populations is represented by **double arrows** and **green blocks** represent inhibition to gene flow, being it complete on the last stage. Reproductive isolation (R.I.) is absent on the onset of the speciation process and complete towards the end. Genotypic clustering, lineage sorting and overall genome divergence pattern also vary along the speciation continuum (represented by gradient bar), exhibiting strongly contrasting patterns in the beginning (white) and end (black). Image adapted from Wu (2001); Nosil et al. (2008); Feder et al. (2012).

According to this model, in order to understand the earliest stages of speciation a distinction should be made between two classes of loci: those that directly affect differential adaptation (speciation genes/islands; Fig. 3) and those that do not (neutrally evolving genes; Wu, 2001). The speciation process can then be divided in four stages (Wu 2001; Feder *et al.* 2012; Fig. 3). At stage I of the speciation continuum, population differentiation has taken place at a small number of loci that are under selection, being therefore responsible for functional divergence. Despite the high levels of overall gene flow, recombination is locally reduced in these genes because they are directly subject to strong divergent selection. Upon secondary contact, gene exchange would be extensive. However, at the loci of functional divergence gene flow would be restricted. At stage II, a very substantial portion of genomic regions would remain shared and undifferentiated. Populations at this stage are given the subspecies status. At this point, if ecological conditions shift, for

example, populations may still fuse upon massive hybridization, instead of continuing to diverge (Mayr, 1963). Contrarily, at stage III, the diverging groups have passed the point of no return. However, in parapatric species, a narrow hybrid zone may still exist. At this point, the accumulation of "speciation genes" and the extensive LD affecting many loci distributed along the genome, resulted in extensively divergent (between populations) and co-adapted (within population) gene complexes. The groups should now be different in aspects of reproductive biology, sexual behavior, and/or morphology. Despite of behaving as two different species, the genomes are not reproductively isolated. Throughout the speciation continuum, stage progression can happen faster if genetic or divergent hitchhiking takes place (Feder et al., 2012), especially if the accumulation of divergence extends to genes that promote assortative mating. These phenomena help to extend the local selection, at speciation islands, to neighbor genomic regions (Feder et al., 2012). If stage IV is ever achieved, a genome-wide barrier to gene flow is attained, and the two gene pools cease sharing alleles through breeding, thus giving rise to two new reproductively isolated branches of the tree-of-life.

Notwithstanding, progression along the speciation continuum is not constant, and speciation can go back and forth, or even be arrested at intermittent stages, resulting in variation in both intensity and type of barriers that isolate incipient and sister species (Seehausen et al., 2014). In many cases, particularly in ecological speciation (Nosil, Harmon, & Seehausen, 2009), strong reproductive isolation may never evolve.

1.2.1 Ecological Speciation

Divergent or disruptive selection, arising from habitat heterogeneity, has been highlighted recently as a dominant force driving population divergence (R. Butlin et al., 2012). When barriers to gene flow, arising between populations, result from ecologically-based divergent selection, that process is defined as ecological speciation (Schulter, 2001).

This model of speciation differs from other models in which the evolution of reproductive isolation involves key processes other than ecological selection, such as genetic drift, events of hybridization and population demography (Coyne & Orr, 2004; Rundle & Nosil, 2005).

Notwithstanding, selection can also be a driving force of non-ecological speciation when not resulting from of individual-environment interactions, and/or when it is not divergent between environments.

Ecological speciation predicts that populations became locally adapted such that, if an individual from one population finds itself in the habitat of the other, then its fitness is reduced. For that reason, the alleles for local adaptation, which

are unfit in the alternate environment, tend to remain within the population in which they originated, leading to divergence (Wu 2001; Nosil et al. 2009). Consequently, ecologically divergent pairs of populations will exhibit greater levels of reproductive incompatibility than ecologically similar pairs of populations (Seehausen et al., 2014).

This process is based on three essential mechanisms: an ecological source of divergent selection, a form of reproductive isolation, and a genetic mechanism which links the first two (Rundle & Nosil, 2005).

Environmental differences causing divergent selection can include habitat structure, climate, resources, and both predators or competitors present (Coyne & Orr, 2004; Schulter, 2001). Although these have been implicated in a number of speciation events in nature, the research focus is not homogenic regarding these environmental variables, and additional cases are needed in more studying systems (Rice et al., 2011; Rundle & Nosil, 2005; Seehausen et al., 2014). For example, predator-generated divergent selection has been implicated in the evolution of reproductive isolation in just a few cases (e.g. Jiggins et al. 2001; Nosil 2004).

Other promotors of ecological selection involve sexual selection and biotic interactions (Rundle & Nosil, 2005). Regarding the first, due to its direct involvement in mate recognition, it can be a commanding force in the evolution of reproductive isolation (Panhuis et al. 2001). Spatial variation in natural selection on secondary sexual traits (Lande 1982) and mating or communication systems (Boughman 2002) has been recorded in different environments. As an example, allopatric populations of *Anolis cristatellus* lizards, occupying two different bioclimatic regions, showed divergence on dewlap design, a trait important in social communication and mating. The trait diverged between populations such that it increases signal detectability in each habitat (Leal & Fleishman 2004). A similar pattern was found for sympatric populations of *Anolis* species and predation and sexual selection seem to be the main drivers of divergence, thus illustrating the interaction of different ecological factors (Muñoz et al., 2013).

As described for the overall speciation process, divergence in ecological speciation often varies continuously, and the degree of phenotypic divergence can vary quantitatively, as can the extensiveness of reproductive isolation (Fig. 3). Studies, in various taxa, including reptiles (Nosil et al., 2008; Muñoz et al., 2013; Rosenblum et al., 2010), support the predictions of the ecological speciation model, and demonstrate that local adaptation, in response to selection across ecological gradients, can generate and maintain phenotypic diversity, even with elevated levels of gene flow.

By complementing ecological studies of natural populations with the new tools

brought by the genomic era, it will be possible to achieve a deeper understanding of how ecological speciation proceeds, from initial adaptation to different environments to incompatible genomes (Rice et al., 2011).

1.3 LIZARDS: A FINE REPRESENTATION OF TREE-OF-LIFE'S DIVERSITY

Lizards are fascinating from an evolutionary perspective, for this group colonizes a wide variety of ecological niches comprising a wide diversity of morphologies and life strategies (Pianka & Vitt 2003), being able to be as small as *Coleodactylus amazonicus,* which could drown on a raindrop, or as big as the last living dragon: *Varanus komodoensis*. Varying from legless shapes to full quadruped, lizards can run, crawl, climb completely vertical surfaces, walk on water and even glide. Tails can be lost to distract predators and grown back, used as a whip or as a fifth prehensile limb.

Colour pattern adaptations in reptiles are remarkable, with plentiful cases of complex background matching, aposematism, gaudy sexual signals, polymorphism and exquisite colour change ability (Mats Olsson, Stuart-Fox, & Ballen, 2013). The plethora of adaptations depict a long evolutionary history, marked by the interaction with both abiotic and biotic components of their ecosystem.

The origin and diversification of lizards is closely related with continental drift. In the Cretaceous, when Gondwana broke apart, some groups like gekkonids and skinks dispersed widely, while others displayed a more limited dispersal, the second being the case of Lacertidae which nowadays exhibit Palearctic distribution (Pianka & Vitt 2003).

1.3.1 ECTOTHERMY AND THERMOREGULATION

The physiological differences of reptiles compared to other tetrapods have important ecological consequences. Due to their ectothermy a lizard's distribution is always influenced by the climatic landscape. From hunting to digestion (Wang *et al.* 2002), from courting (Paranjpe et al., 2014), to sperm production and egg development (Wapstra, 2000), and immunity (Paranjpe et al., 2014; Sandmeier & Tracy, 2014), all physiological functions of reptiles are temperature dependent (Huey 1982; Pianka & Vitt 2003).

Predator evasion efficiency is also impacted by external temperature (Mori & Burghardt 2004; Angilletta, 2009), and it has been shown that thermal constraints shape refuge use in Schreiber's lizards (*L. schreiberi)*. These lizards seem to adapt refuge use depending on external temperature by balancing the physiological costs

of being at low temperatures with the risk of emerging with low escape performance (Martín & López, 2010b).

Thus, thermoregulation affects phenotype and reproductive performance, being directly linked to individual fitness. Metabolism rates and thermal tolerance may differ between species and populations, reflecting the strong selective pressures on behaviour, physiology and life history generated when environmental temperatures vary over space and time, leading to local adaptations (Scheers & Van Damme 2002; McMillan *et al.* 2011; Grigg & Buckley 2013).

1.3.2 COLOUR AND COLOUR PATTERNS

Besides environmental and climatic factors, phenotypic characters, such as body size and color pattern on the lizard's skin, are also determinant to heat gain and thermoregulation (Clusella Trullas, van Wyk, & Spotila, 2007).

Color and pattern are one of the most diverse traits contributing for distinction among vertebrates, often varying drastically between closely related species (Muñoz et al., 2013), or between populations (Rosenblum, Hoekstra, & Nachman, 2004). Coloration is thus an excellent trait to assess how morphological variation arises. It is one of the primary modes of the interaction organism-environment, implicated in a variety of biological processes including mate choice, predator warning, and mimicry and crypsis, consequently having dramatic effects on fitness (Hoekstra, 2010).

Two major functional requirements are intimately linked with color patterns: physiology and communication. Regarding physiology, pigmentation may be involved in thermoregulation or UV protection and, since these functions cannot be decoupled, a certain colour pattern may serve multiple roles, such as dark colouration which can simultaneously contribute to UV protection, thermoregulation and crypsis (Rudh & Qvarnström, 2013). Concerning communication, when intraspecific, is generally connected with sexual selection. When the visual signal receiver is of the same sex, coloration can be used to impress or scare competitors, ascertaining dominance for territory and/or mates (M Olsson, 1994; Stapley & Whiting, 2006). However, when the receiver is the opposite sex, then the function of sexual dimorphic coloration is usually to attract a mate for reproduction (Cooper & Burns 1987; Bajer *et al.* 2010).

On the other hand, when communication is interspecific, then natural rather than sexual selection is acting, and coloration is either cryptic (Muñoz et al., 2013; Rosenblum, Römpler, Schöneberg, & Hoekstra, 2010) or aposematic (Brodie & Janzen, 1995), in order to escape from or warn visually hunting predators, respectively, or it can even be both (Beck, 2005).

Coloration in reptiles results from complex interactions between light and integument structure. It is produced by a combination of reflection and scattering of light (structural coloration) and by absorption of light by chemical pigments, stored within dermal chromatophore cells (reviewed in Olsson *et al.* 2013; Fig. 4)

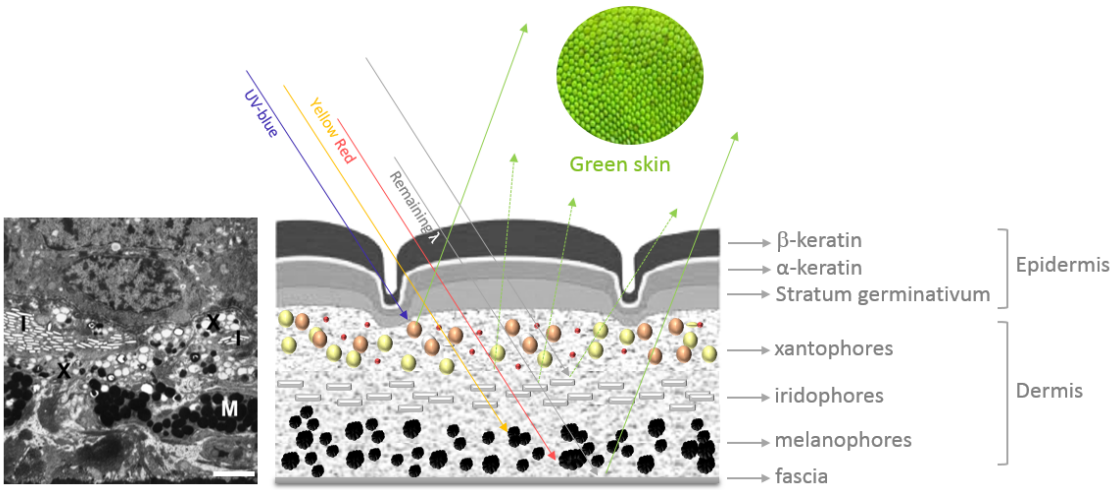

Figure 4. Lizard skin and colour production. A Electron photomicrograph of histological preparation of lacertid green skin. **I** iridophores, **X** xantophores, **M** melanophores; scale bar: 2 µm (adapted from *Kuriyama et al. 2006)* **B** Schematic representation of reptile green skin. Light passes through the epidermis into the dermal layer where it is differentially absorbed by the pigments and is reflected back to the outside, minus the wavelengths absorbed. Light which passes through the melanophores without being absorbed is reflected by the fascia integumental layer. Dashed arrows represent light scattering by iridophores. (adapted from Chang *et al.* 2009).

The upper-most dermal layer contains xanthophores, which enclose yellow-red pigments. Beneath lie iridophores, containing colourless guanine crystals which reflect and scatter light. The resulting structural colour depends on both size and spacing of the crystals. The deepest layer comprises melanophores, which contain eumelanin and absorb all light wavelengths. Bellow the layer of melanophores there is a layer of silvery-white connective tissue (fascia) separating skin from muscle (Fig. 4).

In order to produce green coloration, for example, light passes through the epidermis and short wavelengths (UV-blue) are absorbed by xanthophores, while longer wavelengths (yellow-red) pass through the iridophores and are absorbed by the melanophores. The remaining light, which is not absorbed by melanophores, is reflected by the underlying fascia. This results in the scattering and reflection of middle green wavelengths, and thus we see a green lizard (Mats Olsson et al., 2013).

Many reptiles possess ultraviolet colour patches, often involved with male-male contest, signaling fighting ability (Whiting *et al* 2003; Font *et al.* 2009; Bajer *et al* 2011). These structural colours are generated both by interaction with pigments in chromotaphores and the scattering of light in the integument. In general, the spectral purity of structural colours is increased by increasing melanin within the underlying melanophore layer. In *Anolis conspersus*, the near absence of pigments in xantophores reduces the absorbance of short wavelengths (UV-blue). This, associated with higher concentration of melanin within melanophores, ensures that any light passing through iridophores is absorbed and thus, the only reflected light is the UV, appearing dark blue to UV-insensitive human eyes (Macedonia et al 2000).

Colouration can also signal individual age, readiness for breeding and health condition, since it can be deeply impacted by other physiological systems. For example, structural colours may be influenced by the effects of developmental stress or nutritional status on iridophore organization, by testosterone mediated melanin expression, or both (Quinn & Hews, 2003; Cox et al 2005).

Evaluating colour patterns can be critical when testing for ecological adaptation (Endler, 1990), and since colour signals are composed by multiple wavelengths, which can be higher and/or lower than the visible spectrum (400 – 700 nm; Fig. 5), it would be biased to assess it exclusively through human eyes or ordinary color photography. Spectrometry equipment is currently used in order to obtain objective and repeatable quantification of colours, both in field and laboratory studies.

Color spectrum can be decomposed into brightness, chroma, and hue (Endler, 1990). These features are associated with physical properties of light (Fig. 5). Hue is what we commonly refer to as colour, it is largely dependent on the dominant wavelength of light that is emitted or reflected by the animal. Thus, the same hue, such as blue, can vary in brightness or chroma, producing different tones of blue (Fig. 5 right). Brightness can be defined as overall color intensity, and chroma is a measure of purity, or saturation. Chroma depends on how much light of other wavelengths is mixed with the focal color (hue). Highly chromatic colors contain maximum hue, with negligible impurities such as white, black or gray mixed in the tone (Endler 1990; Grill & Rush, 2000; Fig. 5 right).

A reptile skin colour can fluctuate with any of these factors. This variation may result in quantitative characteristics, which can vary across populations or be correlated with other phenotypic variables, such as body mass and body condition in males (M Olsson, 1994; Thorpe, 2002).

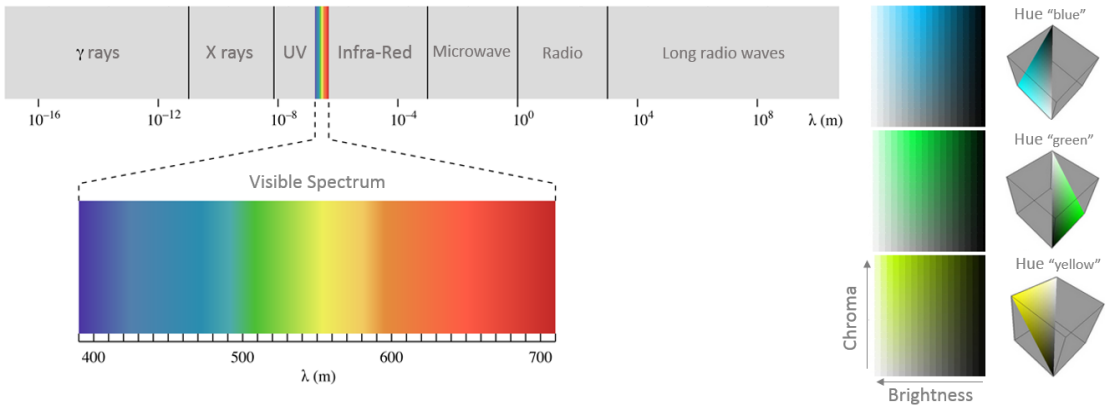

Figure 5. Colour Theory. **left** The electromagnetic spectrum comprising the visible spectrum, which the Human eye perceives, higher and lower wavelength radiation, such as UV perceived by diurnal lizards; **rigth** colour componentes Brightness(B), Chroma(C) (arrow points toward higher values),and Hue, commonly disgnated "colour". Each Hue is contained in a plan of the RGB cube and varies with different values of B and C.

The mechanisms of production and variation of reptile coloration are well characterized at structural level (Fig. 4), and squamate color has been studied in detail from ecological, physiological and systematic perspectives. However, the genetic architecture of color traits is still poorly understood, and the molecular basis of color evolution in this group is still undercovered (Kronforst et al., 2012; Rosenblum et al., 2004).

Even though studies of adaptive color variation and polymorphism have become popular, as models for explore the genetics of natural selection, thus far molecular research consists mainly of association studies with melanocortin- 1 receptor (Mc1r) as a candidate gene. Mc1r has conserved function among vertebrates, regarding melanine production (Hoekstra, 2010) and genome walking techniques have been used to develop various species-specific primers (Rosenblum et al., 2004). Current research shows different mutations in the Mc1r accounting for convergent cryptic phenotypes (V. L. Nunes, Miraldo, Beaumont, Butlin, & Paulo, 2011; Rosenblum et al., 2010), but other melanin-based coloration traits exist which are not directly associated with Mc1r (Corso, Gonçalves, & de Freitas, 2012; Micheletti, Parra, & Routman, 2012).

Less is known about the genetics underlying red/yellow and blue /green colour variation in reptiles. Unraveling the genetics of blue/green coloration could be challenging due to the nature of structural colors, which are determined by the interaction between melanin layers and the number, size, and spacing of reflecting platelets in iridophores (Kuriyama et al., 2006), thus involving various integument structures and cell types.

Different approaches, such as genome-wide association studies, could bring new insights regarding the genetic basis of colour pattern variation in reptiles, and melanism is an excellent focal phenotype since many reptile species have evolved melanic dorsal coloration.

Despite the wide range of features making reptiles, more specifically, diurnal lizards, promising models for research on ecology, coloration genetics, local adapatation and speciation (Pianka & Vitt 2003; Camargo *et al.* 2010; Olsson *et al.* 2013), this group is still far behind in in the application of population genomics and genome sequencing, with the first lizard genome only recently sequenced (Alföldi et al., 2011). In the present study an NGS technique is applied to a non-model lizard species, aiming to further comprehend its evolutionary course in the Iberian Peninsula. Despite being previously investigated regarding population genetics and structure, this is the first genomic approach of *Timon lepidus*.

1.4 *TIMON LEPIDUS*, EUROPE'S OCELLATED LIZARD

Timon lepidus (DAUDIN 1802), previously denominated *Lacerta lepida*, underwent taxonomic revision with the upgrade of *Timon* from subgenus to genus. Based on morphology, karyotype and phylogenetic characters the genus *Timon* forms a monophyletic clade, including four species of ocellated lizards: *Timon pater* and *Timon tangitanus*, both native from Northern Africa, *Timon princeps*, native from Middle east (Ahmadzadeh, Carretero, Harris, Perera, & Böhme, 2012; Harris, Arnold, & Thomas, 1998; Harris & Carretero, 2003) and *Timon lepidus*, native from Europe.

Timon lepidus, is one of the biggest lacertids and the only species of European ocellated lizard. Presenting Mediterranean distribution, it can be found in southwestern Europe, with wide distribution in the Iberian Peninsula, also occurring in some Atlantic islands along Spanish and Portuguese coasts, ranging from sea level up to altitudes of 2500m. Populations exist in southern, south-central and western France, and in extreme northwestern Italy (Castroviejo & Mateo, 1998; Mateo and Castroviejo, 1991; Mateo *et al.*, 1996). Some insular and Italian populations are on the verge of extinction and habitat loss is causing a general trend for decreasing population effective size. Being so, *Timon lepidus* is classified as nearly threatened (IUCN red list 3.1).

Four subspecies of *T. lepidus* are recognized, based both on morphological and molecular evidence (Castroviejo & Mateo, 1998; Mateo, 1988; Paulo et al., 2008; Nunes, 2011): *Timon lepidus oteroi* (Castroviejo & Mateo 1998) is an insular subspecies, endemic to the island of Salvora in northern Spain. The remaining three possess parapatric distribution, within the Iberian Peninsula (Fig. 6). *Timon lepidus*

lepidus (Fig. 6 B, D) occurs in the majority of the species range, associated with typically Mediterranean climate. This subspecies is replaced by *Timon lepidus ibericus* (LÓPEZ-SEOANE 1884) (Fig. 6 *A, D)* in the north-western corner of the peninsula, where the climate is temperate oceanic, with abundant annual rainfall (Fig. 6 F) and the lowest amount of sunshine hours registered in the peninsula.

Contrastingly, on the south-eastern coast, associated with the Betic mountain ranges, inhabits *Timon lepidus nevadensis (BUCHHOLZ 1963)* (Fig. 6 C, D). This region is also marked by differences in bioclimatic variables, characterized by reduced and irregular amounts of annual precipitation (Fig.6 F) and higher mean annual temperatures (Fig. 6 E). Despite also being a Mediterranean bioclimate, it is a xeric oceanic region, with predominance of sparse shrub-like vegetation with large portions of exposed soil (Mateo & Castroviejo, 1990; Hodar et al., 1996).

An ecological northwest-southeast cline is thus generated by the variation of bioclimatic variables, such as temperature and precipitation (Fig. 6 E, F), which in turn affect the vegetation cover, proprieties of the soil and, most likely, the invertebrate fauna, which constitutes *Timon lepidus* main dietary resource (Bruun *et al.* 1993).

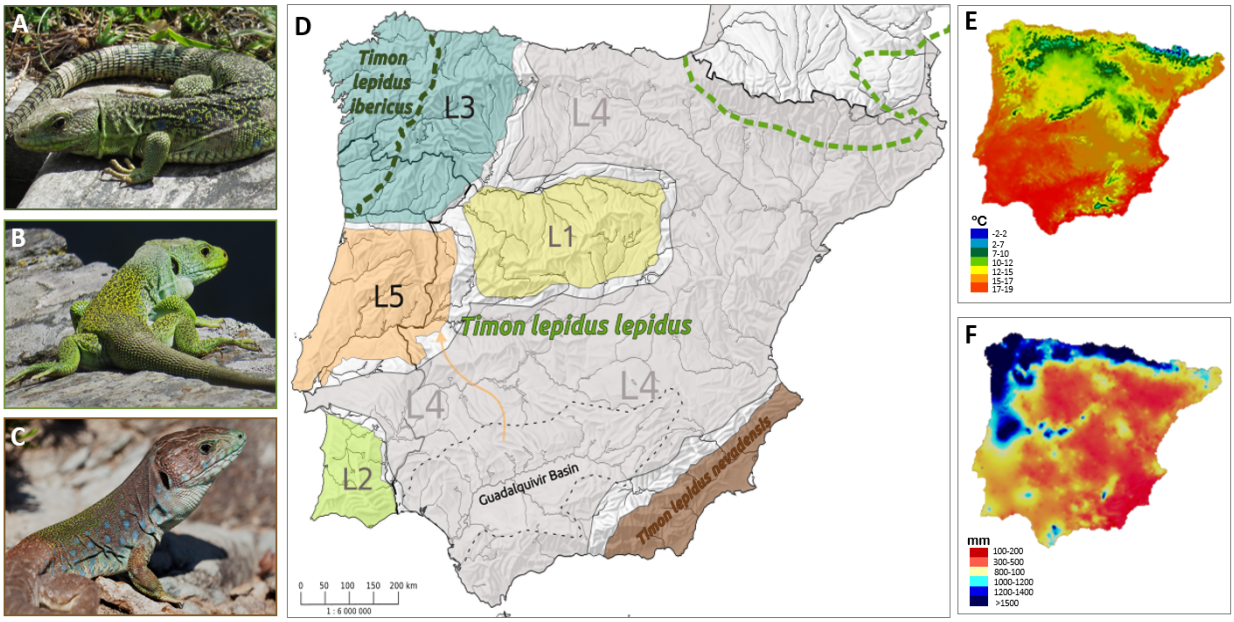

Figure 6. Subspecies, phylogeography and ecological clines of *Timon lepidus*. A *T.l.ibericus*, from Galiza **B** *T.l. lepidus*, from Serra da Estrela **C** *T.l.nevadensis* **D** Iberian peninsula with mitochondrial phylogroups represented by different shaded colours. Subspecies ranges are limited by dashed lines as dark green: *T.l.ibericus*, light green: *T.l. lepidus*. Guadalquivir basin is delimited by a dark grey dashed line, and the orange arrow represents the movement of ancestral L5 lizards from the glacial refugium to the current distribution area (adapted from Miraldo et al. 2011). **E** mean annual temperature **F** mean annual precipitation (adapted from Nunes 2011).

The ultimate source of heat for *Timon lepidus* is the sun. Their corporal temperature of activity ranges from 21ºC to 35ºC and, as heliotherms, they seek out patches of direct sunlight exposure in their habitat, in order to bask and thermoregulate. This characteristic, associated with the fact that morphological and genetic divergence occurs between the three Iberian mainland subspecies, strongly suggests that the contrasting ecological conditions along the distribution range may be leading to local adaptation and promote the speciation process (V. L. M. Nunes, 2011).

1.4.1 GENETIC VARIATION AND PHYLOGEOGRAPHY OF *TIMON LEPIDUS* IN IBERIAN PENINSULA

Studies have been performed on the genetic variability of *Timon lepidus* (*T.l.*) since 1988 (Mateo 1988). The pioneer research was performed recurring to allozymes and pointed *T.l. nevadensis* as the most divergent clade (Mateo 1988; Mateo *et al.* 1996). Later, with additional genetic data, from both mitochondrial and nuclear markers, the phylogeny and phylogeography of the genus is uncovered and related with the evolution of the western Mediterranean region and the Messinian salinity crisis (Paulo et al., 2001; Paulo *et al.* 2008). The Phylogeography and demographic history within the Iberian Peninsula is also assessed (Miraldo, Hewitt, Paulo, & Emerson, 2011) and *T.l. nevadensis* is confirmed as the most divergent subspecies, with the cladogenetic event estimated to occur approximately 9.4 Ma ago (Miraldo et al., 2011; Paulo et al., 2008).

These studies also detected further genetic structure and geographical subdivisions among *T. lepidus* populations, uncovering an evolutionary history deeply influenced by vicariance events and the climatic oscillations of the later Pleistocene (Miraldo et al., 2011). Six mitochondrial lineages, with non-overlapping distribution, are identified (Fig. 6 D). *T.l. nevadensis* presents a unique lineage, exclusively found in its distribution range (Fig. 6 D, in brown). Contrarily, *T.l.ibericus* does not possess an exclusive lineage associated with the subspecies range, it is contained within the clade L3 (Fig. 6 D, in blue), and *T.l.lepidus* spans all lineages from L1 to L5.

The phylogroup L3 (Fig. 6 D, in blue), comprising populations form both *T.l.ibericus* and *T.l.lepidus*, occupies the north-western corner of the peninsula. Despite currently being geographically close to the phylogroup L5 (Fig. 6 D, in orange), L3 forms a monophyletic group with L2 (Fig. 6 D, in green), and the currently disjunct distribution, with the intervening region ocupied by L5, can be explained by vicariance events taking place during the Middle Pleistocene (0.82-2.27 Mya; Miraldo *et al.* 2011).

During ice age periods, phylogroups L1, L4 (Fig. 6 D, in grey) and L5 were probably concentrated in refugia on the south-eastern zone of the Guadalquivir

basin. With the subsequent climatic amelioration, during the interglacial period, L5-bearing lizards were able to expand from the refigium, and thus colonize the geographic gap existent at the time between the L2-L3 lineage (Fig. 6 D).

During these ecological challenges, the demographic history of populations was shaped by alterations in population effective size, migration rates, splitting and admixture of populations and thus, the evolutionary history of *T.lepidus* in the Iberian Peninsula is consistent with cyclic processes of population fragmentation, followed by allopatric divergence within the glacial refugia, and subsequent expansion and new environment colonization.

The present thesis is focused on the two subspecies presenting the latest process of divergence and a fainter pattern of differentiation: *T.l.lepidus,* from populations of L5 and L3 phylogroups, and *T.l.ibericus*.

1.4.2 ON *TIMON LEPIDUS* PHENOTYPE AND ITS VARIATION ALONG THE ECOLOGICAL CLINE

Timon lepidus is a really interesting species, when regarded from a morphological scope. The intraindividual variety of colour patterns and scale types can be astonishing, showing regional specificity of different shapes, arrangements and colours with different body parts (Fig. 7). Body coloration in this species figures prominently in descriptions of intraspecific ontogenetic and geographic variation (Mateo & Castroviejo, 1991; Mateo & López-Jurado, 1994).

As the common name (Ocellated lizard) implies, the presence of eyespots (ocelli), on the flanks of adult individuals, is the trademark trait of the species, which has been dazzling naturalists for a long time (Lopez-Seoane, 1884). The eyespots present nearly circular motifs, with contrasting colours, and emit in the UV spectrum (Font et al., 2009), thus appearing blue to a human observer (Fig. 7 G, H).

Dorsal skin is composed by small and dense non-overlapping oval scales, without apparent anterior-posterior polarity, forming an irregular reticulated pattern of green over a black background (Fig. 7 E). Some of the pattern can extend towards the throat skin, but it is fainted, and the predominant color of this area is green, with the loss of great majority of the black background (Fig. 7 F)

Timon lepidus presents sexual dimorphism, with males differing from females mainly in head and body proportions (Pérez-Mellado, 1998). On top of that, sexual dichromatism has been reported, with females being less brightly coloured and having fewer eyespots, with lower UV reflectance values than males (Arnold 2002; Font *et al.* 2009). A link between immunity and coloration in males was also discovered, with males with higher immunological competence showing flanks that are more greenish, darker, and with higher chroma, thus more saturated (Martín & López, 2010a).

Both body proportions and chromatic differences can be related with sexual selection (Paulo 1988; Font *et al.* 2009; Martín & López 2010).

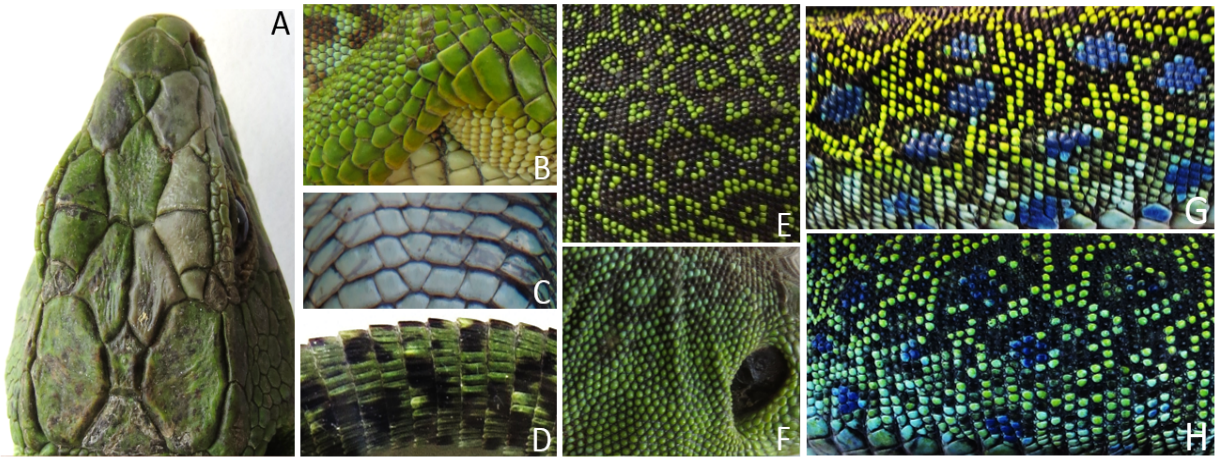

Figure 7. Intraindividual scale diversity and colour patterns of *Timon lepidus*. **A** Poligonal non-overlaping scales, typical of the top of the head, **B** overlapping assimetric scales present on limbs, **C** overlaping ventral scales which are more regular in shape and organized in transversal rows, **D** Tail possesses keeled scales which come to a shaped ridge, forming rings, **E** Dorsal skin composed of smaller and denser oval non-overlapping scales, and the typical black and green reticulated pattern, **F** Throat skin with faint to absent reticulated pattern and usually green, **G** Flank skin with typical male eyespots, well defined and usually larger and in higher numbers than **H** female eyespots.

Thus, while flank regions are under sexual selective pressures, at the same time, dorsal regions tend to be under natural selection, from both visual predator pressure and thermoregulation requisites. Opposing forces can origin to simultaneously diminish dorsal conspicuity, and enhance background matching, and become laterally more conspicuous, to optimize intraspecific communication (Macedonia et al. 2002; Stuart-Fox et al 2003; Husak et al 2006). The interplay of this can thus impact the phenotype and individual fitness.

Despite of intrapopulational variation in dorsal colour pattern, visually recognizable differences in pattern disposition (Mateo, 1988) and melanin-based colouration (Mateo 1988; Nunes *et al.* 2011) were associated with *Timon lepidus* subspecies, so that each presents a distinct colour phenotype associated with a specific bioclimatic region of the species range (Mateo & Castroviejo, 1990). Actually, the three subspecies of the Iberian Peninsula mainland were initially recognized exclusively based on morphological divergence (Mateo & Castroviejo, 1990). Notwithstanding, The degree of subspecific differences in colour pattern was later shown to be consistent with the level of genetic differentiation showed by mitochondrial and nuclear DNA (Miraldo et al., 2011; V. L. M. Nunes, 2011; Paulo et al., 2008)

As said before, *T.l. nevadensis* (Fig. 6 C) presents clear distinction form the remaining subspecies, at both phenotypic and genotypic levels. This subspecies coloration pattern is remarkably contrasting, with absent or faded dorsal pattern, overall dominance of brown scales and, regarding the eyespots, they rarely present the black contour found in the other subspecies (Mateo & Castroviejo, 1990; Mateo & López-Jurado, 1994). A statistical association with a mutation in the Mc1r gene was discovered, shedding light on the genetic basis of the phenotypic differences of *T.l. nevadensis* (V. L. Nunes et al., 2011). This mutation is possibly responsible for a phenomenon of convergence of melanin-based colouration also found in other background matching lizard species (Rosenblum et al., 2010).

However, clear patterns of morphological distinction and association with Mc1r gene variants are lacking for *T.l. ibericus* (Fig. 6 A). Being so, the genetic basis of the described increased melanization in this subspecies, when comparing with *T.l. lepidus*, is still uncovered.

T.l. ibericus are described as smaller individuals bearing darker dorsal coloration, with predominance of black scales and transversal arrangement of the dorsal pattern (Mateo & Castroviejo 1990; Mateo & Castanet 1994). Body size is important for multiple dimensions of an organism's biology, including physiology, ecology, and life history traits. Thus, this trait is likely under various types of selection simultaneously and within species, body size–environment relationships can arise due to local adaptation and/or phenotypic plasticity (Phillimore *et al.* 2010, 2012). On top of that, body size and reproductive strategies are known to vary across thermal environments (Ashton and Feldman 2003; Pincheira-Donoso *et al.* 2008; Muñoz *et al.* 2014).

Finally, dentition is another morphological trait varying with subspecies. The number and morphological specialization of teeth varies along the southeast-northwest cline, with *T.l. ibericus* presenting higher number and more homogeneous, regarding size and symmetry, teeth (Mateo 1988; Castroviejo & Mateo 1998). Associated with this phenotype, slight variation in dietary resources was described (Mateo & Lopez-Jurado 1997; Castilla 1989). Coleoptera are the main prey for ocellated lizards, and in populations from *L. l. lepida*, in central Spain, Scarabidae beetles were the most abundant dietary resource in these lizards diet. However, Gastropoda are especially abundant in humid regions and have a relevant contribution to *L. l. iberica* diet (Mateo 1988; pers. observation during field work of the present thesis).

Altogether, these facts point towards a scenery of ecological adaptation that could explain the taxonomic pattern within this species, in Iberian Peninsula, and hints from different genetic markers point in that evolutionary direction (V. L. M.

Nunes, 2011). Thus, *Timon lepidus* represents an excellent model to assess questions regarding local adaptation and the speciation continuum.

1.5 The omics era: revisiting the long-lasting question of speciation with new tools

Biology is currently entering the 'omics' era, and next-generation sequencing (NGS) techniques are revolutionizing life sciences (Ellegren, 2014). NGS refers to high-throughput sequencing technology, which has become simultaneously less time consuming and more affordable, when comparing to previously applied sequencing methods. Such technology allows researchers to obtain huge amounts of genomic data from non-model organisms by sampling the genome much more densely, thus allowing the observation of patterns of genetic variation resulting from the full range of evolutionary processes acting across the genome (Narum, Buerkle, Davey, Miller, & Hohenlohe, 2013; Stapley et al., 2010).

We are currently able to study organisms that are relevant for evolutionary questions that are also distantly related to model species, which often possess sequenced whole genomes (Hudson, 2008). Actually, since ecological speciation research is focused on variation within and between populations, whole genome sequencing will rarely, if ever, be the goal of such projects. Instead, methodology to approach this type of questions usually requires the development of a set of polymorphic markers (Rice et al., 2011). However, the development of these datasets for population genetic analyses can be complicated, when studying wild populations, and this has effectively excluded most species from research focus (J. L. Davey & Blaxter, 2010). The traditional detection of markers imposes the screening of large numbers of individuals, and with traditional sequencing, this is both time consuming and expensive, becoming overwhelming when multiple sets of populations must be assessed.

Restriction site-associated DNA sequencing (RADSeq) overcomes this problem for it allows the discovery and assay of markers, simultaneously, thus providing the ability to record genetic polymorphism across thousands of loci (Baird et al., 2008; J. L. Davey & Blaxter, 2010; J. W. Davey et al., 2011). The library preparation protocol for RADSeq consists of digesting genomic DNA with restriction enzymes, with posterior tagging of each individual sample with barcode sequences. Tagged individuals can then be pooled and sequenced with Illumina NGS, covering from 0.1 to 10% of the genome, depending on organism's characteristics. This "complexity reduction" approach to genome-wide sequencing offers significant advantages compared to other common NGS approaches (J. W. Davey et al., 2011; Miller,

Dunham, Amores, Cresko, & Johnson, 2007) making it a particularly attractive approach for the study of population diversity in organisms lacking reference genomes or possessing complex evolutionary histories (Rowe, Renaut, & Guggisberg, 2011).

By using methods such as the previously described in natural populations, the general importance of divergent selection in driving speciation can be better understood. Outlier and cline analyses can be used to pinpoint loci showing signatures of divergent selection (Rice et al., 2011) and genotype-phenotype association mapping and the understanding of intraspecific variation are just two of many applications of Single Nucleotide Polymorphisms (SNPs) discovery and genotyping resulting from RADSeq (Rice et al., 2011; Rowe et al., 2011).

RADSeq is thus a cutting edge technology and has been so far successfully applied in studies such as conservation genomics in Bumblebees (Lozier, 2014), demography and phylogeography of penguins (Trucchi et al., 2014), and in ecological speciation studies in both sticklebacks (Baird et al., 2008) and plants (Andrew & Rieseberg 2013; Stölting *et al.* 2013), contributing with further insight into the genomic patterns of divergence during the speciation process.

1.6 Objectives and the three-way approach

The present thesis is focused on assessing the putative ecological speciation occurring between the two northern Iberian subspecies of *Timon lepidus*: *Timon lepidus lepidus* and *Timon lepidus ibericus*. The aim is to assess if the currently described taxonomy is in accordance with newly collected genomic and phenotypic data, and if bioclimatic causes, generating ecologic-based divergent selection, may be the main driver of the differentiation between the two.

For this, a three-way approach considering genomic, phenotypic and environmental data is applied, in order to give further insight regarding questions such as I) if the two subspecies are in advanced or incipient speciation stage, II) if genomic divergence is associated with bioclimatic environmental differences of the subspecies range, III) if genomic signatures of natural selection are present at the subspecies level and finally, if IV) there is phenotypic data contributed to the further understanding of divergence between these subspecies.

The data to assess population variability and speciation pattern in the genome consists of a dataset of thousands of genome-wide SNPs, which were obtained recurring to RADSeq NGS and analyzed with bioinformatics tools.

Phenotypic data focuses on morphological characteristics of body measures and colour pattern. These were assessed both by digital photography and reflectance

measurement methods, with some of the phenotypic traits known to be involved in intraspecific communication, thus putatively involved in sexual selection.

2. METHODS

2.1 ETHICAL STATEMENT

Field sampling was carried out under the license nº474/2014/CAPT emitted by Instituto da Conservação da Natureza e Florestas in Portuguese territory, and under the license 086/2014 issued in 06/06/2014 by Conselleria de medio ambiente território e infraestruturas de Xunta de Galicia, for Galiza territory.

The animals were kept for a limited period of time for tissue collection, with stress being reduced to the minimum.

All procedures took into account measures of field hygiene preventive of disease spreading: the sampling equipment was always sterilized recurring to alcohol and flame between individuals, and the traps, and all material that contacted with individuals, cleaned with veterinary sanitizer (Virkon®).

2.2 LIZARD SAMPLING

Sampling season was held from 11/05/2014 to 10/07/2014. Lizards were captured with Tomahawk traps, and tissue from the tail tip was collected from a total of 41 adult individuals and 2 juveniles. Tissue was preserved in 95% ethanol. The four sampling areas in the Iberian peninsula spanned two previously described mitochondrial (mtDNA) clades (Miraldo *et al.* 2011), with Galiza (GAL; 43°30'7.92"N 8°19'2.21"W), Gerês (GER; 41º43'16,7'N 008º06'49,2''W) and Montezinho (MTZ; 41º56'16,30''N 007º00'57,19''W) belonging to mtDNA clade L3, and Serra da Estrela (SE; 40°26'13.42"N 7°30'47.29"W) representing the L5 clade. These poulations represent two of the currently described subspecies: Timon lepidus lepidus (MTZ and SE) and Timon lepidus ibericus (GAL and GER). An individual was sampled in Botica (BOT; 41º39'40,1''N 007º52'28,8''W), a region in the area comprised between GER and MTZ populations. Total sampling number per population are as follows: for 11 GAL, for 9 GER, for 11 MTZ and for 12 SE. A total of 67 museum specimens from Museu Nacional de Historia Natural e da Ciência, Lisbon, were sampled for morphometrics and location of capture, in order to enhance sampling numbers for phenotypic analysis.

All individuals were sexed and Snout-to-vent length (SVL) was measured to the nearest 1mm with a ruler. Morphological measurements of head width and length were performed with calipers (to the nearest 0.1 mm). Head length was the greatest horizontal distance between the tip of the snout and the posterior side of the parietal scales. Head width was the greatest horizontal distance between the

external sides of the parietal scales. Measurements were always performed by the same researcher to avoid entropy on the data.

Dorso, flanks and throat of every individual, wild-caught and museum specimen, was photographed with a Canon SX50 HS.

2.3 DNA EXTRACTION AND RAD-SEQ

Muscle rings from the lizard's tail were hydrated with ddH_2O and skin and bone were removed, with the extraction protocol being applied only to the muscle tissue isolated from the sample. Genomic DNA was then extracted with DNeasy BNlood & Tissue Kit (Qiagen) with RNaseA (Qiagen) treatment.

RAD library preparation protocol followed the one described by (Etter *et al.* 2011). DNA quantification to determine molar concentration of the libraries was performed with *Qubit*™ fluorometric quantitation. RAD sequencing for this project was carried out with Illumina technology by the gene pool (Edinburg, UK) using the restriction enzyme *Sbf*I. Enzyme choice was based on estimated proportion of CG (0,4) and C-value of *Timon lepidus* genome (2,35), fold coverage (100) and plexity (42).

FastQC v0.11.2 software was applied in order to control for the sequence quality and the primary analysis pipeline implemented was the software PyRAD v2.16.1 (Eaton 2014).

2.4 SNP DATA ANALYSIS

Summary statistics for polymorphic SNPs were calculated with *Genepop* 4.2 (Rousset, 2008), and filtered with a custom script (https://github.com/CoBiG2/RAD_Tools/blob/master/BioGenepop.py) that relies on the Biopython project (Cock et al., 2009) module "PopGen". All *p*-values were corrected with a FDR test to account for multiple testing. Expected and observed heterozigosity and AMOVA analysis were performed with Arlequin 3.5.1 (Excoffier & Lischer, 2010). Identity by descent analysis was performed recurring to R package ADEGENET (https://github.com/thibautjombart/adegenet).

Histograms depicting genomic divergence between populations were constructed in based on Fst values per loci in pairwise populations calculated in *Genepop* 4.2. Differential genotypic and allelic frequencies were assessed with in-house developed scripts (https://github.com/Telpidus/misc_plotters) based on loci information calculated in *Genepop* 4.2.

In order to remove the effect of evolutionary history, denounced by mtDNA lineage, which could mask patterns of speciation and adaptation within the L3

mitochondrial clade, a SNPs matrix was generated where Serra da Estrela (SE) population was excluded from both the analysis of selection detection and environmental associations with genotype.

GphoCS v1.2.2 was used to access migration rates between populations. The input for this software was generated using in house developed python3 script (loci_and_vcf_to_GphoCS.py @https://github.com/CoBiG2/RAD_Tools) and the software was run with 1.000.000 mcmc iterations with remaining parameters default. Outputs were analyzed in Tracer v1.6.

All file format conversions in order to generate specific inputs for different programs were performed with PGDSpider (Lischer & Excoffier, 2012).

2.5 Outlier detection and environmental association

In order to distinguish between neutral and non-neutral loci, the polymorphic genotyped SNPs were scanned for Fst outliers using *LOSITAN* (Beaumont and Nichols, 1996; Antao et al., 2008).

The applied parameters were maximum simulation number (1000) confidence interval of 0.99 with options of Neutral mean FST and forced mean FST. False discovery rate (FDR) correction had to be applied post exporting the loci list file, with an in-house developed script (https://github.com/CoBiG2/RAD_Tools/blob/ master/FDR.R; commit 786ca75). The Fst-outlier analyses compares the distribution of Fst values across loci with the distribution expected in the absence of divergent selection for the same average differentiation. A locus with an Fst value that exceeds expectation is likely to be influenced by divergent selection, either on the locus itself or on a linked locus.

Following the results of *LOSITAN*, individual subsets of loci under positive and balancing selection, and neutral loci where generated to investigate population structure and differentiation patterns regarding only these markers under different evolutionary paths. Subsets where generated using python3 and Shell code developed in house (https://github.com/CoBiG2/RAD_Tools.git; VCF_converter.py). These subsets where applied downstream in software STRUCTURE, EIGENSOFT 5.0 and Blast2GO.

Environment variables used to conduct the genotype-environment association were obtain from WorldClim database (Hijmans *et al*. 2005) and processed using in-house developed code (https://github.com/StuntsPT/Misc_GIS_scripts). PCA of environmental variables was conducted in R.

Associations of SNP alleles to environmental and geospatial variables were performed with BayEnv2.0, which tests for allele frequency variation along different

population environments. Variables include values of temperature and precipitation, both seasonal and annual, altitude and sunlight, which are expected to have impact on natural history of these populations, since *T. lepidus* is an heliothermic species. An environmental association was considered to be significant when the Bayesian factor was superior or equal to 10, and the corresponding Spearman correlation was superior to 0,35 or -0.35.

SNPs with association to environmental variables were considered non-neutral and a dataset was generated including both SNPs under putative positive selection (detected by Lositan software) and SNPs with environmental associations. This is referred to as the non-neutral dataset throughout this study.

2.6 POPULATION GENETIC STRUCTURE

Population genetic structure was inferred from both total dataset comprising all populations and excluding SE, and for the individual subsets of markers under different evolutionary forces previously described.

For each of the mentioned datasets, patterns of population structure were investigated using the software *STRUCTURE* 2.3.4 (Pritchard et al., 2000). The ideal value of "K" was inferred using *Structure Harvester* (Earl & vonHoldt, 2012) with 30 replicates of 3000000 iteration runs with a "burn-in" value of 50000 (K = 1 – 6). All runs were performed with the "admixture" model.

Distruct 1.1 (Rosenberg, 2004) was used to produce the STRUCTURE plots in vectorial format. The value of *K* with the highest Δ*K* according to Evanno *et al.* (2005) was assumed to be the most probable number of clusters.

Population structure and clustering pattern was also assessed recurring to principal components analysis of the genomic information, with EIGENSOFT 5.0 (Patterson et al., 2006). This software is based on formal significance tests, eigenanalysis, and the graphs presented in the results section always comprise the eigenvectors which significantly explain the majority of variance (all p-values < 0.05).

2.7 PHYLOGENOMIC TREES

A maximum likelihood method was applied with RAxML v7.4.9 (Stamatakis, 2014), with partitioned model analysis, based on bootstrap method (number of bootstrap replications: 1000) using the model "GTRGAMMA". An in-house developed script was developed to convert the input to RaxML and generate a partition file. Trees were visualized in FigTree v1.4.2.

2.8 Functional Annotation of RAD-tags

Functional annotation of the RAD-Seq filtered results was obtained based in NCBI databases. For gene assignment, KEGG (Kanehisa et al., 2008) and GO annotation were applied. Rapsearch2 similarity searches were locally conducted against non-redundant ("nr") peptide database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/ downloaded at January 15, 2015 including all "nr" GenBank CDS translations + PDB + SwissProt + PIR+PRF), with e-value cutoff of 10^{-5}. The output was submitted to an in-house developed script Rapsearch2XML (https://github.com/Nymeria8/Rapsearch2Xml), and subsequently to Blast2GO (Conesa et al., 2005) software for enzyme identification, metabolic pathway assignment and functional annotation using GO terms.

2.9 Reflectance

Reflectance of dorso and throat of every wild-caught individual was measured from 400 to 700 nm, comprising the visible spectrum, using a PP Systems-UniSpec-SC Spectral Analysis System. The probe was held in a 90º angle to the skin, and reflectance was always measured by the same person.

Dorso was measured in 4 spots on the right flank along spine. Throat was measure in one spot bellowe ear membrane. Reflectance was calculated relative to a white standard and mean reflectance was summarized over 6 nm steps ("binned"; Grill & Rush 2000) before statistical analysis.

intraindividual measurements were highly repeatable within each spot (intraclass correlation coefficients, $r>0.90$, $P<0.0001$ in all cases), we calculated the mean values of the three measures. The same procedure was used to produce population average spectra (intraclass correlation coefficients, $r>0.89$, $P<0.001$ in all cases).

Spectrum and spectral segment analysis followed the methods described by (Endler, 1990). Spectral data followed the premises of normality and homoscedasticity and being so, analysis of Variance (ANOVA) with post hoc Tukey-Kramer for unequal sample sizes were performed in spectrum segment analysis to determine differences between populations.

Analysis of background matching followed the methodology in (Endler, 1990) and was conducted for both population average and for individual animals that were observed to exhibit basking behaviour on the collected substrate samples. Since individual values did not differ significantly from population values, only the last are shown in results. The collected substrates are representative of the principal basking substrate available for basking in each habitat. A transect of 10

reflectance measurements per substrate was performed in loco, following the same methodology applied for the reptiles.

2.10 COLORATION VARIABLES

A phenotype data matrix was constructed with individual information of varibales of Throat hue, chroma and brightness; Dorso hue, chroma and brightness; SVL; eyespot number, eyespot area; head morphometrics and melanization index.

To assess melanization index, $1cm^2$ squares where cropped from dorsal photographs, and all whole scales inside that area where counted by colour, in a total of 84 individuals: 28 wild sampled and 56 museum specimens. Melanization index is defined as the porportion of green scales of the total scales in that area (Mateo, 1988).

Eyespot area was measured using ImageJ software, and total number of eyespots per individual was counted in individual flank photography. UV patch area and number was thus assessed from color photography which has been proven to be a reliable method (Thorpe and Richard, 2001).

Phenotypic data did not pass the Shapiro-Wilk test for normality and non-parametricWilcoxon test was applied, in R, to look for differences between groups. False discovery rate was applied to all tests, also in R. Since SVL varied significantly between populations (p-value= 0.0004772), the remaining morphometric variables that could be influenced by body size were normalized for this effect.

3. RESULTS

3.1 RAD-TAG SEQUENCING

A total of 408.629.034 ($4x10^8$) Illumina sequenced reads were acknowledged for initial analysis. During demultiplexing: assigning reads to individuals based on their barcode tag, 7.997.306 reads were discarded due to having more than two mismatches in their respective barcode sequence, a phenomena that hinders the assignment of the sequence to a specific individual.

Posterior to quality filtering, 335.888.736 reads were retained, with an average coverage of 8.192.408,2 (±817049.4 SE) reads per individual.

After clustering, a set of 7.460.727 loci was obtained, with an average of 20.752.885,7 (±2289526,7 SE) sites per individual, of which 0.23% are polymorphic.

Of the 41 individuals sequenced, six were excluded (2 from GAL, 1 from GER, 2 from MTZ and 2 from SE) due to low sequence coverage and excessive missing data.

After filtering the missing data per loci and per individual and discarding loci with a minor allele frequency below 0.05, the resulting matrix comprised 1704 SNPs and 35 individuals (9 from GAL, 8 from GER, 9 from MTZ, 8 from SE and 1 from BOT).

In order to remove the effect of evolutionary history, denounced by mtDNA lineages, a second SNP matrix was generated, with the same parameters as the first, but where Serra da Estrela (SE) population was excluded. This matrix comprised a total of 3908 SNPs and 27 individuals. Roughly twice the SNPs of the matrix including SE. This result reflects the divergence of SE population, regarding the ones of the mitochondrial clade L3 (GAL, GER and MTZ), since a total of 2204 markers were not being considered SNPs in the first matrix due to lack of representation in SE.

3.2 GENOMIC DIVERSITY

All populations were analyzed for measures of genomic diversity (Fig. 1). Observed Heterozygosity (Ho) ranged from 0.074 in GAL to 0.197 in SE. Expected Heterozygosity (He) ranged from 0.292 in GAL to 0.412 in SE. GAL population was also the one expressing the highest value of Fis (0.758; Fig. 1).

This result, depicting GAL as the least diverse population contrasting with SE, is further confirmed by the Identity by state analysis of individual relatedness (Fig. 2). In this analysis GAL population shows the highest levels of relatedness among individuals, with SE expressing the lowest. This emphasizes the overall lowest diversity found in GAL population and the highest values of genomic diversity found

in SE, which is in accordance with previous research regarding GAL population comprising genetic and demographic data.

The dataset exclusively comprising SNPs evolving neutraly (neutral) and the one comprising exclusively the markers expressing either selection signatures or environmental association (non-neutral) were both analysed for population (Fig. 1 heatmap) and individual (Fig. 1 curves on the right) differentiation.

Both neutral and non-neutral markers are in accordance regarding GAL and SE being the pair of populations expressing higher genomic divergence (Fst = 0.384 for neutral, and Fst = 0.51 for non-neutral SNPs).

This is not unexpected since this is the population pair geographically farther apart, and belonging to a different taxonomic group: with GAL belonging to *T.l. ibericus* subspecies and SE to *T. l. lepidus*. Therefore, a reduction in gene flow is to be expected.

When considering exclusively non-neutral SNPs, the overall values of population differentiation rise (Fig. 1) showing higher values of Fst, depicted by darker shades of blue on the heatmap. Once again, GAL and SE are the pair of populations expressing higher genomic divergence. This overall value increment is also expected, since these markers are under evolutionary forces which alter allele frequency within populations, and thus contribute to divergence.

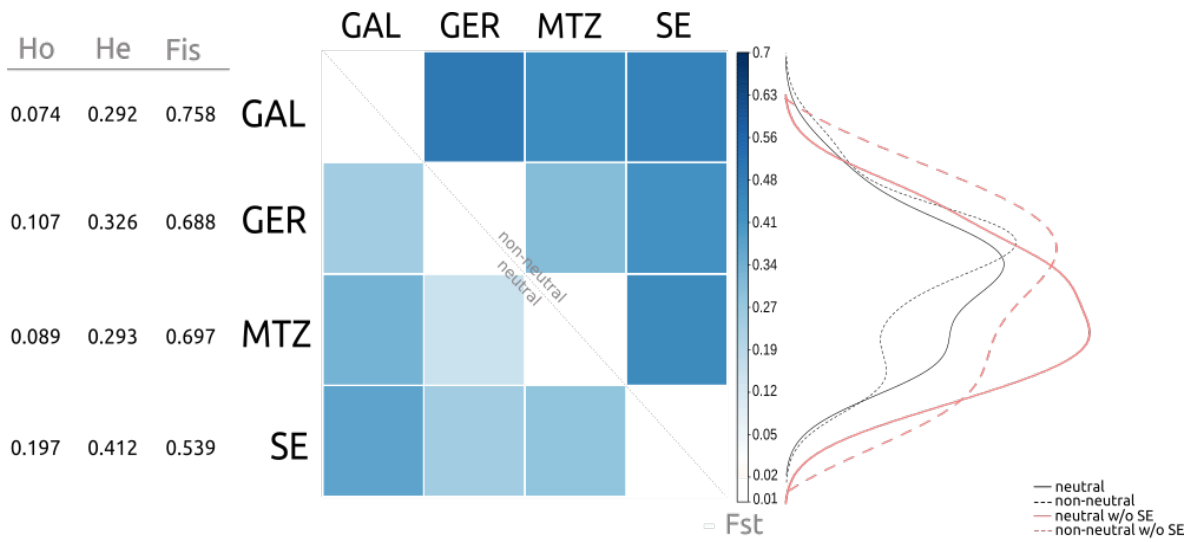

Figure 1. Population genomic diversity. On the left, observed (**Ho**) and expected (**He**) values of heterozigozity and inbreeding coefficient (**Fis**) for all studied populations (GAL, GER, MTZ, SE). On the center, Fst heatmap with superior diagonal comprising non-neutral SNPs and lower diagonal comprising exclusively neutral markers. On the right global Fst curves are show for both data sets with SE (**black lines**) and without SE population (**red lines**). **Dashed lines** represent SNPs showing signatures of selection or environmental association (non-neutral) **full lines** represent neutral SNPs.

Likewise, when analyzing general Fst profile curves (Fig. 1 on the right) a peak shift towards higher values is observed when considering exclusively non-neutral markers (Fig. 1 dashed lines). This pattern is observed in both datasets: with SE population (Fig. 1 black lines), and thus allowing for evolutionary history effect, and without SE population (Fig. 1 red lines), thus excluding the said effect.

Regarding the dataset without SE population, the impact of evolutionary history in population divergence is also patent in the observed peak shift of both neutral (Fig. 1 red continuous line) and non-neutral (Fig. 1 red dashed line) curves towards lower levels of Fst. When excluding SE we are looking exclusively to populations with shared mitochondrial DNA phylogroups and close phylogeographic history, and thus the divergent effect of different evolutionary history is absent.

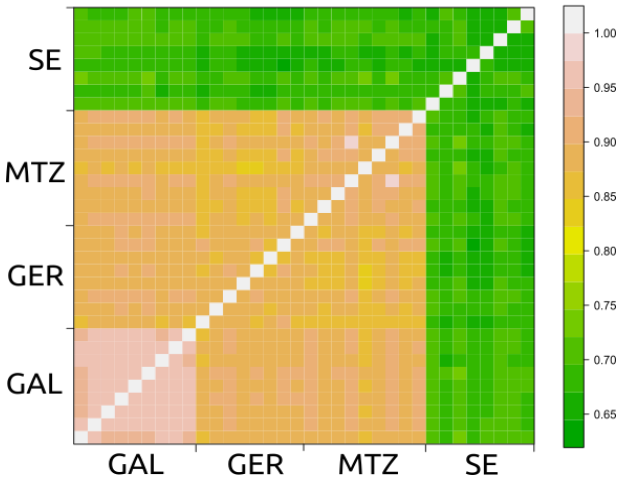

Figure 2. Identity by state analysis of individual relatedness. Each column in the heatmap represents an individual, and each square is a pairwise comparison of individuals. Populations are delimited by vertical dashes. Relatedness value increases up the colour scale so that 1.00 = totally related, as seen in the diagonal. Lighter shades of orange stand for more related individuals and darker shades of green stand for more distinct individuals.

3.2 Genomic patterns of divergence across all population pairs

Comparisons of the distribution of genomic differentiation across all molecular markers were performed pairwise, for all four populations (Fig. 3). Percentil 75 and 90 were marked on the histograms (dashed lines) in order to visualize the approximate proportion of SNPs putatively under positive selection (around 25%), and the 10% of SNPs showing elevated values of genomic divergence.

Values of missing data range from 5,2% (SE:GER comparison) to 57,9% (GAL:MTZ comparison) for different pairwise comparisons. Being so, the minimum number of

SNPs used for a pairwise analysis was 716, for GAL:MTZ, which is still a fair amount of data to assess overall genomic divergence pattern.

As expected, regardless of population pair, the majority of SNPs exhibited low values of Fst, reflecting the great number of SNPs which are evolving neutrally and/or being exchanged between populations.

However, in every pairwise comparison with SE, a shift of the peak towards higher values of Fst is clear, once again depicting the differentiation of this population, bearing different mitochondrial DNA lineage, in relation to the remaining populations. The most divergent pair of populations, with percentile 90 at 0.56 Fst and the highest number of SNPs fixed (values of Fst >0.9), was SE:GAL (Fig. 3 B). This result is in accordance with the herein described results regarding genomic diversity, posing these are the more differentiated pair.

Even between SE and MTZ (Fig. 3 C), which are assigned to the same subspecies (*T. l. lepidus*) the peak is shifted, with the majority of loci expressing Fst values of approximately 0.3, and 9.4% of the markers with Fst values superior to 0.5.

When comparing GER with GAL (Fig. 3 E), two populations assigned to *T.l.ibericus*, 52,5% of the SNPs express Fst values between 0 and 0.1, with 6.7% of loci expressing Fst values superior to 0.5, thus lower frequency for the same divergence values found within the *T.l.lepidus* subspecies.

Despite being assigned to different subspecies both GER and GAL (*T.l. ibericus*) comparisons with MTZ (*T. l. lepidus*) show high frequency of SNPs expressing really low Fst values (Fig. 3 D, F). GER:MTZ are the only population pair with 0% of loci presenting values of Fst > 0.9 (Fig. 3 D).

This 'L-shaped' frequency distribution of genetic differentiation across loci in the genome is typical of populations experiencing high levels of gene flow.

The bimodal profile of the curve correspondent to SE:GER (Fig. 3 A) and SE:MTZ (Fig. 3 C) comparisons is fairly uncommon to obtain, and it depicts an unexpected excess of SNPs expressing low levels of Fst, between these populations.

When focusing on a finer scope on these comparisons, and analyzing the subset of SNPs with values of Fst lower or equal to 0, which are binned together in the histogram, a pattern arises showing that the same SNPs express contrasting values of Fst depending on the populations being compared (Fig. 4 A, B).

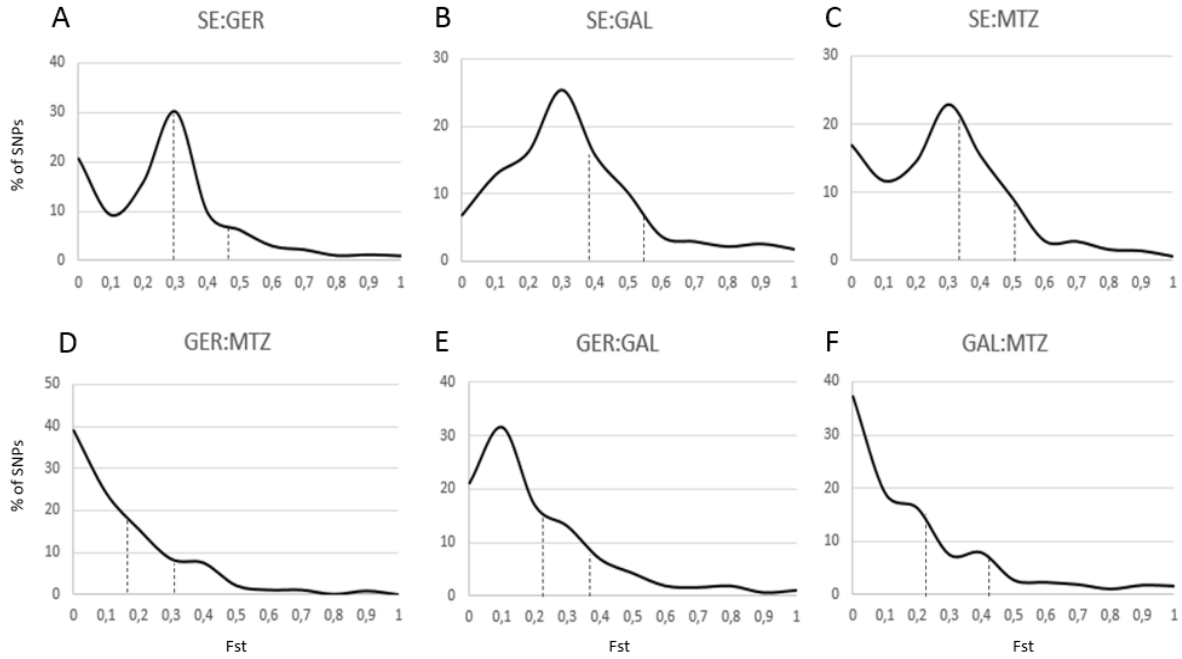

Figure 3. Genomic patterns of population divergence. Histograms depict population divergence measured by Fst values of pairwise comparisons. Dashed lines mark percentil 75 and percentil 90. SE and GAL populations belong to *T.l.ibericus* and MTZ and SE populations belong to *T.l.lepidus* subspecies.

A great number of SNPs expressing abnormal low values of Fst in the SE:MTZ (Fig. 3 A, green) and SE:GER (Fig. 3 B green) comparisons, show an increment in Fst values when comparing SE with GAL (Fig. 4 A, B in blue). The exact same pattern is obtained when analyzing SE:MTZ versus GAL:MTZ (Fig. S1 A, C), and SE:GER versus GAL:GER (Fig. S1 B, D). The average of Fst shifts from -0.06, when comparing SE with MTZ, to 0.10, when comparing SE with GAL instead (Fig. 4 A, E).

A portion of SNPs maintain low values of Fst in the alternate comparison (Fig. 4 A, B in blue) and these should be the ones normally expected to present low values.

However, this shift in pattern of Fst distribution values is not observed when considering all the remaining SNPs, for the same comparisons (Fig. 4 C, D). In this case, the pronounced contrast between the same pairwise comparisons is lost, and the average of Fst does not vary significantly: from 0.29, when comparing SE with MTZ, to 0.30 when comparing SE with GAL instead (Fig. 4 C, F).

This pattern may be suggestive of similar selection pressures being felt on SE, MTZ and GER populations that would modulate the allele frequencies in a convergent manner, thus giving rise to an excess of loci with low Fst values when comparing these populations pairwise. Taking into account the general habitat

characteristics SE, MTZ and GER would correspond mountain populations while GAL corresponds to a costal habitat.

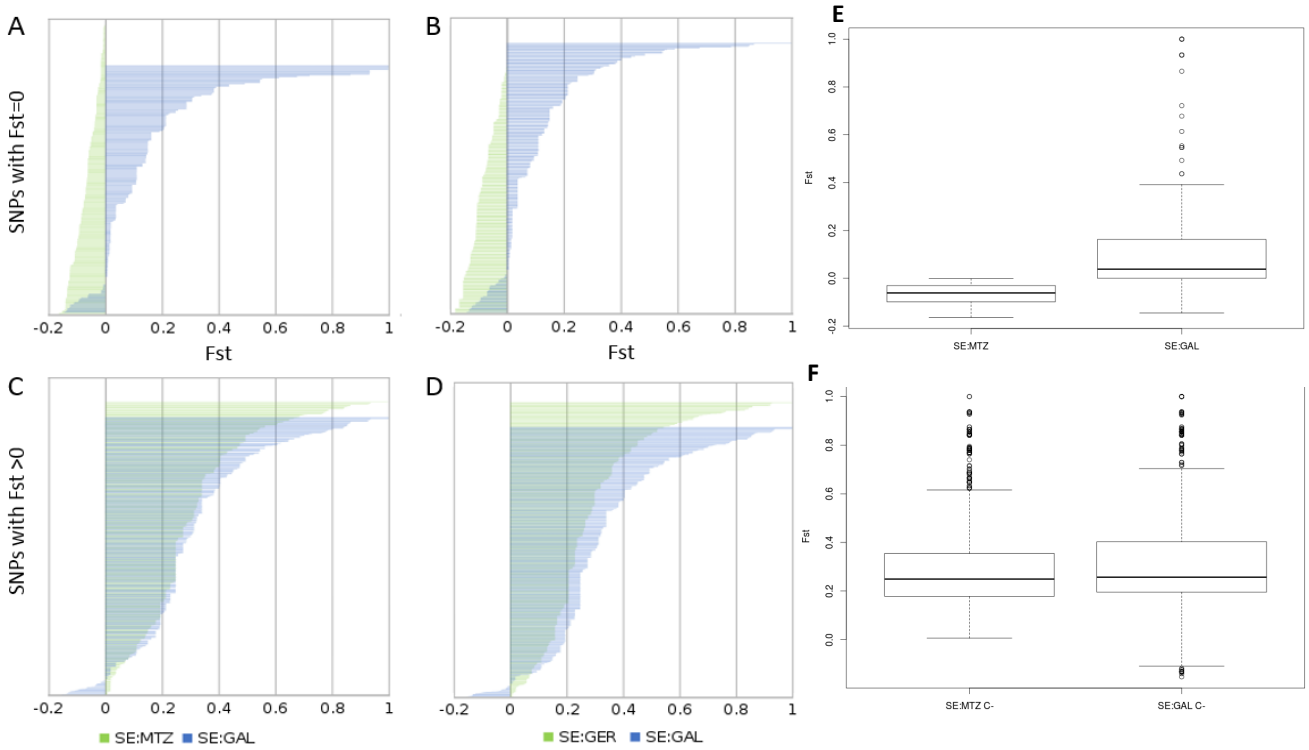

Figure 4. Fst values for individual SNPs presenting Fst lower or equal to zero (**A** and **B**) or Fst superior to zero (**C** and **D**) in population pairwise comparisons. To each SNP corresponds a green and a blue bar. Green bars depict mountain:mountain comparisons while blue bars represent mountain:coast comparisons for the same population pair. **A** and **C** correspond to SE:MTZ and SE:GAL and **B** and **D** to SE:GER and SE:GAL comparisons. Box-plots **E** and **F** depict changes in Fst values referring to the patterns observed in **A** and **C**, respectively.

3.3 Detection of selection signatures in the genome of *Timon lepidus*

Detection of selection was performed for two datasets: one including all the study populations, comprising a total of 1704 SNPs, and a second in which SE population is excluded from the analysis, comprising a total of 3908 SNPs. In both datasets, the majority of loci express neutral evolutionary pattern (67.19% for all populations and 70.95% without SE; Fig. 5).

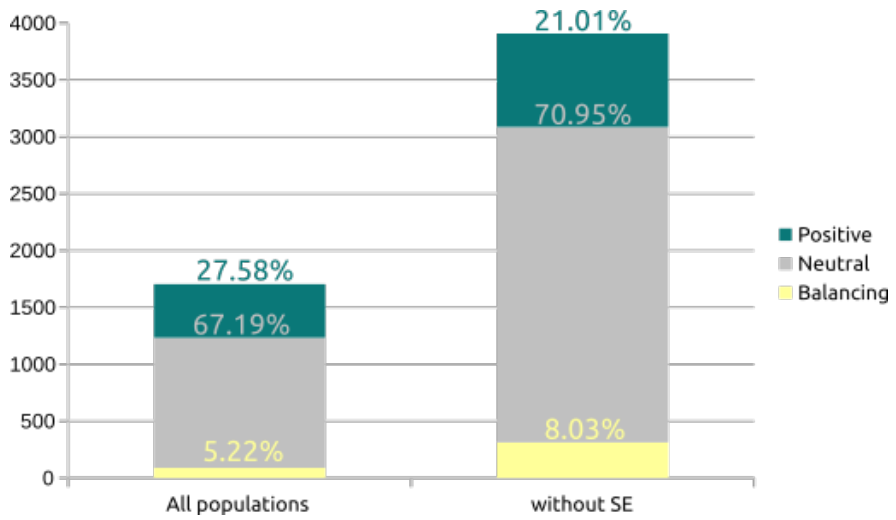

Figure 5. Detection of Selection signatures in the genome of *Timon Lepidus*. On the left data regarding a dataset including all populations, **on the right** data excluding SE population. Percentage of loci evolving under selection or neutrality is indicated with respective colours.

The relative proportion of SNPs under putative positive selection is lower for the dataset without SE (21.02%), comparing with the one with all populations (27.58%).

These results enable us to produce sub-datasets of SNPs exclusively under positive selection (470 for all populations, and 821 without SE), exclusively under balancing selection (89 for all populations, and 314 without SE) and SNPs evolving under neutrality (1145 for all populations, and 2773 without SE). This allows a partitioned and more precise analysis of the genomic data, in which patterns are not confused by the different evolutionary forces acting on individual molecular markers.

3.4 Genomic structure considering the different acting evolutionary forces on all studied populations

The Principal components analysis (PCA), for all four populations, regardless of being performed with a dataset including all loci (1704 SNPs; Fig. 6 A), exclusively neutral (1145 SNPs; Fig. 6 B) or exclusively positively selected loci (470 SNPs; Fig. 6 C) shows a well-defined separation of SE individuals in relation to the remaining three populations, which form a single cluster. The exception arises when performing the PC analysis exclusively with loci under balancing selection (89 SNPs; Fig. 6 D). In this case, all the populations mingle together, with clustering or divergence pattern completely absent. This is expected since balancing selection would promote the existence of a loci in heterozygosity, thus maintaining the polymorphism and hindering the divergence between populations.

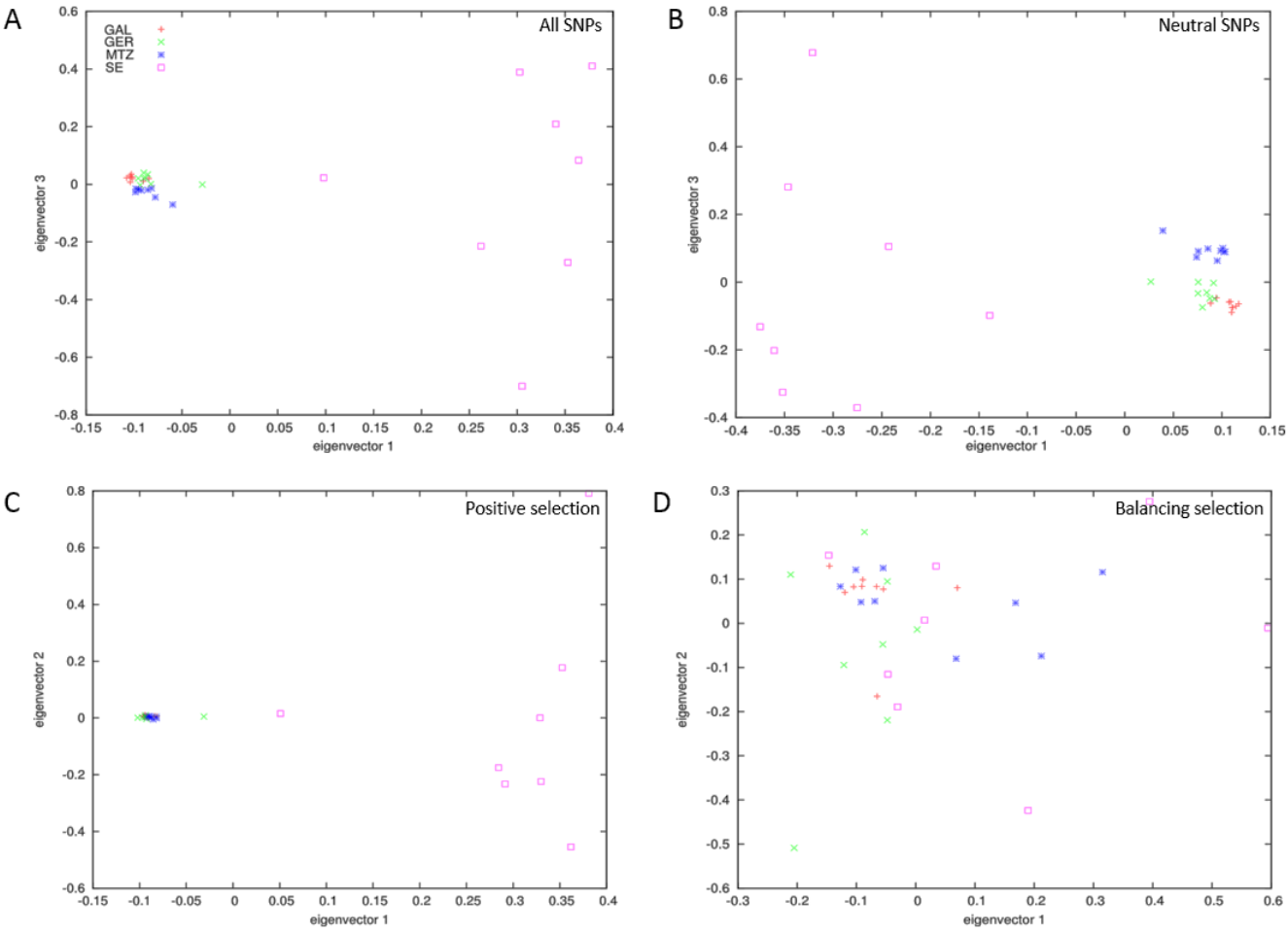

Figure 6. Genomic PC analysis for all populations comprising subsets of molecular markers experiencing different evolutionary forces. **A** All SNPs independent of evolutionary force **B** SNPs evolving under neutrality **C** SNPs under positive selection **D** SNPs under balancing selection.

Structure analysis for all four populations, comprising all 1704 loci, shows a pattern in two clusters (K=2; Fig. 7 up) as the most probable, according with the method described by Evanno et al. (2005). Once again, SE population forms an individual cluster with the remaining three populations being assigned to a second cluster, reflecting the effects of the differential evolutionary history patent in the mitochondrial lineages of these populations.

When observing K=3, a second cluster emerges within SE, reflecting the genetic variability within that population, with the other three populations remaining assigned to the same cluster. However, when we analyze the pattern for K=4 (Fig. 7 down), despite not being the one statistically selected by Evanno's method, relevant biological information arises. When four clusters are considered, a new cluster appears associated with GAL, showing introgression with both GER and MTZ, although with different intensity. GAL, now in a uniform cluster, seems to admixture with GER more intensely than with MTZ. Although the pattern is not clearly reflecting the taxonomic division of the populations, other factors should be taken into account such as the demographic history of GAL population and the occurrence of gene flow between GER and MTZ.

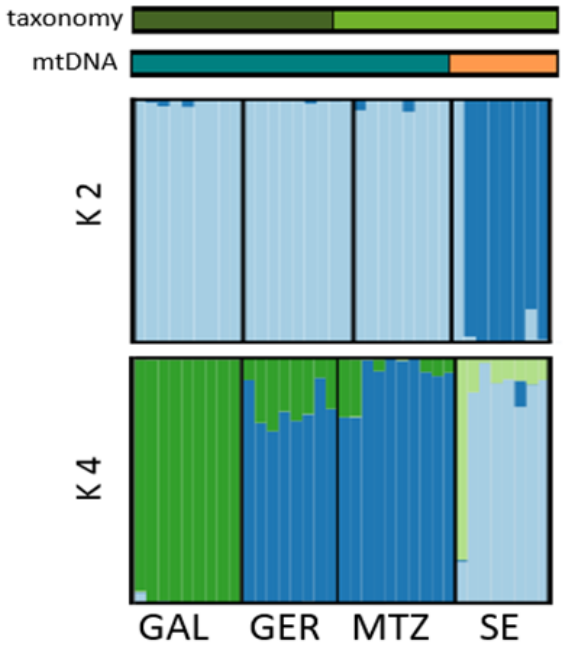

Figure 7. STRUCTURE analysis of all SNPs available for the four populations. Galiza (GAL), Gerês (GER), Montezinho (MTZ) and Serra da Estrela (SE). To each individual corresponds a vertical bar representing the probability of assignment to each cluster (indicated by different colors). Two-cluster pattern was selected by Evanno method as being the more accurate. Current taxonomy (Light green represents *T.l.lepidus* and dark green *T.l.ibericus*) and mtDNA clade (Clade L3 in green, clade L5 in orange) are shown on top.

Population structure is assessed based solely on neutral evolving markers (Fig. 8). The structure pattern is similar to the clustering pattern when analyzing all markers simultaneously (Fig. 7). Population structure in two clusters (K=2) is the most probable, once more with SE assigned to a single cluster and the remaining populations to a second (Fig. 8 left). However, when analysing K=3 (Fig. 8 right), we again see that a new cluster appears associated with GAL, showing higher levels of introgression with GER and lower with MTZ.

The fact that the neutral pattern resembles the pattern shown when analyzing all markers, independently of the evolutionary forces acting on those, is not surprising, since as we have seen (Fig. 5) the majority (1145) of SNPs is evolving neutrally, thus impacting the final pattern.

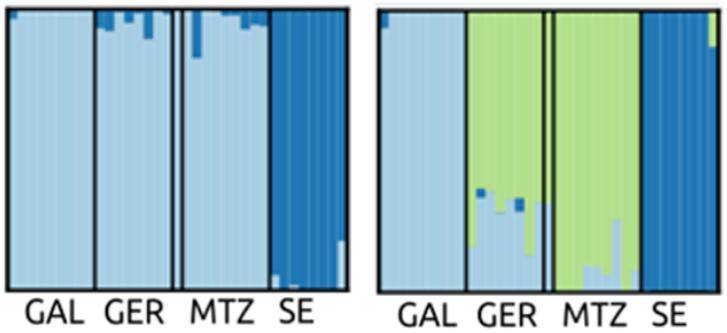

Figure 8. STRUCTURE analysis of neutral SNPs for all populations. Galiza (GAL), Gerês (GER), Montezinho (MTZ) and Serra da Estrela (SE). To each individual corresponds a vertical bar representing the probability of assignment to each cluster (indicated by different colors). **left** Two-cluster pattern (K=2) was selected by Evanno method as being the more accurate **right** K=3. Bar between GER and MTZ corresponds to one individual sampled in a location between these two populations (BOT, see methods).

If we assess population clustering pattern, in a finer genomic scope: analyzing only SNPs putatively under positive selection (Fig. 9), the most probable number of clusters is again two, with one containing exclusively SE, and the other englobing the remaining three populations (Fig. 9 A), as seen in the PC analysis (Fig. 6 D). Results for higher number of clusters still retain this same pattern (Fig. 9 B, C). This result, once again reflects the impact of evolutionary history on population genomics pattern, which may be influenced by the SNPs fixed due to drift, falsely assigned to positive selection by Lositan software.

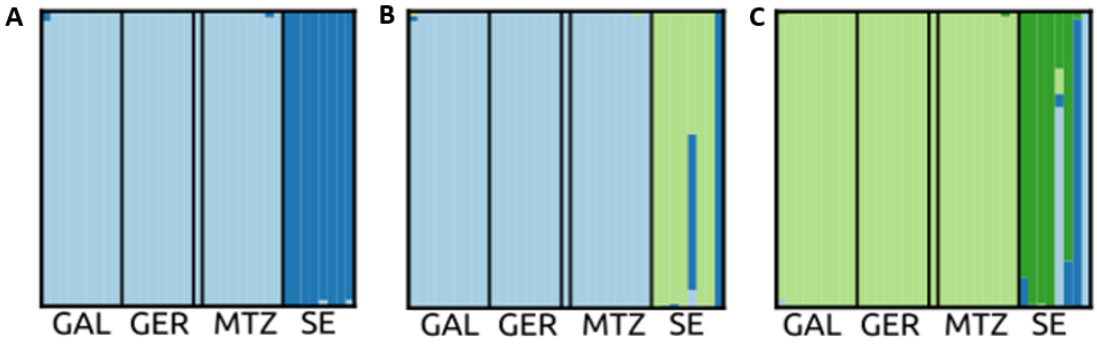

Figure 9. STRUCTURE analysis of SNPs under positive selection for all populations. All patterns show constantly SE population differentiated from the remaining populations, independently of clustering by **A** K=2, **B** K=3 or **C** K=4. Bar between GER and MTZ corresponds to one individual sampled in a location between these two populations (BOT, see methods).

As expected, when regarding SNPs under putative Balancing selection (Fig. 10), the population clustering pattern is lost, as seen in the PC analysis (Fig. 6 D).

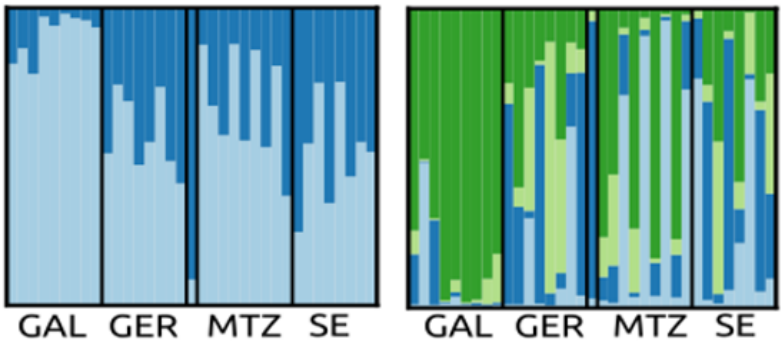

Figure 10. STRUCTURE analysis of SNPs under blancing selection, for all populations. When analyzing these markers population structure is lost both for **left** K=2 and **right** K=4 Bar between GER and MTZ corresponds to one individual sampled in a location between these two populations (BOT, see methods).

3.5 Adaptation and Selection within L3 populations: when evolutionary history is shared

PC analysis of the populations sharing mitochondrial lineage (Fig. 11) revealed three separated clusters, except when analyzing exclusively SNPs under balancing selection (Fig. 11 D). In this analysis, clustering is lost and all populations appear mingled as expected since balancing selection maintains polymorphism across populations, inhibiting divergence.

Clustering patterns shown by PCA are similar to those found with STRUCTURE analysis.

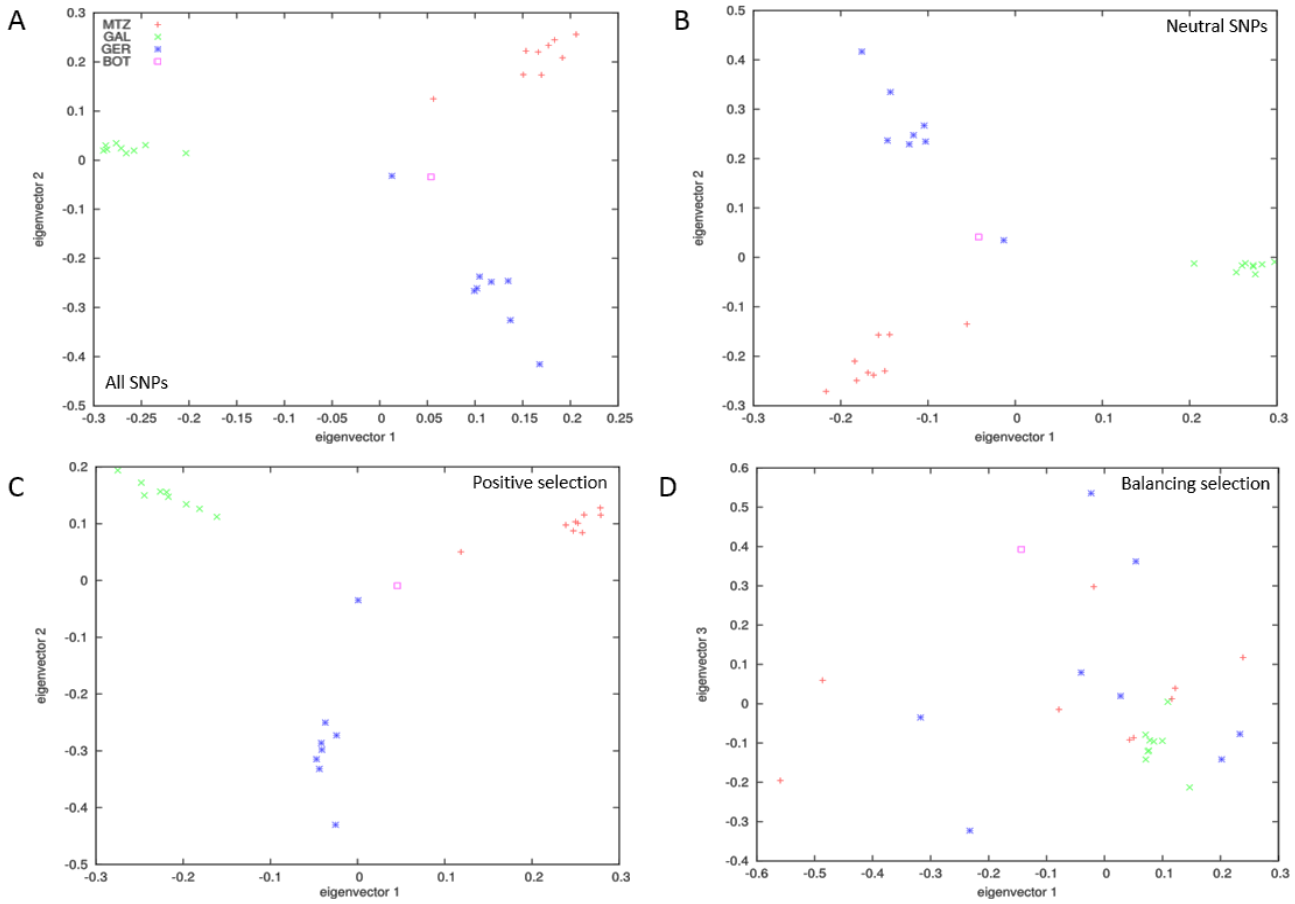

Figure 11. Genomic PC analysis for L3 populations comprising molecular markers experiencing different evolutionary forces. A All SNPs independent of evolutionary force, **B** SNPs evolving under neutrality, **C** SNPs under positive selection, **D** SNPs under balancing selection.

According with the method described by Evanno et al. (2005), population structure when analyzing exclusively SNPs evolving neutrally (Fig. 12 left) mirrors the clustering pattern of SNPs under putative positive selection (Fig. 12 right), with three clusters one assigned to each population.

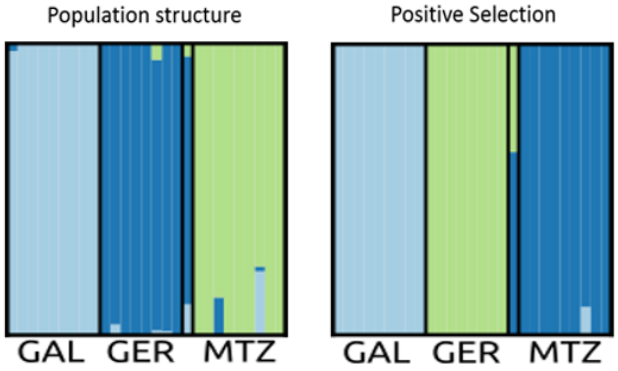

Figure 12. Population structure and clustering pattern when analyzing SNPs exclusively under positive selection. On the left population structure depicted by neutrally evolving loci. **On the right** clustering pattern depicted by loci under putative positive selection. Three clusters arise in both analysis suggesting local adaptation of populations.

If we take into account the taxonomic division of these populations, it would be expected to find a structure of K=2 where GAL and GER would be grouped in one cluster, since the same subspecies (*T.l.ibericus*) is attributed to these two populations, and MTZ (*T.l.lepidus*) comprising a distinct cluster. However, even with K=2 the pattern observed is GAL isolated from GER and MTZ, for both positively selected (Fig. 13 A) and neutral SNPs (Fig. 13 B), thus defying the expected taxonomic association.

This pattern may be reflection of both demographic history and strong local adaptation of these populations.

Regarding the SNPs under putative balancing selection the clustering corresponds to K=2 (Fig. 13 C), with total loss of the clear separation pattern between populations, as expected for markers under this evolutionary pressure.

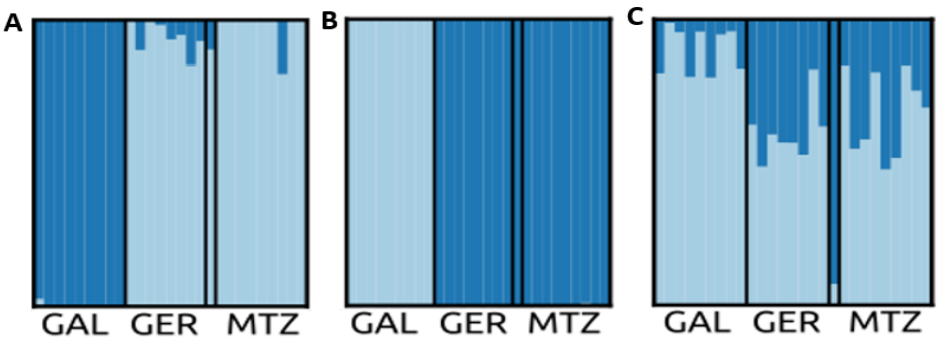

Figure 13. Clustering pattern of L3 populations when analyzing loci under different evolutionary forces. **A** SNPs under putative positive selection **B** SNPs under neutral evolution **C** SNPs under balancing selection, for populations sharing evolutionary history.

3.6 Evironmental variables

PC analysis comprising bioclimatic and geospatial variables, for all populations, shows four clusters, with SE and GER being the pair of populations more closely aggregated (Fig. 14 left). PC1 explains 93.7% of the variance, and Temperature seasonality, Altitude and mean annual sun duration together explain 85% of the variance in PC1.

When excluding SE from the analysis, thus focusing exclusively on the environment of populations from the L3 mitochondrial lineage (Fig. 14 right), there is a clear clustering pattern differentiating all populations. PC1 explains 94.8% of the variance, with Temperature seasonality, Altitude and mean annual sun duration together explaining 85.7% of the variance in PC1.

PC2 explains around 5% of the variance in both cases, and Precipitation of Wettest Quarter, Precipitation of Coldest Quarter and Annual Precipitation together explain 67.9% and 60.3% of the PC2 for the analysis excluding SE and the one comprising all populations, respectively.

This pattern is similar to the one found with genomic data, where each population is assigned to a cluster and thus, once again, suggestive of local adaptation.

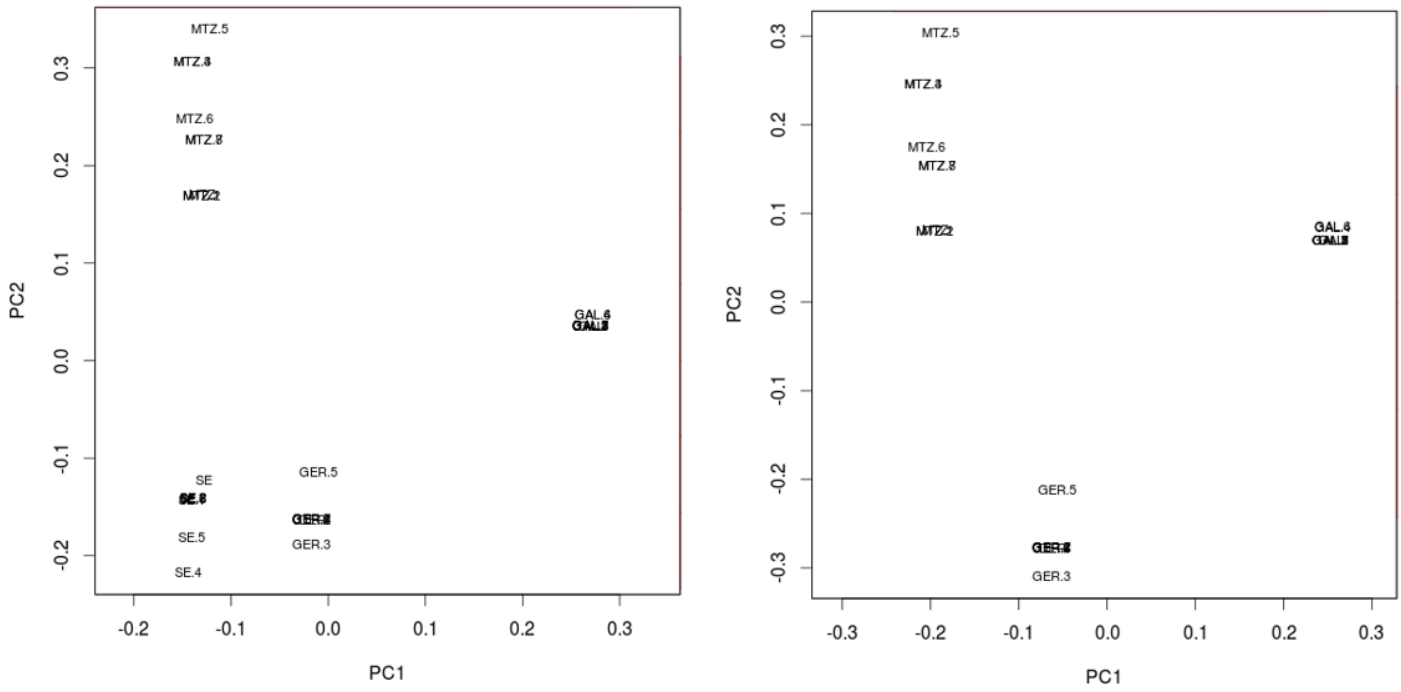

Figure 14. PC analysis of bioclimatic and geospatial variables for all populations (**left**) and of population exclusively belonging to L3 mtDNA clade, thus excluding SE (**right**).

3.7 GEA: GENOTYPE-ENVIRONMENT ASSOCIATIONS

Genotype-Environment associations were assessed for the populations sharing evolutionary background (L3 populations), thus excluding possible confounding effects of differential evolutionary history from SE. Allele frequency variation along different population environments was tested for associations with 22 bioclimatic and geospatial variables.

From the 3908 analyzed SNPs, a total of 123 SNPs (3.15%) showed significant associations with environmental variables. Various SNPs show association with multiple variables resulting in a total of 295 genotype-environment associations (Table 1), being the majority (175) associations with precipitation related variables

(Fig. 15), specifically Precipitation of Warmest Quarter, with 99 associated SNPs (Table 1).

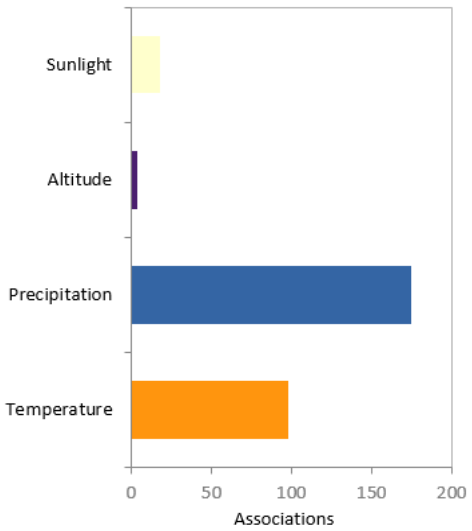

Figure 15. Number of associations per principal category of bioclimatic variables. Majority of associations were found for Precipitation related variables (blue) followed by Temperature related variables (orange).

From the 22 bioclimatic and geospatial variables analyzed, associations were found with 14. It was common for a SNP to have association with more than one of the 14 variables, and this is not unexpected since some variables show statistically significant correlation.

Table 1. Genotype-Environment associations found for 3908 SNPs of GAL, GER and MTZ populations. A total of 295 associations were found. Further description of variables in appendix.

Variable		Description	# Assoc SNPs
Temperature	YmeanT	Annual Mean Temperature	20
	IsoT	Isothermality (BIO2/BIO7) (* 100)	17
	Tseason	Temperature Seasonality (standard deviation *100)	20
	mTcm	Min Temperature of Coldest Month	3
	Tyrange	Temperature Annual Range (BIO5-BIO6)	1
	meanTWetQ	Mean Temperature of Wettest Quarter	4
	meanTDryQ	Mean Temperature of Driest Quarter	12
	meanTwQ	Mean Temperature of Warmest Quarter	1
	meanTcQ	Mean Temperature of Coldest Quarter	20
Precipitation	precDrym	Precipitation of Driest Month	46
	precDryQ	Precipitation of Driest Quarter	30
	precwQ	Precipitation of Warmest Quarter	99
Altitude	Altitude	Altitude in m	4
Sunlight	SunDu	Mean annual Sunlight duration (hours)	18

3.8 NON-NEUTRAL SNPS

A dataset comprising 944 non-neutral SNPs, i.e. SNPs under putative positive selection, SNPs showing environmental association, or both simultaneously, was created prior to selection detection and GEA analysis.

Both STRUCTURE (Fig. 16 B) and PC analysis (Fig. 16 A) on this dataset reflect the same clear three-cluster pattern found when considering exclusively neutral SNPs (Fig. 13 B), as well as those exclusively under positive selection (Fig. 13 A).

The fact that neutral and non-neutral genomic information show consistent patterns is reinforcing the idea of local adaptation, independently of taxonomic attribution of these populations.

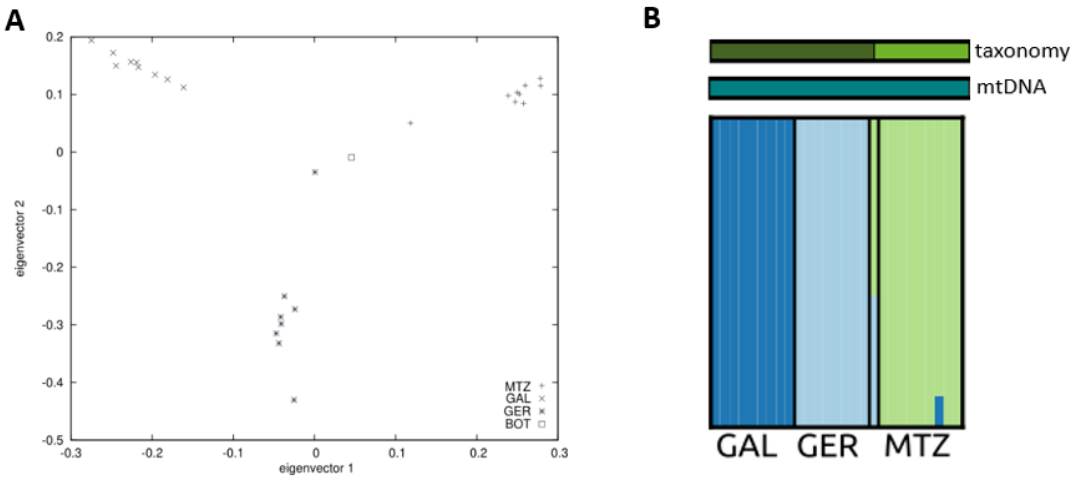

Figure 16. Clustering pattern of a subdataset of 944 non-neutral SNPs, thus under putative positive selection, showing environment associations, or both. **A** PC analysis and **B** STRUCTURE analysis of the genomic subset. Current taxonomy and mtDNA clade are shown over STRUCTURE plot in **B**. Both analysis show a pattern in three clusters, suggestive of local adaptation.

3.9 ENVIRONMENTAL ASSOCIATIONS UNDER POSITIVE SELECTION

From the 123 SNPs that showed significant association with environmental variables, 37 were also shown to be under putative positive selection (Fig. 17). These SNPs are truly interesting when considering the evolutionary scenery of ecological speciation.

The majority of these SNPs show association with more than one variable simultaneously, and SNPs from the same RAD-tag, or loci, show equal association profiles to the same variables (Fig. 17).

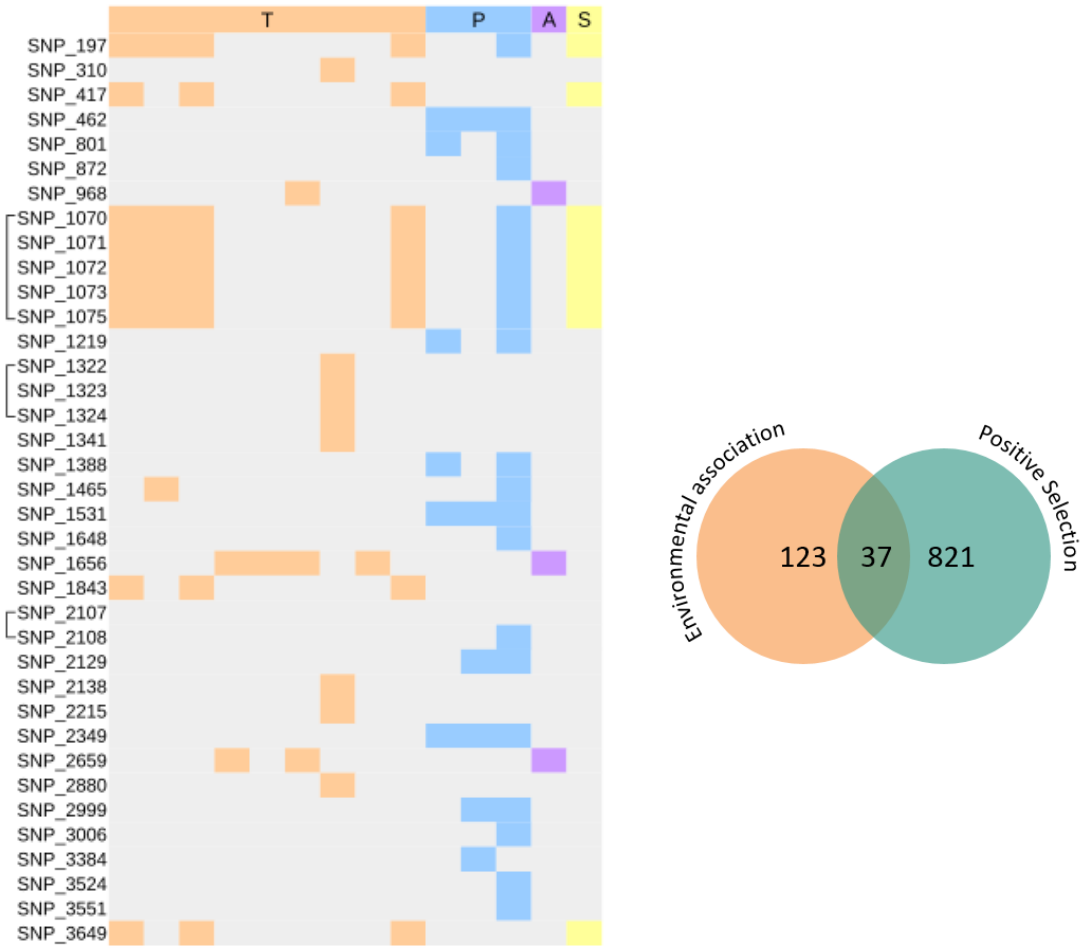

Figure 17. Genotype-Environment association. Association map **on the left** presents the 37 SNPs under putative positive selection on rows and columns correspond to environmental variables grouped by principal category of Temperature (**T**; orange), Precipitation (**P**; blue) Altitude (**A**; purple) and Sunlight (**S**; yellow). Colored squares mark association and grey means no association. SNPs on same loci are grouped by square brackets and show similar profiles of associations. Venn diagram **on the right** shows total numbers of SNPs under putative positive selection, showing environmental association and both simultaneously.

Regarding the population clustering when analyzing the subset of SNPs simultaneously associated with environmental variables and under putative positive selection, both PC (Fig. 18 A) and STRUCTURE (Fig. 18 B) analysis show a clustering pattern that mirrors that of the previous analysis of both neutral and non-neutral marker, with each population assigned to an individual cluster. Once again in a pattern suggestive of local adaptation.

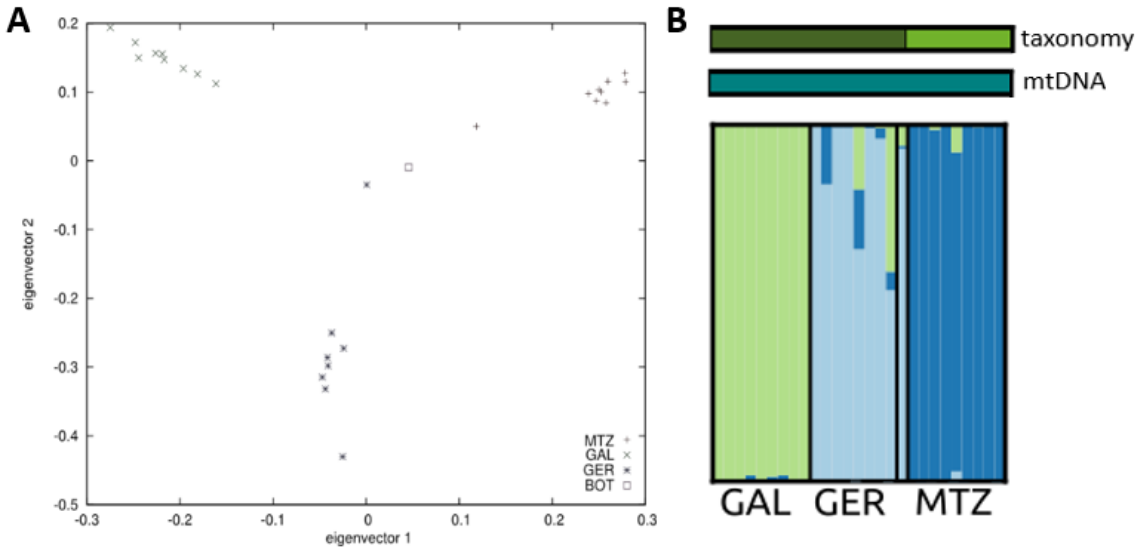

Figure 18. Clustering pattern of SNPs simultaneously associated with environmental variables and under putative positive selection. PC analysis (A) and Structure analysis (B) both depict a pattern in three clusters. Current taxonomy and mtDNA clade are shown over STRUCTURE plot in B.

The genotypic frequencies for these 37 SNPs vary differentially.

Seven out of 37 (18.91%) showed a pattern concordant with the taxonomic distinction of the populations, thus showing clear genotypic frequency differences between MTZ (*T.l. lepidus*) and GER and GAL (*T.l.ibericus*; case examples in Fig. 19 A, B)

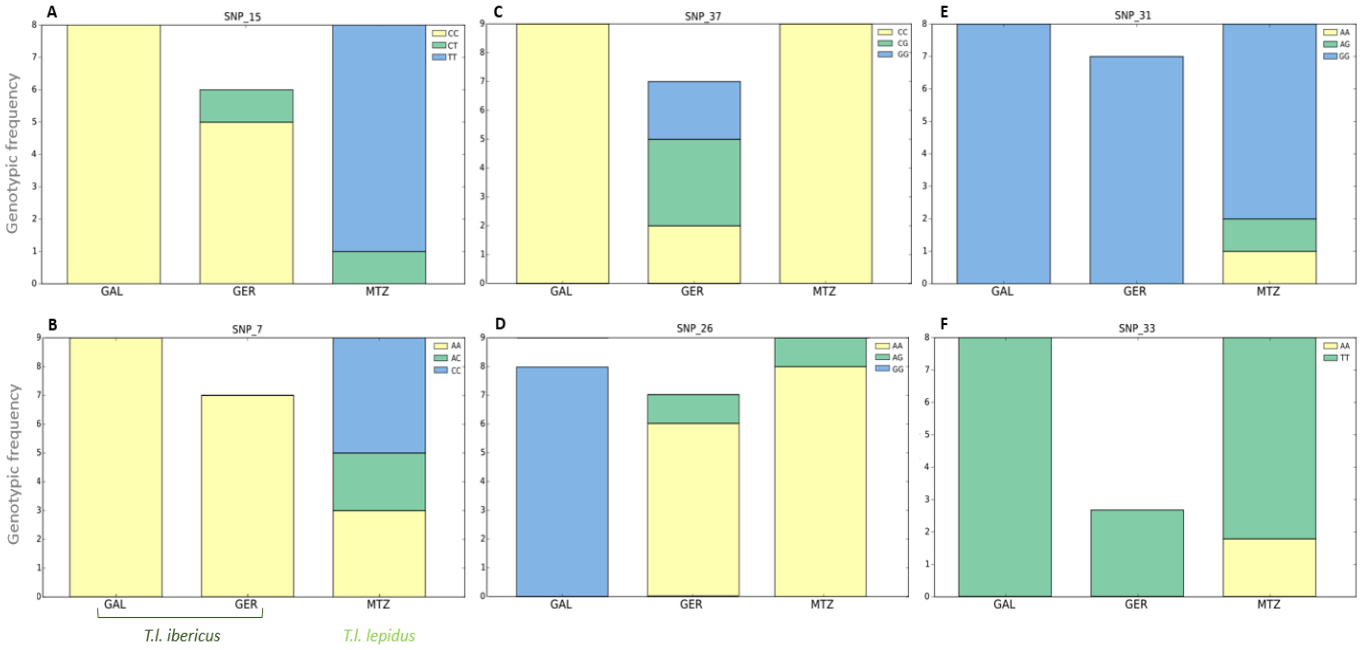

Figure 19. Differential genotypic frequencies on L3 populations. Examples of differential allele frequency variation between *T.l.lepidus* and *T.l.ibericus* (A and B), allele frequencies differential in GER population (C) allele frequencies differential in GAL population (D) and allele frequencies non-differential between all three populations. Homozygotes depicted in yellow and blue, Heterozygotes depicted in green.

Six and Five SNPs showed differential genotypic differences for GAL (case example in Fig. 19 D) and GER (case example in Fig. 19 C) populations, respectively. However, the majority of SNPS (51, 35%) showed patterns of genotypic frequencies that were not significantly different between populations (case example in Fig. 19 E, F), with the majority of the individuals across populations expressing the same allele in homozigoty.

3.10 DOMINANT PATTERN OF ALLELE FREQUENCY VARIATION

In order to get the number of SNPs which are related with the current taxonomic distinction between GER and GAL (*T.l.ibericus)*, and MTZ (*T.l.lepidus)*, the number of SNPs with allele frequencies equal or superior to 80%, 85% and 90% within each subspecies, compared with other groups of populations, was assessed.

Regarding the comparison based upon the taxonomic arrangement of populations: MTZ (*T.l.lepidus*) versus GALGER (*T.l.ibericus*), 25 SNPs were found to have allelic frequencies equal or superior to 80% in *T.l.lepidus* and 32 in *T.l. ibericus* (Fig. 20, in grey).

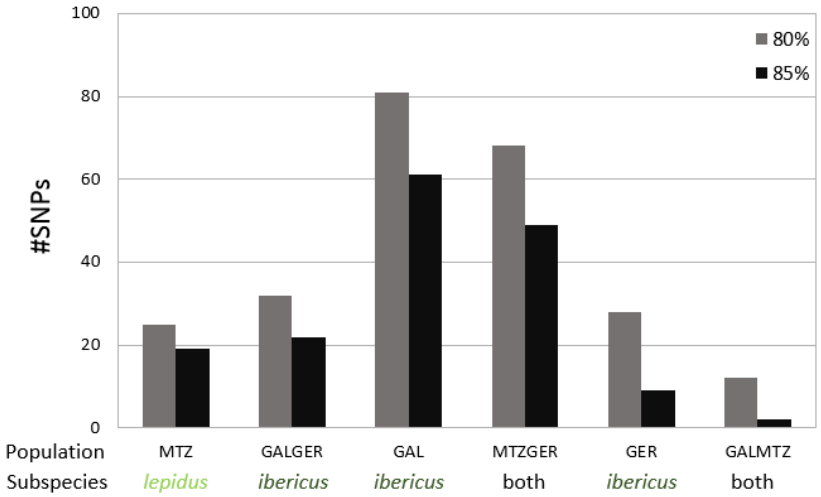

Figure 20. Number of SNPs with allele frequencies equal or superior to 80% and 85% in different populations and population groups. Subspecies according to current taxonomy is indicated under each population, or population group, designation.

When rising the allelic frequency to 85% (Fig. 20, in black), a subset of the previously discovered SNPs is uncovered, with 19 SNPs expressing allelic frequencies equal or superior to 85% for *T.l.lepidus* and 22 for *T.l.ibericus*.

The majority of SNPs with high allele frequencies is obtained when comparing GAL, which belongs to *T.l. ibericus*, with MTZGER, which groups populations from both subspecies. A total of 81 SNPs are found to have allelic frequencies equal or superior to 80% in GAL, and 68 in the MTZGER grouping. When raising the allelic frequency threshold to 85%, 61 SNPs are found matching that condition in GAL and 49 in MTZGER.

No SNPs were found with allelic frequencies equal or superior to 90% in any of the populations, or groups of populations, and so unique alleles for the subspecies are absent.

When applying PC analysis on the subset of SNPs showing differential allele frequencies between *T.l.lepidus* and *T.l.ibericus* the obtained pattern is, as expected, two clusters corresponding to the subspecies (Fig. 21 A). However, when applying the same analysis to the subset of all SNPs resulting from the differential allele frequency analysis, the dominant pattern is once again three distinct clusters (Fig. 21 B) instead of the two clusters representing GAL and MTZGER, as could be expected from the higher number of SNPs found to reflect this distinction.

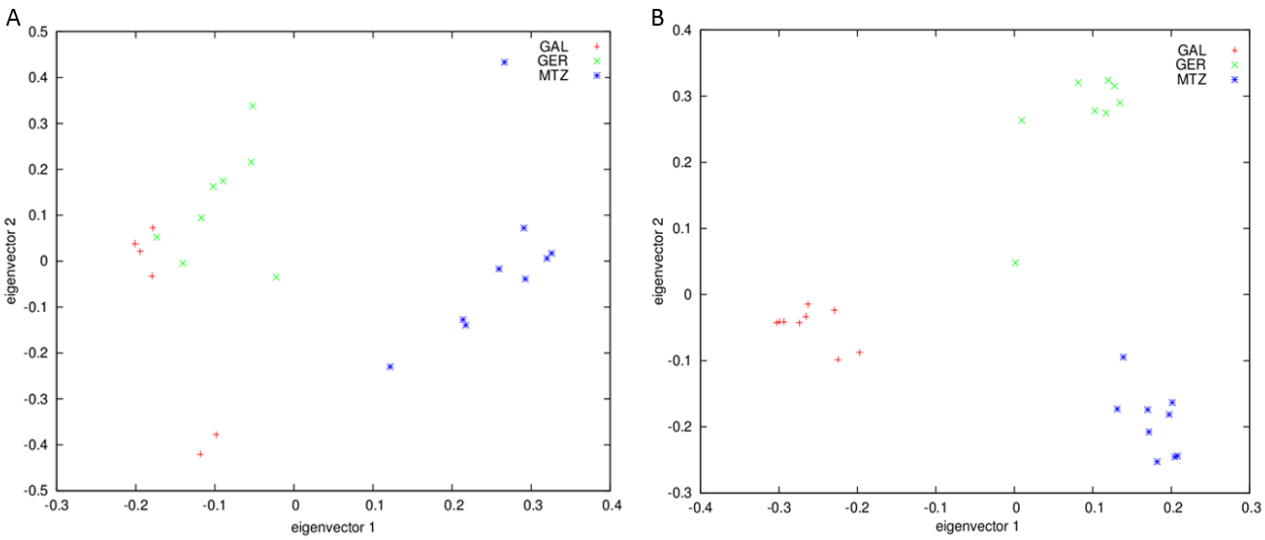

Figure 21. PC analysis of SNPs allelic frequencies equal or superior to 85% within different population groups. **A** SNPs with allelic frequencies varying differently between subspecies, according to current taxonomy. **B** All SNPs expressing differential allelic frequencies on any of the designated groups, depicting dominant pattern in three clusters.

AMOVAs were performed for grouping of populations following the actual taxonomy in order to uncover the percentage of variation explained by that criteria (Table 2). All the analysis showed that majority of variation is contained within populations, thus among individuals. Percentage of variation among subspecies always presented negative values, independently of considering SE population in the *T.l.lepidus* group. Once again, taxonomic grouping of populations proved to be insignificant regarding genomic differentiation.

Table 2. Percentage of variation attributed to differences between subspecies, among populations and among individuals. All tests were significant (p<0.05)

	% variation
Within L3: GAL GER versus MTZ	
Between subspecies	-12.56
Among populations	34.37
Among individuals	78.19
All populations: GAL GER versus MTZ SE	
Between subspecies	-5.51
Among populations	29.08
Among individuals	76.43

3.11 PHYLOGENOMIC ANALYSIS

The tree calculated based on the dataset comprising all studied populations (Fig. 22 A), segregates four clades. SE is the population expressing higher genetic variability, shown by longer branch lengths. Contrastingly, GAL is the population presenting lower levels of genetic variability, in accordance with previous results of population genomics and identity by descent analysis (Fig. 2). All four clades are supported by high bootstrap values ranging from 86 for GER to 100 for GAL and SE. However, the relationship between the mentioned clades is not well defined, as depicted by relatively low bootstrap values in the center of the tree. These results do not support the current subspecies taxonomy, according to which lineage sorting would be expected between GAL GER (*T.l.ibericus*) and SE MTZ (*T.l.lepidus*).

When constructing a tree based on the dataset comprising populations sharing evolutionary history (Fig. 22 B), three clades segregate, supported by a bootstrap value of 94. Clade support values range from 72 for MTZ to 100 for GAL. Contrarily to the tree comprising all populations, topological relationship between the mentioned clades is well supported, thus implying that these populations are

separated, also not showing signs of lineage sorting accordant with current taxonomy.

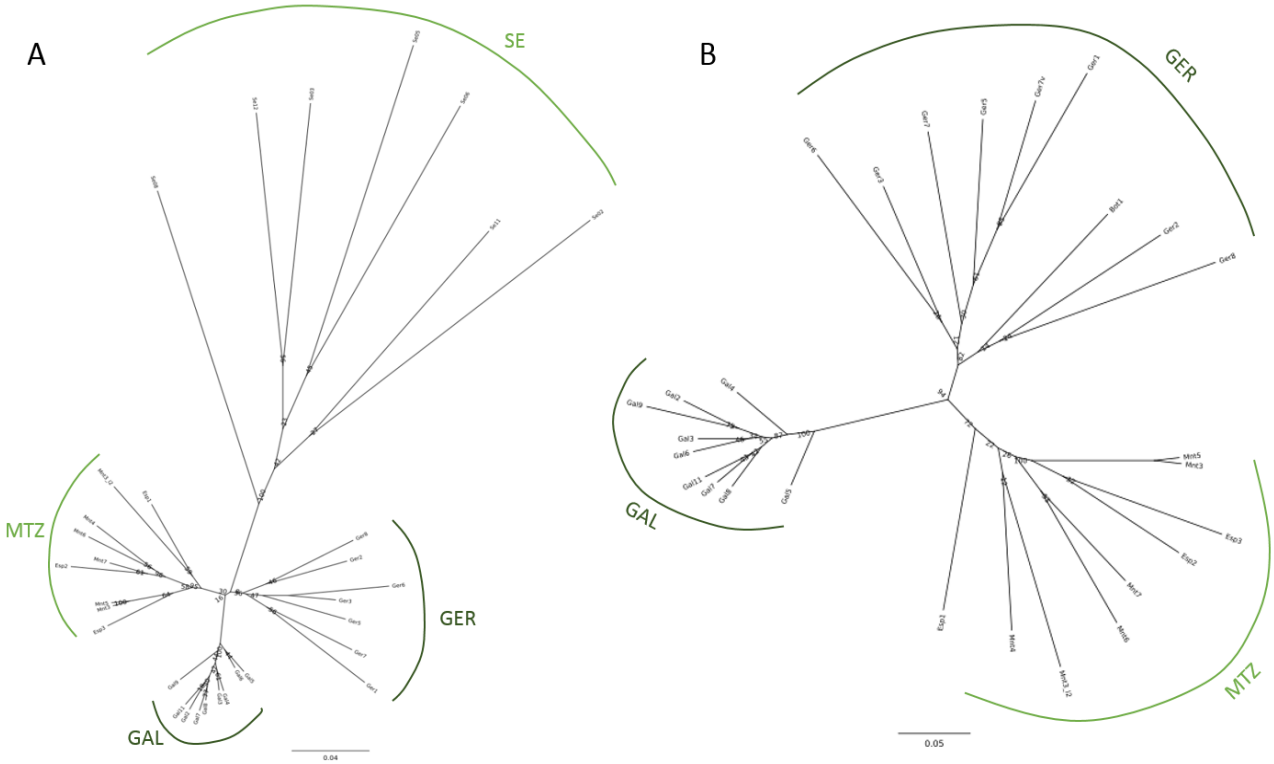

Figure 22. Phylogenomic trees of all studied populations (**A**) and populations sharing evolutionary history (**B**). Galiza (GAL) and Gerês (GER) are *T.l.ibericus* populations, and Montezinho (MTZ) and Serra da Estrela (SE) are *T.l.lepidus* populations. For enlarged version with visible bootstrap values see supplementary material (Fig. S2 and S3).

3.12 Annotation of RAD-seq loci

Functional annotation of the all SNP-bearing sequences that passed quality control resulted in a total of 363 hits, with the great majority associated with reptile genetic information.

Kegg annotation identified five enzymes from the fatty acid biosynthesis pathway, and three from the biotione metabolism pathway. These pathways can be involved in thermoregulation-related processes and are thus interesting as putative selective targets. However, the annotation of sequences possessing SNPs under positive selection or expressing environment association did not match any Kegg annotations, with no known enzymes matching the sequences containing this subset of SNPs. Likewise, no specific hits were found for any of the main GO terms categories of biological process, cellular component and molecular function associated GO terms.

3.12 SKIN REFLECTANCE OF LIZARDS FROM ALL STUDIED POPULATIONS

Reflectance spectra for the four populations show emission peaks corresponding to wavelengths of green coloration for both dorso (Fig. 23 A), and throat (Fig. 23 B). Despite the slightly different curve profiles of the spectra, no statistic significant differences were found between populations for both dorsal and throat spectra.

Both spectrum profiles and segment analysis for males and females separately, per population, did not show significant differences neither between sexes nor populations (Fig. S4).

Segment analysis of dorsal (Fig. 23 C) and throat (Fig. 23 D) population spectra, which translates into values of hue, chroma and brightness, also failed to show significant differences between populations. Consequently, no significant differences were found between reflectance phenotype of populations of the described subspecies.

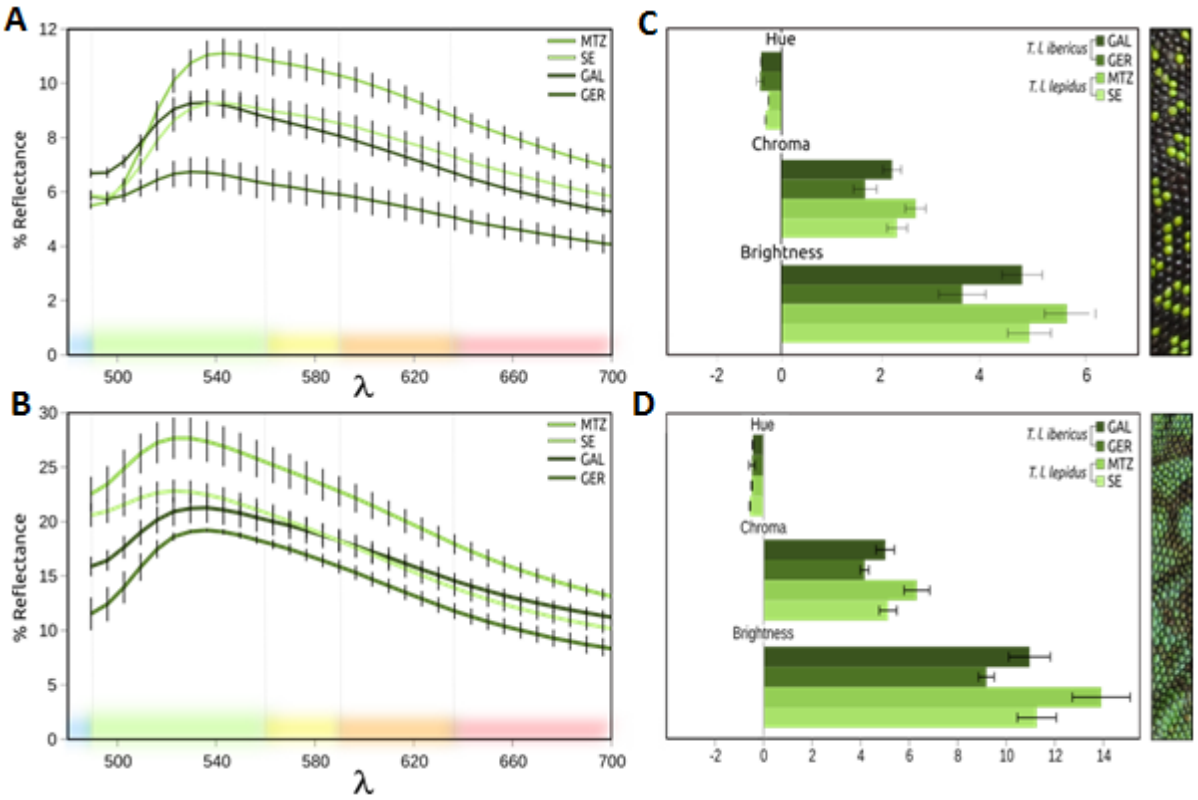

Figure 23. Dorso and throat reflectance analysis of the four populations of *Timon lepidus*. A Dorsal reflectance spectra for all populations **B** Throat reflectance spectra for all populations. Vertical lines indicate error bars. Population corresponding line colour is shown on graphs: *T.l.lepidus* populations are represented by lighter shades of green, *T.l.ibericus* by darker shades of green. Colors on xx axis correspond to reflected wavelength **C** Dorsal spectrum segment analysis for all populations **D** Throath spectrum segment analysis. Hue is commonly called color, chroma corresponds to color

saturation and brightness to the amount of white or black mixed in the colour (hue). Photography on the right correspond to dorsal (up) and throat skin (down).

3.13 BACKGROUND MATCHING HYPOTHESIS

Values of Hue, Chroma and Brightness from dorsal skin of animals and substrate were compared to assess background matching. Euclidean distances were calculated as a proxy for conspicuity between lizard and background where they were observed to exhibit basking behavior, thus being exposed to visual predators (Table 3).

Table 3. Background matching analysis for all populations. Numbers represent Euclidean distances calculated for brightness, chroma and hue of lizards and basking substrates. The higher the number the most conspicuous the animal relative to substrate.

	SE granite	SE shale	GAL wood-walk	GER granite	MTZ granite	MTZ shale
GAL	26,59	193,33	5,75	24,49	7,30	5,62
GER	26,67	193,37	5,82	25,82	8,62	6,94
MTZ	24,32	192,34	3,47	23,47	6,27	4,59
SE	25,17	192,73	4,32	24,32	7,12	5,44

Regarding exclusively natural backgrounds, individuals from all populations exhibit higher distance to the Serra da Estrela (SE) shale substrate, and lower values for Montezinho (MTZ) substrates, either granite of shale (Table 3). This predicts that, independently of native population, all individuals would present higher levels of background matching in Montezinho, thus being less conspicuous to visual predators.

If we take into account the anthropogenic background were individuals were observed to bask, values of background matching were also very low for Galiza's (GAL) foot-walk wood, for individuals from all populations, including GAL.

These results suggest that populations are not locally adapted towards higher levels background matching on native habitat.

3.14 Morphometric and pattern colouration differences between populations

A total of 40 adult wild-caught individuals were analyzed for phenotypic differences of morphometry and colour-pattern-related variables between populations.

Sampling number ranged from 6, for GER population, to 12, for SE population.

Significant differences in body size (SVL) were found between GAL and MTZ populations. These two populations share evolutionary history, both belonging to L3 mtDNA clade, but belong to different subspecies, with GAL being attributed to *T.l.ibericus* and MTZ to *T.l.lepidus*.

Significant differences were found between populations regarding melanization index. SE population exhibits higher melanization index when compared to both GAL (W=17 p-value=0.01) and MTZ (W=9 p-value=0.002). This result means that individuals from Serra da Estrela (SE) tend to possess higher quantity of green scales relative to black scales, for the same area of dorsal skin, when compared to GAL and MTZ.

Montezinho (MTZ) population also showed significant differences in eyespot area comparative to SE (W= 129, p-value= 0,001) and GAL (W= 13, p-value= 0,009) populations. Individuals from MTZ population have larger blue eyespots on flank skin, when compared with GAL and SE individuals. However, number of eyespots does not differ between populations.

3.15 Phenotypic differences between subspecies

The majority of the museum specimens (91.04%) belonged to populations attributed to *T.l.lepidus* subspecies. Being so, a total of 18 adult *T.l.ibericus* (17 wild caught and 1 museum specimen), and 50 adult *T.l.lepidus* (23 wild-caught and 27 museum specimens) were comprised in this analysis.

The two subspecies were found to show significant differences regarding morphometric variables of SVL (W=214.5, p-value= 0.003), head width (W=263.5, p-value= 0.01) and head length (W=254.5, p-value= 0.009). These results are in accordance with previous descriptions of the current taxonomy, where *T.l.ibericus* is said to be smaller.

Regarding coloration measures, despite no significant results being found when analyzing population individually, differences seem to arise in coloration metrics once populations are grouped according to current taxonomy. Significant differences were found between *T.l.lepidus* and *T.l.ibericus* in Dorsal (W= 23, p-value= 3.2307e-05) and Throat (W=222, p-value= 0.01) hue. This means that,

despite both subspecies been green, as showed in the reflectance spectra (Fig. 23 A, B), they may vary in the shade of green coloration exhibited in both dorsal and throat body areas.

Differences were also found in melanization index between the two subspecies (W= 217, p-value= 0.005). This result indicates that the proportion of black to green scales between the two subspecies varies, with *T.l.lepidus* presenting higher melanization index, thus showing higher number of green relative to black scales, for the same area of dorsal skin, as described in previous studies. However, when considering populations individually SE, showed significantly higher melanization index than both GAL and MTZ, being the second attributed to the same subspecies, denoting inter-population variability on this phenotypic trait.

No significant differences were found between the two subspecies regarding both eyespot number and area.

3.16 PHENOTYPIC DIFFERENCES BETWEEN MITOCHONDRIAL CLADES

When filtering the phenotype matrix per mitochondrial DNA clade, a total of 32 adult individuals belonging to clade L3 and 29 belonging to clade L5 were analyzed for phenotypic differences.

Differences in morphometric variables were significant for SVL (W=298, p-value= 0.047), head width (W= 268, p-value= 0.016) and head length (W=256, p-value= 0.01) when comparing both clades. L5 individuals, which belong to *T.l.lepidus* subspecies, are bigger than the ones from L3, which, depending on population range can be either *T.l.lepidus* or *T.l.ibericus*.

Regarding colouration-related variables, significant differences arise in Throat hue (W= 219, p-value= 0.008) and melanization index (W= 130.5, p-value= 0.0009) with L5 individuals showing higher melanization index, thus presenting a higher proportion of green scales for the same area of dorsal skin compared to L3 individuals.

3.17 OVERALL PHENOTYPIC CLUSTERING PATTERN

PC analysis comprising phenotypic information, for all studied individuals: both wild-caught and museum specimens, does not show clear clustering pattern neither according with taxonomy nor population (Fig. 24 left).

PC1 explains 80.1% of the variance, and body size variables of SVL and head measures together explain 75.7% of the variance in PC1.

When excluding museum specimens without population attribution from the analysis, thus focusing exclusively on wild-caught individuals (Fig. 24 right), no clustering pattern emerges as well, with individuals from different populations mingling together. PC1 explains 76.1% of the variance, with eyespot area, number of eyespots and SVL explaining 85.8% of the variation in PC1.

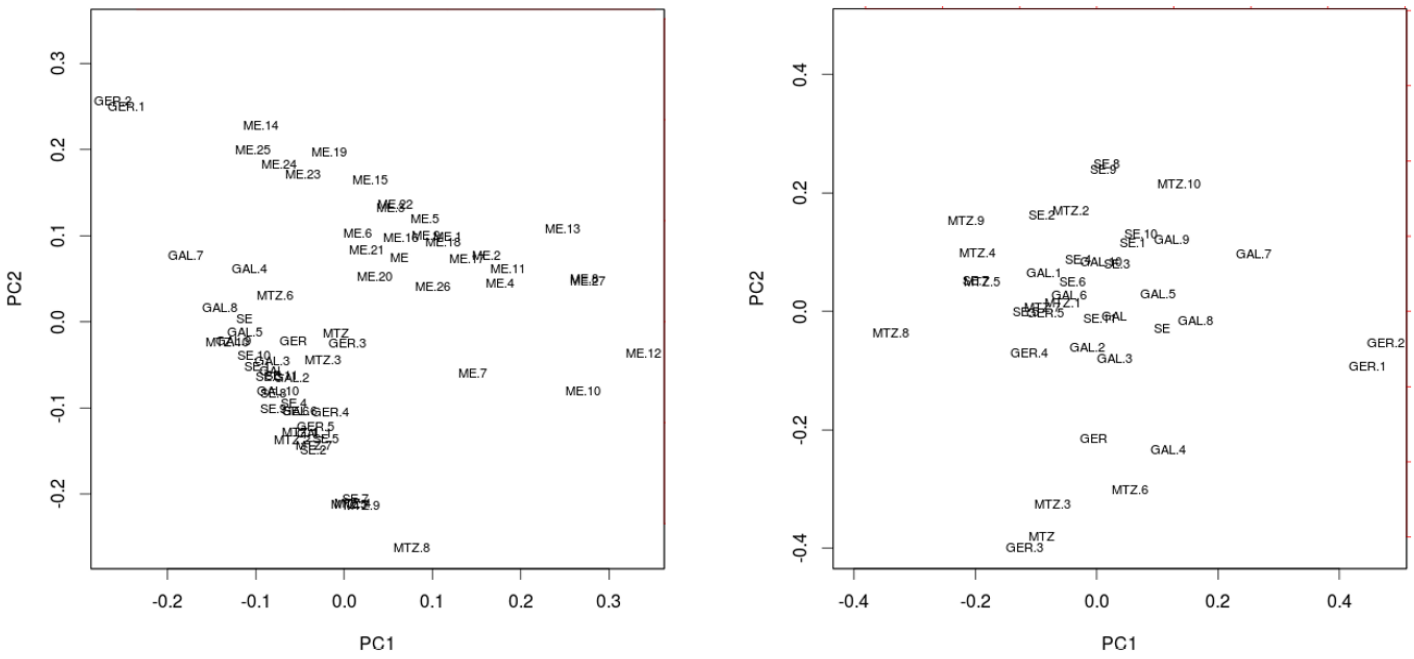

Figure 24. PC analysis of phenotypic variables for all individuals (**left**) and of exclusively wild-caught individuals, thus excluding museum specimens (**right**).

4. DISCUSSION

4.1 DISENTANGLING THE PHYLOGEOGRAPHY OF *TIMON LEPIDUS* AND THE EFFECTS OF DIFFERENT EVOLUTIONARY FORCES ON POPULATION DIFFERENTIATION PATTERN

The distribution of ancestral haplotypes of Serra da Estrela (SE) population, and others from the same mitochondrial clade, indicates that during ice age periods, these were probably concentrated in refugia on the south-eastern zone of the Guadalquivir basin. There, populations diverged, in allopatry, and during the subsequent interglacial period colonized the geographical area of the current range of the population (Miraldo *et al.* 2011). This has deep impact on the perception of the genomic differentiation pattern. When analyzing all four herein studied populations, SE appears as the most divergent, masking the differentiation among the remaining populations of the L3 clade, which share a different evolutionary history.

The differential evolutionary history of Serra da Estrela also influences the genomic detection of selection signatures. Lositan software performs the detection of selection with an algorithm that takes into account the relation between loci Fst and heterozygosity. The unique evolutionary history of SE, would lead to a lot of markers expressing high Fst values due to fixation by drift. Therefore, when excluding this population from the analysis, a considerable amount of false positives for selection are also eliminated, enabling us to further investigate the selection signatures among the remaining populations, which were previously masked.

Even in the absence of selection, divergence is expected to vary due both to stochasticity of genetic drift and the complexities of population history and demography (Seehausen *et al.* 2014). in complex environments it may be difficult to disentangle the roles of demographic history and selection (Savolainen *et al.* 2013) and the effects of the first appear to play a part in *Timon lepidus* divergence pattern.

Galiza (GAL) population is the one situated in the NW extreme of the bioclimatic cline that crosses the Iberian Peninsula. This population appears differentiated from the remaining, both when analyzing all molecular markers for all populations in a four-cluster pattern, and when analyzing the L3 populations, in a two-cluster pattern.

This is unexpected when taking into account the taxonomic arrangement of the populations, according to which GAL would be expected to group with GER in the genomic analysis, since both populations are attributed to *T.l.ibericus* subspecies.

GAL individuals appear to possess low levels of genetic diversity, depicted by low values of heterozygosity, high inbreeding coefficient, which is further confirmed by high relatedness between individuals, and short length of phylogenomic branches. This result is in accordance with previous research on this population, comprising both genetic and demographic evidence (Miraldo *et al.* 2011; Nunes 2011).

Altogether, these data may reflect a smaller effective population size and different demographic history for this population (Kirk & Freeland 2011; Jones *et al.* 2012), probably involving bottlenecks due to range contraction events caused by the glaciations.

Additionally, GAL has 61 SNPs with allelic frequencies equal or superior to 85%, while the other populations from the same mtDNA clade, GER and MTZ, only possessed 9 and 19 SNPs in that condition, respectively. This could be a pattern generated by the impact of genetic drift, which would be more intense in a small population, thus shifting allelic frequencies differentially near to fixation.

Besides the demographic history, another fact that can contribute for the lack of clustering of GAL and GER population, is evidenced by the high levels of introgression between GER and MTZ. This is depicted both by the highest rate of migration found between these populations (Fig. S5), by the "L-shaped" Fst pairwise curve, and further supported when analyzing the structure of all populations with four clusters. The sharing of genetic material between GER and MTZ will tend to homogenize these populations, thus leading to a higher differentiation from GAL.

Moreover, GAL also presents an elevated level of gene flow with MTZ, rather than with GER, with which it shares taxonomy. This could be explained by differential migration between these populations.

The migration route from Gerês towards northern Galiza, and *vice versa*, is a demanding one, for a lot of water lines cross the coast line. This hydrographic arrangement generates a series of riverine barriers, which are known to inhibit gene flow and shape genetic structure in lizards (Pounds & Jackson 1981; Lamborot 1991; Boumans *et al.* 2007).

Being so, both differential demographic history and migration may be two factors contributing to the population differentiation pattern which is discordant with current taxonomic distribution ranges of these populations.

4.2 THE PATTERN OF LOCAL ADAPTATION OVERRULES THE CURRENT TAXONOMY AND PUTATIVE UNDERLYING SPECIATION PATTERN

Both neutral and selective processes can lead to phenotypic and genomic differentiation (Kirk & Freeland 2011; Seehausen *et al.* 2014). Being so, identify signatures of adaptive genomic differences, and individually assessing the acting evolutionary forces is crucial to further understand the divergence pattern between populations.

After excluding the confounding effects of differential evolutionary history, and assessing the genomic signatures of selection within L3 populations, the loci found to be under putative positive selection comprise about 21% of the polymorphic SNPs surveyed. This indicates that multiple loci are under selection and involved in population-pair divergence. These results are in accordance with NGS-based genome scans of sympatric species, which generally report genomically wide-spread and highly heterogeneous divergence varying on a very local scale (Jones *et al.* 2012; Gagnaire *et al.* 2013; Keller *et al.* 2013)

Non-neutral markers are known to provide better insight regarding genetic diversity, local adaptation or evolutionary potential than their neutral counterparts (Kirk & Freeland, 2011). However, even though the majority of loci (around 71%) is evolving neutrally, as expected, the genomic divergence pattern is consistent with three population clusters arising also when analyzing these.

The only situation in which this pattern is lost is when analyzing loci under putative balancing selection, which is expected since this selective force favors the existence of alleles in heterozygosity, thus homogenizing the allelic frequencies between populations.

The fact that non-neutral markers show a clear pattern of differentiation, between the three populations sharing evolutionary history, strongly suggests a pattern of local adaptation. Local adaptation is the fine-tuning of populations to their local environment, which can vary in both biotic and abiotic factors, via natural selection (Savolainen *et al.* 2013). Persistent environmental gradients may impose divergent selection, such that populations evolve differences in morphology, physiology, behavior, or life history that provide a fitness advantage under those local conditions (Kawecki & Ebert 2004). While this pattern is common for markers evolving under divergent selective pressures, it is not common to observe when analyzing exclusively neutral loci.

However, gene flow will not necessarily ensure that non-adaptive genes are continually exchanged between populations (Kirk & Freeland 2011). Even though

differentiation of neutral markers is primarily driven by stochastic processes, whereas that of non-neutral markers is driven by both selective and stochastic processes (e.g. Galindo *et al.* 2009), natural selection can also influence the distribution of neutral markers (Kirk & Freeland 2011). This pattern can arise when divergent selection is sufficiently strong to inhibit gene flow, which can potentially result in genome-wide differentiation of populations, even in geographic proximity (Freeland *et al.* 2010).

However, while some population divergence can occur even with moderate gene flow, strong divergence requires very low gene flow, and is promoted, for example, when incompatible mutations arise between populations, and when allopatric divergence occurs prior to secondary contact (Nosil & Flaxman 2011).

Despite the past of the studied populations being marked by episodes of divergence in allopatry and range reductions, the herein results also show that gene flow occurs between populations. Furthermore, if selective pressures were strong enough to collaterally prevent gene flow in neutral markers, it would be expected to find a considerable amount of fixed alleles on those populations. This is not confirmed for the studied populations since no loci were found with allelic frequencies equal or superior to 90%. Altogether, these facts make the scenery of strong selection inhibiting gene flow, and promoting divergence in non-neutral loci, highly improbable.

A more parsimonious explanation for this pattern could be related to the method used to assess the optimal number of clusters in the Bayesian analyses. Despite the fact that the Evanno method indicates three clusters as the most probable value of K explaining the structure of these populations, when we analyze the two cluster pattern, GAL appears attributed to one individual cluster, with loss of structure between GER and MTZ, which share a common cluster when K=2. This scenario is probable due to the known demography of GAL, which, in a scenario of bottleneck or reduced effective population size, could be highly impacted by drift generating this pattern in neutral loci. This is further supported by the fact that GAL possesses a high number of SNPs with differentially high allelic frequencies.

Another effect related with software could be influencing the data. Despite the relative percentages of loci detected to be under positive and balancing selection and the ones evolving neutrally being expected, the individual proportions seem to be exaggerated, namely the amount of loci putatively under selective forces. In other studies in which Fst outlier approaches were applied, percentages on the order of 1% of the studied loci were found to be under positive selection (Jones *et al.* 2012). This leads to the suspicion of over estimation of selective forces by the software, which could lead to false attribution of balancing selection to loci that are

actually neutral, thus losing the homogenizing pattern when analyzing the putatively neutral loci, which are actually biased towards higher Fst values. Thus, the interpretation of the different subsets of markers should be carefully analyzed and further investigation is needed in order to understand the discordance in the neutral marker pattern.

A fair number of molecular markers were found to be associated with environmental variables (3.15%), mainly regarding precipitation and temperature. When analyzed for differentiation pattern, these markers show once again a clustering pattern in which all populations are clearly distinct. Furthermore, temperature seasonality, mean annual sunlight duration and altitude are the bioclimatic and geospatial variables explaining the majority of the differentiation between habitats. All these are known to influence ecological adapatation in heliothermic lizards (e.g. Castilla *et al.* 1999; Scheers & Van Damme 2002; Yang *et al.* 2014, 2015) and thermal adaptation is known to lead to divergence in reptile populations (Muñoz *et al.* 2014). Being so, it seems probable that ecological differences are driving the divergence between the studied populations of *Timon lepidus*, however, not in accordance with the current described distribution range of the subspecies, but in a local pattern.

Together, environmental heterogeneity and different demographic and evolutionary histories should be the main shapers of the local adaptation pattern.

4.3 Cues for repeated ecological adaptation

When analyzing the population pairs presenting abnormal bimodal Fst curves, cues for a scenario of repeated ecological adaptation emerge.

If we take into account the natural environment of these populations, comparison of SE with MTZ and GER, corresponds to pairwise comparisons between mountain environment populations, for all these inhabit mountain range altitudes higher than 800 meters asl. Conversely, when comparing any population with GAL, which was sampled at 7 meters asl, corresponds to a contrast between mountain and coastal environments.

This pattern arising from similar versus alternative environmental comparisons may be resulting from similar selection pressures being felt on mountain environments. This parallel selective pressure would modulate the allele frequencies of those populations, thus giving rise to an excess of loci with low Fst values. Being so, instead of contributing to the differentiation between populations, these loci are at very similar allelic frequencies in similar environment populations.

According to this scenario, the prediction is that the same SNP that exhibits low Fst value in mountain-mountain comparisons should then exhibit higher Fst values

when comparing the contrasting environments, i.e. mountain-coastal, for the common selective pressure is not present in the last. This is exactly what is depicted upon analyzing the loci with Fst values lower or equal to 0, and it is supported by the fact that the same pattern is absent when analyzing the remaining SNPs not contributing to the bimodal distribution.

Many traits in plants and animals evolve repeatedly in response to similar environmental conditions, suggesting that they are adaptive and shaped by natural selection (Wake et al., 2011). For example, marine three-spine sticklebacks colonized and adapted to a large number of new freshwater environments at the end of the last ice age. This generated a pattern where geographically farther apart populations, with lower levels of gene flow, were more similar genetically due to parallel environmental selective pressures, in the fresh-water habitats (Jones *et al.* 2012). This is not an improbable scenario to have occurred with *Timon lepidus*, for this species also may have colonized mountain tops during interglacial periods (Paulo *et al.* 2008; Miraldo *et al.* 2011), especially in the north of Iberian Peninsula were range contractions should have been more intense, and the conditions in mountain ranges would be fairly harsh for an heliothermic lizard during ice ages.

Thus, it seems that divergence between *T. lepidus* populations is overall spread in the genome, with a lot of loci implied in local adaptation, while other loci are being equally selected in similar environments probably leading to repeated adaptive evolution.

The fact that the pairwise comparison between GER and MTZ does not produce the same bimodal Fst curve profile, despite both being mountain populations, is most probably linked with the great amount of gee flow that seems to occur between these two populations. This would give rise to the observed “L-shaped” curve instead (Feder *et al.* 2012).

Once again environmental local conditions, such as those of the microclimate effects of altitude, seem to have an impact in the pattern of population differentiation, adding to the complexity to the evolutionary history of these populations. Genes underlying repeated adaptive evolution in natural populations are still largely unknown (Jones *et al.* 2012) and this is thus an interesting line of research, which could be assessed by conducting altitude transects on phenotypic and genomic information in these populations of *Timon lepidus,* in order to identifying the genetic and genomic basis of repeated evolutionary change in natural populations.

4.4 Background matching is not the main driver of local adaptation

Body colouration in heliothermic squamates is under strong natural selection and diurnal reptiles experience intense selection for substrate matching to diminish vulnerability to visual predators (Norris 1965; Rosenblum 2004). Unlike the eyespots, dorsal surfaces tend to be exposed at all times, especially when basking, and so this part of the body surface may be expected to be more influenced by the requirements of crypsis (Thorpe 2002). On top of that *T. lepidus* is predated by a great variety of animals, constituting the main dietary resource of birds of prey in some regions (Mateo, 1988). Being so, if predatory pressure was one of the factors driving local adaptation regarding pattern colouration phenotype, it would be expected to find individuals to better match basking backgrounds of their native living range, than that of other populations. This prediction is not corroborated by our results, and background matching regarding spectral segments even seems to reach high level in anthropogenic substrates as those seen in Galiza.

Evidence of behavioural compensation of colour conspicuousness has been found in desert lizards (Norris, 1967). In some species, individuals compensated behaviorally for lack of background matching by maintaining a close distance to shelter and faster escape response. *Timon lepidus* were also observed to bask constantly near their refugia, quickly escaping when approached by ground dwelling predators (*pers.obs.*). This strategy may be costly to ectotherms if the refuges are located in microhabitats with shady and cold thermal conditions, such as rock crevices, because body temperature of ectothermic prey will decrease below preferred levels in short amount of time (Polo et al., 2005), which may negatively affect physiological and locomotor performance. However, *Lacerta schreiberi*, a closely related species to *Timon lepidus*, seem to consider physiological costs of being at low temperatures and also the risk of emerging with low escape performance when deciding refuge use (Martín & López 2010), thus balancing the cost of thermal constraints and escaping.

Together, these observations suggest that the selective pressure for crypsis should not be the main driver of population divergence, contrarily to cases reported in other lizards (Rosenblum *et al.* 2010; Muñoz *et al.* 2013). However, this topic needs to be further investigated with analysis comprising information on the UV emission spectrum and pattern analysis, which would complement the colouration analysis.

4.5 PHENOTYPIC DIFFERENCES ARE INCONCLUSIVE REGARDING DIFFERENTIATION BETWEEN POPULATIONS OR SUBSPECIES

According with previous studies (Nunes *et al.* 2011; Mateo 1988), significant differences were found in melanization index between subspecies, upon analysis of 69 adult individuals. However, significant differences in this phenotypic quantification were also found between populations attributed to the same subspecies (MTZ and SE), and between mtDNA clades.

The dorsal pattern of this lizard is composed by the reticulated organization of green and black scales on a black background. The pattern spans the entire dorsal body surface of the individual and has been described to vary along the bioclimatic cline (Mateo, 1988), shifting from the typically reticulated to a more aggregated pattern, where the black scales form bigger continuous areas. Being so, in such a variable pattern comprising a large area of the individual's body, and without taking into account the differential areas of each colour, $1cm^2$ is hardly representative of the entire dorsal area. This variance is illustrated by the intra and inter population variance in this trait depicted in previous studies (Nunes *et al.* 2011). This evidence calls for a different approach to the question of inter-subspecific colouration differences, where the whole dorsal area should be taken into account, as well as pattern organization analysis.

Previous studies of colouration differences between the two subspecies were exclusively focused on human visual assessment. This is the first study analyzing reflectance data for these subspecies and significant differences were found in dorsal and throat hue between *T.l. lepidus* and *T.l. ibericus*. This means that the differences described as lighter or darker morphs of the same color, can actually be different shades of green. This would probably involve different underlying physiological mechanisms, which are worth of further investigation from a histological and developmental perspective that could provide further insight regarding the phenotype-environment relationship, within and between subspecies.

MTZ population showed significant phenotypic differences regarding eyespot area with populations both from same (SE: *T.l. lepidus*), and different subspecies (GAL: *T.l. ibericus*). Montezinho individuals thus have bigger eyespots on flanks, and this is a trait known to influence intraspecific communication in *Timon lepidus* (Font *et al.* 2009) and other species. In natural populations of *Lacerta agilis*, males with relatively larger badges showed higher reproductive success, and badge size was under significant sexual selection (Olsson, 1994). Being so, this trait influences the gene flow between MTZ and these two populations, since males are the primary migrants in this species (Mateo, 1988). Furthermore, in a study of sexually mature male lizards (*Gallotia galloti*), UV markings used in conspecific signaling were

suggested to be more influential than historical separation in determining gene flow (Thorpe and Richard, 2001). Thus, individuals from MTZ could have higher reproductive success on sink populations due to possessing more attractive (towards females) or intimidating (towards other males) eyespots. However, pre-copulatory female choice on male colours seems to be rare in reptiles (Olsson *et al.* 2013) and being so, this is an interesting question to study on controled laboratory experiements that could contribute to the understanding of the tendency of population divergence or homogenization in *Timon lepidus*.

Except for dorsal hue, no other phenotypic characteristic herein analyzed showed differences exclusively between subspecies. Overall, the variance within population phenotypic data seems to hinder the perception of the main pattern of divergence between populations, regarding phenotype.

This result is actually supported by the AMOVA, which points towards the variance being mainly contained between individuals within populations. Moreover, the negative variance components found when analyzing subspecies can indicate that genes from different subspecies are more related to each other than genes from the same subspecies, once again arguing against the taxonomic attribution of the populations.

4.6 Is the tree-of-life branching for *Timon lepidus*?

When relating the speciation process to a phylogenetic tree, the branching of two lineages is not the speciation event, but it rather corresponds to the terminus of that process, when the two lineages become isolated. Population data is still lacking to allow a complete understanding of the isolation moment, but it has been suggested that , in most cases, described species separated by only tens of thousands of years are not real species (Hedges *et al.* 2015). *T.l.ibericus* are estimated to be diverging for about 3 My (Miraldo *et al.* 2011), which could be enough evolutionary time to attain full divergence. However, the extent of interacting forces, and the impact of population history on this process generates a continuum of complexity that cannot be reduced to average time needed to complete speciation. The phylogeny comprising the herein studied populations does not show evidence of linage sorting according with the current taxonomy, and rather highlights the pattern of local adaptation shaped by the individual evolutionary history of the populations.

While *T.l.nevadensis* has complete lineage sorting regarding mtDNA, with a unique mitochondrial clade associated with its distribution range, the same is not found for *T.l.ibericus,* which shares mitochondrial clade with *T.l.lepidus* populations.

Regarding Karyotype, chromosomal alterations are known to be highly correlated with population divergence and being promotors of speciation (Kirkpatrick & Barton 2006). Despite being diverging for 9 My *T.l.nevadensis* do not possess a different karyotype from the nominal subspecies. Contrastingly, variation in the number and position of chromosome nucleolar organizers (NORs) is reported for *T.l.ibericus*. However, this it is a matter of frequency because three karyotypes are known to exist simultaneously in this subspecies, with the only one being unique also not being the most commonly found.

A common garden study performed on the three mainland Iberian subspecies aimed to uncover non-plastic differences in life-history traits and morphometric variables between those (Mateo & Castanet 1994).

Females from *T.l.ibericus* were significantly smaller and attained sexual maturity earlier than the remaining subspecies, also producing smaller eggs. However, the differences on *T.l.nevadensis* body size, normally found in the natural range, did not persist in the common garden.

Small body size associated with a population at the north-western limit of the bioclimatic cline (Pontevedra) can be suggestive of adaptation to climate, for thermoregulation would be facilitated due to the greater surface area–to-volume ratio of small-body individuals (Angilletta *et al.* 2004) and thus, this is a trait likely to be under selection. However, geographic variation in body size is known to interact with a lot of environmental and life-history traits which are not comprised in this study (e.g. Horváthová *et al.* 2013). Moreover, the authors do not present a description of the environmental conditions of the common garden, and only comprised one generation of captivity born individuals. This means first, that the conditions could be favoring the adaptation of one of the subspecies, which are naturally distributed in a wide bioclimatic gradient, and second, that the data could be influenced by maternal effects (Matos 2012). A new study taking the present knowledge on plasticity and adaptation into account, and comprising more than one generation in a gradient of conditions, instead of a unique rearing condition, could contribute with further insights regarding the varying reproductive strategies and life-history traits of the subspecies.

While the distinction between *T.l.nevadensis* and *T.lepidus* is overall well supported by combined evidence of phenotype, genotype and environment, the genetic relationship between *T.l.ibericus* and *T.l.lepidus* is frequently hard to disentangle (Nunes 2011). The taxonomic attribution of GAL and GER populations to a different subspecies from that of MTZ and SE is strongly supported by morphological arguments, and GER population is only putatively attributed to *T.l.ibericus* (Mateo & Castroviejo 1991). The certain, and researched on, distribution

area of this subspecies comprises exclusively the Spanish provinces of Coruña and Pontevedra, being the lectotype (Mertens 1925) an individual originating from Coruña (Mateo 1988; Mateo and Castroviejo 1991).

All these facts, together with the previously debated demographic history of Galiza, suggest that GER population may be falsely attributed to the *T.l.ibericus* subspecies, and that the distribution range must be revised.

Despite all the advances regarding speciation theory and the novelty that the NGS revolution brought to the study of this theme, we still lag in our ability to accurately characterize taxa at lower levels, such as subspecies. This difficulty partly results from the lack of universal acceptance of a species concept. Consequently, subspecies recognition is often based on inconsistently applied criteria such as concordance in multiple, independent, genetically-based traits, geographic and phylogenetic separation but reproductive compatibility, and differences in morphology, behavior, life history or ecology (Sackett *et al.* 2014).

Therefore, the current taxonomy should be reviewed in the light of the speciation continuum, taking into account the pattern of local adaptation expressed by these populations.

4.7 Final Remarks and Future perspectives

The RAD-Seq technology allowed the analysis of thousands of genetic loci simultaneously, across *Timon lepidus* genome, while being time and cost-effective. The present study was the first to apply NGS to this lizard, which does not possess a model-organism counterpart. Until very recently, in the history of molecular evolution, this would not have been possible.

The evolutionary history of populations and the classic taxonomy of this species were assessed in the light of the genic view of the speciation process, and interesting patterns arose. However, discerning process from empirical patterns of NGS divergence is complex, due to overlapping expectations generated by different combinations of evolutionary forces. Nevertheless, broad generalities may still be drawn from analyses of groups of taxa at varying stages in the speciation continuum (Feder *et al.* 2012). Combining knowledge of the natural history of a system with the research for the key factors and traits generating divergent selection, and uncovering how that divergence is distributed in the genome, allows general trends to be ascertained.

Overall, a pattern of local adaptation, driven by ecology of populations, seems to be clearer that the expected pattern of incipient speciation suggested by previous studies and taxonomy of *T.l.lepidus* and *T.l.ibericus*.

This, however, does not exclude the possibility of speciation in this species. Adaptation can eventually lead to speciation, for reproductive isolation can evolve between populations adapting to contrasting environments. Moreover, local adaptation is also an important component of responses to changing environments (Via 2001; Nosil *et al.* 2009). Appreciation of the connection between these two processes began with Darwin, and together with the understanding of speciation and organismal diversity, evolutionary biology also seeks to explain this major feature of the living world, which is the evolutionary process by which populations become better suited to their own environment, through genetic change (Butlin *et al.* 2012).

Although there is evidence of widespread local adaptation in both plants and animals, the genetic basis of this process remains poorly understood (Savolainen *et al.* 2013) which poses *Timon lepidus* once more as a very interesting model to assess hot-topic questions of evolutionary biology.

Future perspectives should look further into the phenotypic divergence of these populations and seek for phenotype-genotype associations, which could provide further insights regarding the need of a taxonomic review.

The contact zone between subspecies is an area of great interest, for the existence of narrow clines and hybrid zones demonstrates that selection can dominate gene flow over quite small scales, allowing parapatric divergence (Turelli *et al.* 2001). Being so, a comprehensive analysis of the gene flow between more populations of *Timon lepidus* could uncover cues regarding both subspecies range and dynamics of genomic divergence.

Other NGS techniques, such as QTL and whole-genome sequencing could complement genome-wide approaches, such as the one implemented in this study, and contribute to the understanding of which genes are the ones being affected by disruptive selective pressures and contributing to local or repeated ecological adaptation.

On a long-term approach, difficulties can arise in assessing sexual selection and hybrid viability in controlled laboratory experiments, due to this lizard's life-cycle duration. However, these have been bred in captivity and even domesticated. Controlled experiments, with individuals controlled for sex and age could provide

valuable insight regarding a lot of aspects, such as the development and physiology of the colouration pattern, life-history traits and reproductive strategies of the species, and experiments with environmental manipulation should provide important data on the ecological adaptation of these populations.

Speciation is a process that takes longer than the human life span, and despite all progress on both theoretical and empirical perspectives of this theme, we are still trying to define and understand the patterns, and disentangling the processes that shape biodiversity. As so, this will continue to be a central topic in evolutionary biology for a long time and deepening the study of adaptation will certainly contribute for the overall understanding of such matters.

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ANEXO 1 - SUPPLEMENTARY INFORMATION

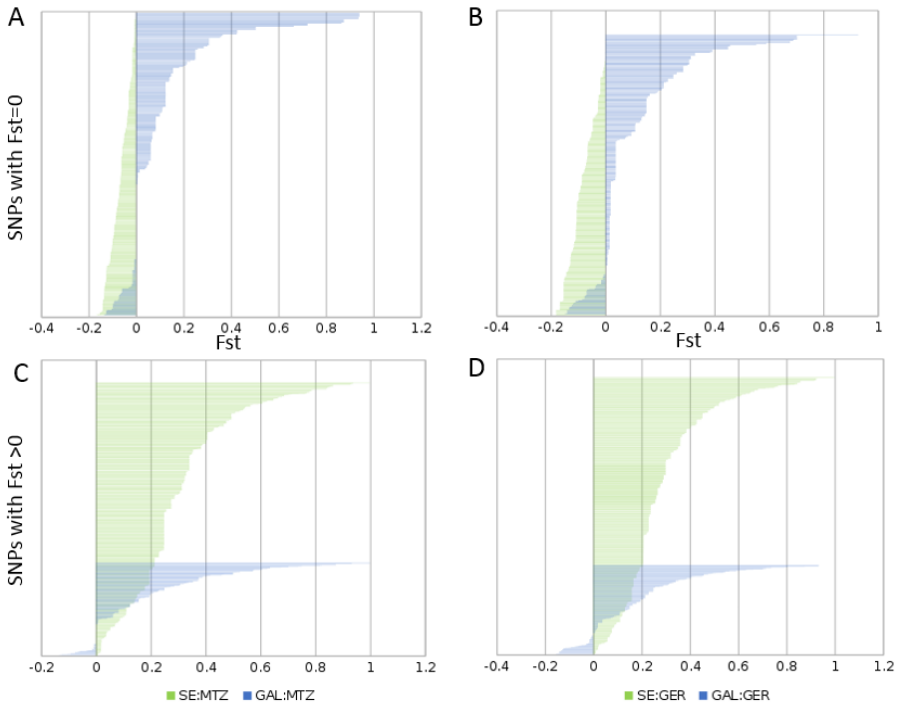

Figure S2 Fst values for individual SNPs in similar (green) and contrasting (blue) environment pairwise comparisons. To each SNP corresponds a green and a blue bar. Green bars depict mountain:mountain comparisons while blue bars represent mountain:coast comparisons for the same population pair. **A** and **C** correspond to SE:MTZ and GAL:MTZ and **B** and **D** to SE:GER and GAL:GER comparisons.

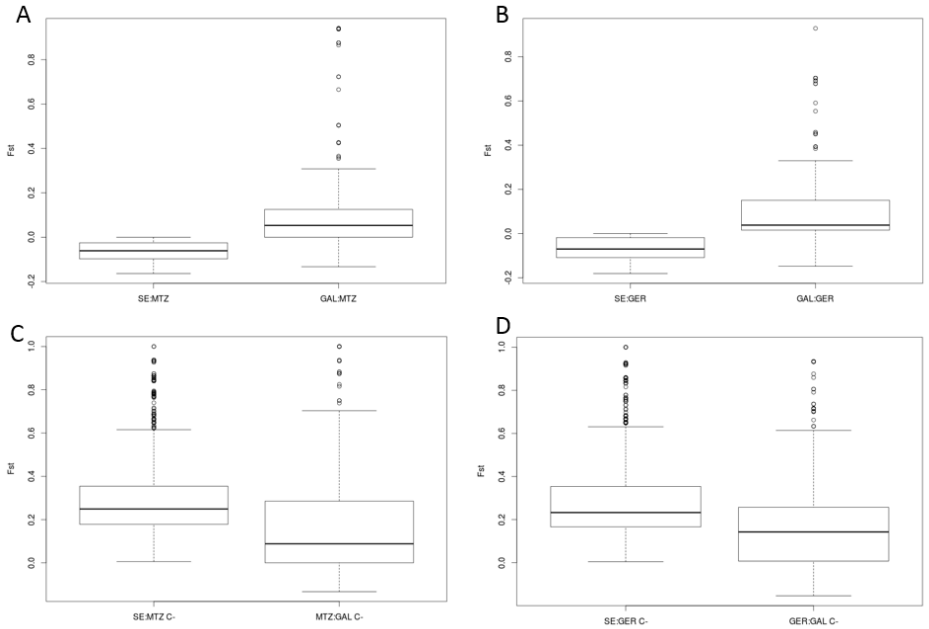

Figure S1.2 **Fst average shifts** when comparing similar (**C D)** and contrasting (**A** B) environments. Letter numeration in the present figures is related to the previous, with equivalent population comparisons being depicted.

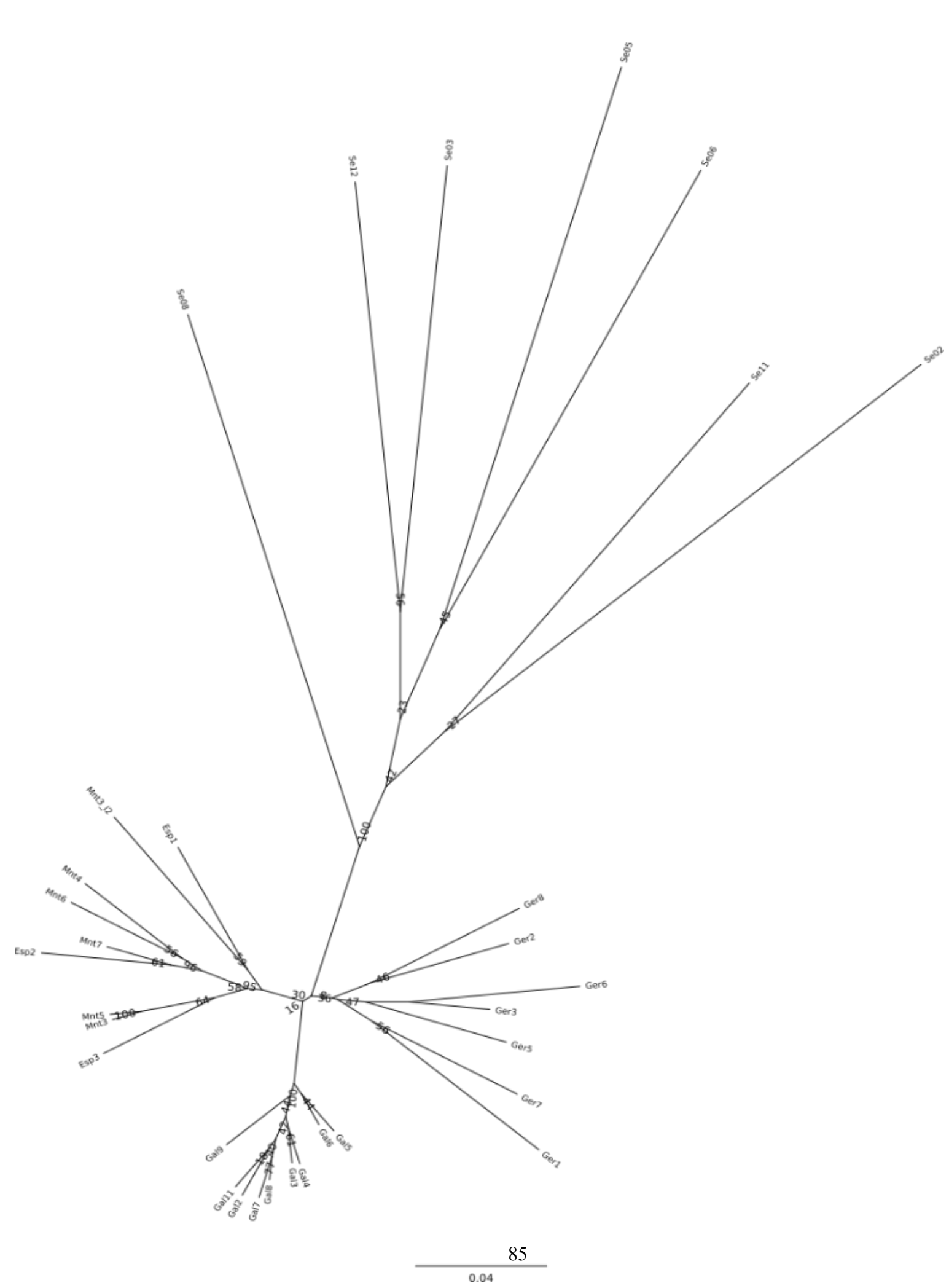

Figure S2 Phylogenomic tree of all studied populations.

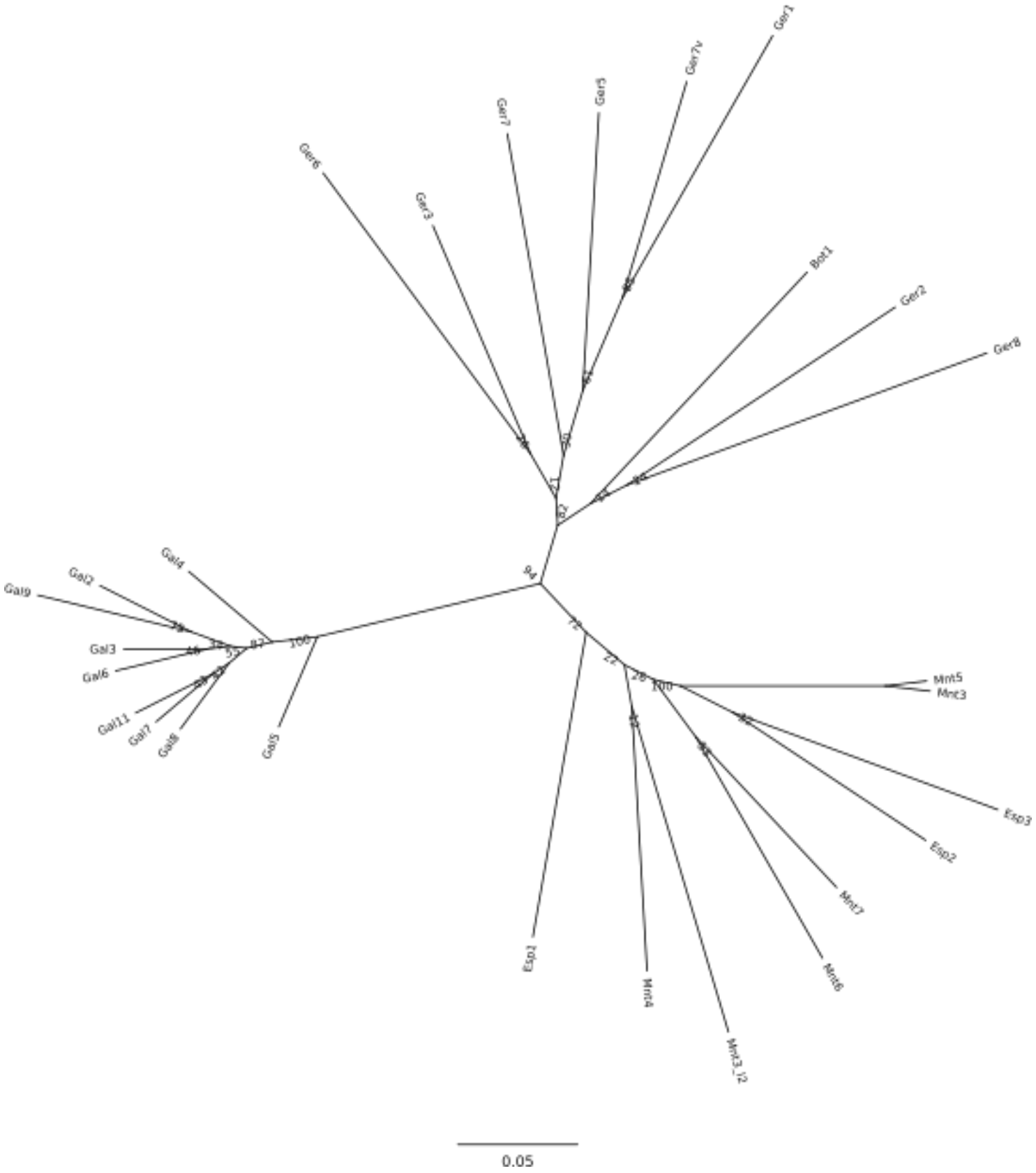

Figure S3 Phylogenomic tree of L3 populations: GAL, GER and MTZ. These share mitochondrial DNA clade.

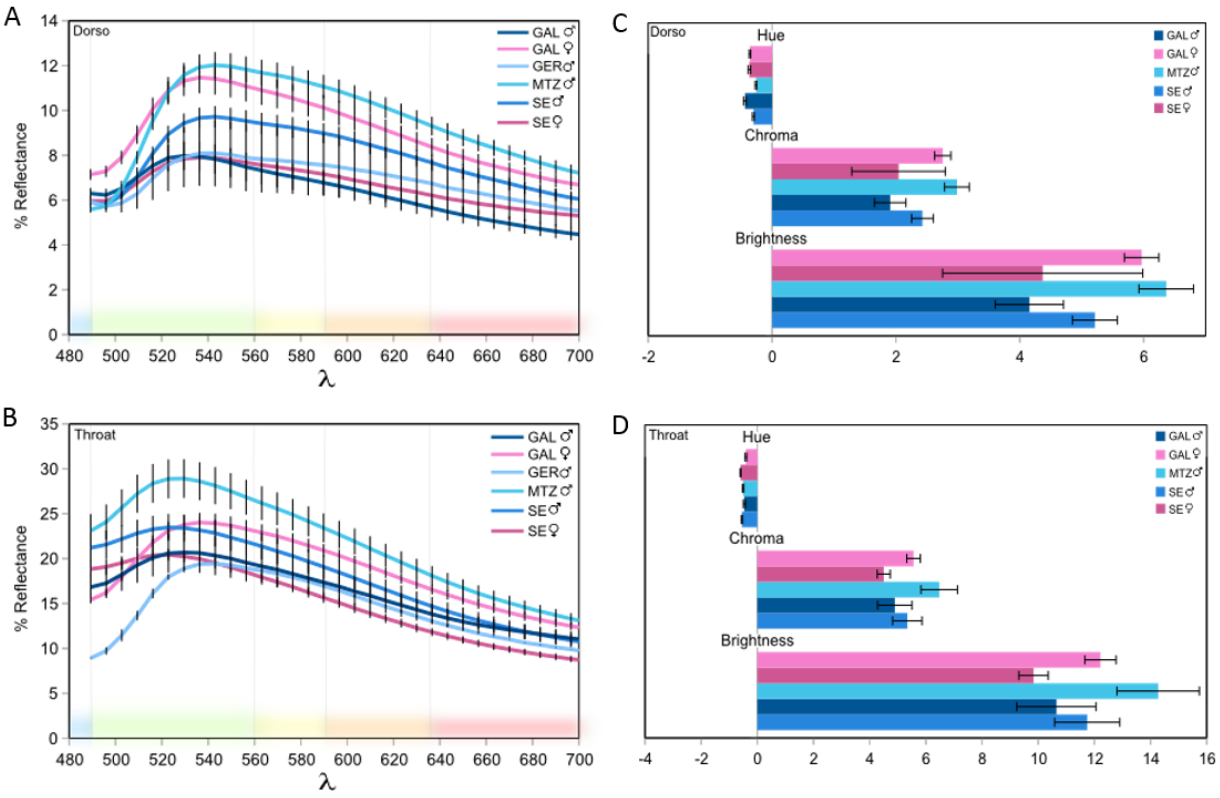

Figure S4 Dorso and throat reflectance analysis of the four populations of *Timon lepidus per sex.* **A** Dorsal reflectance spectra for males and females **B** Throat reflectance spectra for males and females of all populations. Vertical lines indicate error bars. Each sex and population is represented by a line color. Colors on xx axis correspond to reflected wavelength **C** Dorsal spectrum segment analysis for males and females of all populations **D** Throath spectrum segment analysis. Hue is commonly called color, chroma corresponds to color saturation and brightness to the amount of white or black mixed in the colour (hue).

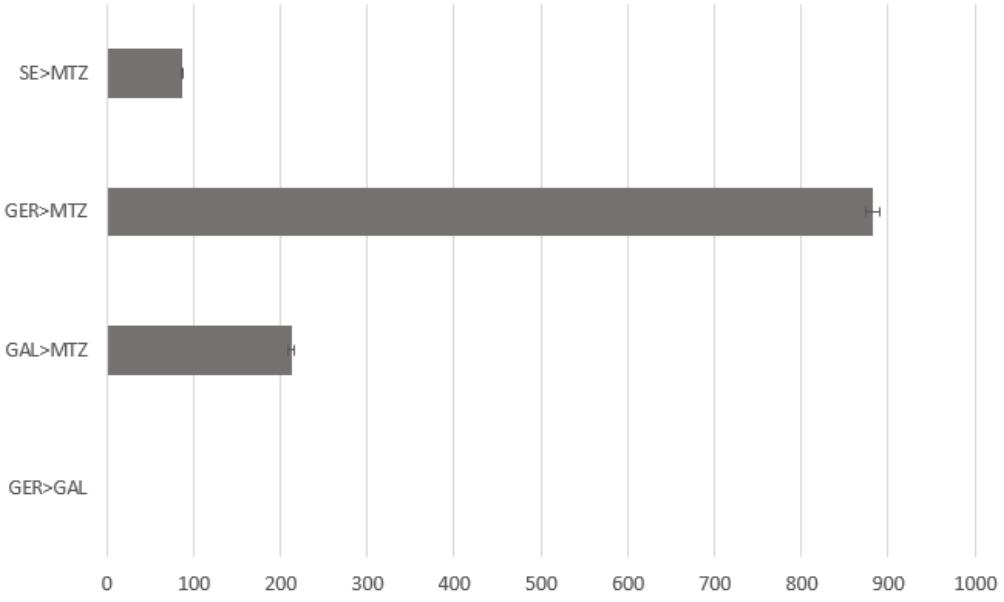

Figure S5 Migration rates between populations inferred with GPhoCs software. Geneflow is modulated by number of migrates per generation divided by estimated mutation rate.

ANEXO 2 – POSTER

Dorsos are green, Eyespots are blue, Does Natural Selection shape you?

Telma G. Laurentino[1], Francisco Pina-Martins[1], Joana Fino[1], Pedro Patrício[2], Octávio S. Paulo[1]

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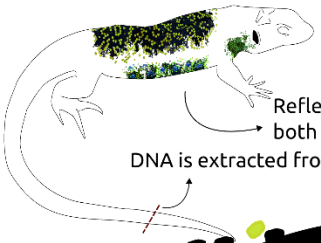
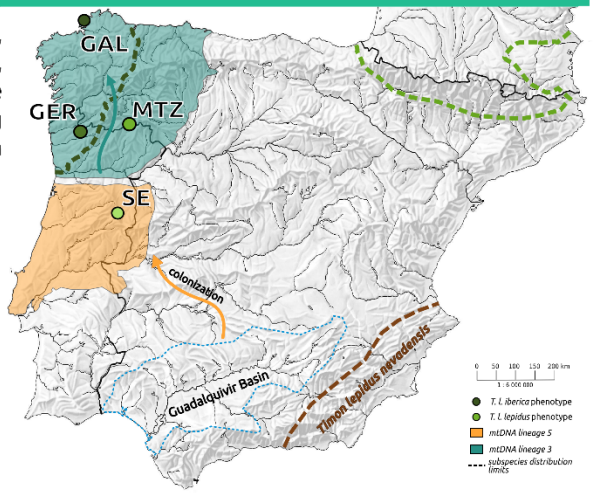
The Eco-Evo History of *Timon lepidus* is shaped by glaciations and interrupted romance!

The Iberian Peninsula is recognized as an important refuge, during ice ages. Here, subsisting subspecies endured processes of range contraction, fragmentation, expansion and admixture, resulting in the ecological and genetic patterns that we see today. *Timon lepidus* persisted during these demanding climatic alterations, diversifying into three parapatric subspecies with morphological variation associated with environmental heterogeneity.



Reflectance was measured along the right flank, both dorsally and in eyespot area.

DNA is extracted from tail tissue to apply RAD-seq technology.

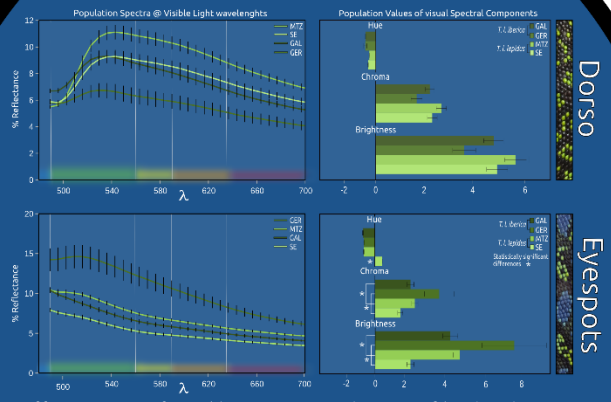


"The four iberian populations studied are GAL (Galiza), GER (Gerês), MTZ (Montesinho) and SE (Serra da Estrela). The first three share mtDNA lineage, and evo history, but SE belongs to a different mtDNA lineage, which results from inter-glaciar colonization from the South-Eastern refuge of Guadalquivir Basin. There, surviving populations would have been diverging in allopatry, during the glacial period."

Phenotype

Colouration deeply influences the life history of an organism, thus impacting individual fitness.

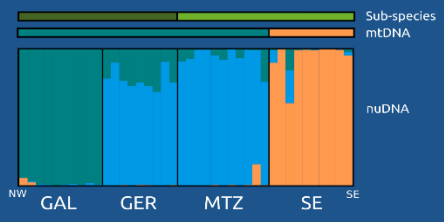
Sexual selection, intraspecific communication, antipredator strategies and thermoregulation are all shaped by colour patterns. Thus, geographically divergent body coloration often appears as adaptive.



Differences are found between populations of both subspecies, in eyespot coloration, that can impact communication.

"Are phenotypic differences promoting subspecies differentiation?"

Genotype



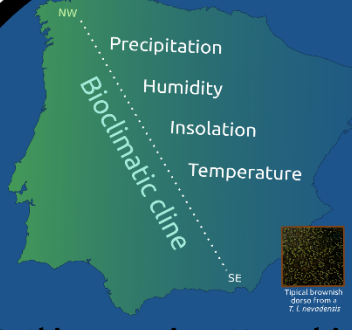
Genomic nuclear data shows discordance with both mtDNA structuring, and phenotype geographic distribution.

MTZ individuals, despite sharing evo history and similar nuDNA pattern with GER, display lower degree of introgression with GAL.

This data suggests a genomic and phenotypic gradient along the bioclimatic cline... feels like Ecological Adaptation!

"Which SNPs are associated with this pattern?"

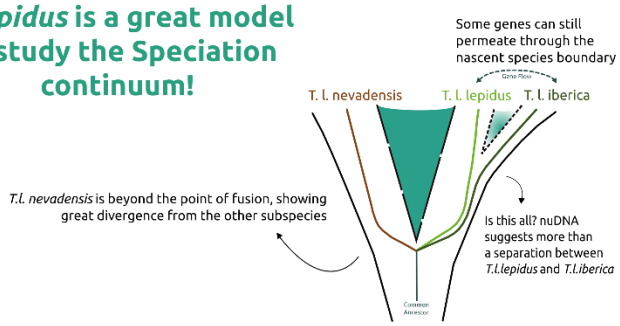
Environment



"Is this genomic pattern driven by environmental pressure?"

Next steps are focused in the understading of the genomic and environmental factors shaping this pattern

T. lepidus is a great model to study the Speciation continuum!




Timon lepidus ibericus, GAL

Whant to know more? For references access this QRcode

Aknowledgments

Thank you to Otília Gato and Patrícia Fernandes for the UniSpec, Marisa X. for her awesome design skills, Zé Conde and Xabi Prieto for sharing amazing lizard spots, and Moises Mallo for the use of Bioruptor.

This work would never be possible without colaboration

ANEXO 3 – TALK ABSTRAT

ENCONTROS SCIENTIA

FCUL, sala 6.2.5, dia 17 Junho às 12h00
Videoconferência na UAc de Angra do Heroísmo e Ponta Delgada (SINF1) às 11h00

Telma Laurentino
Grupo de investigação cE3c: Computational Biology and Population Genomics

Is the tree of life branching? Tales of the speciation continuum by the lizard *Timon lepidus*

Timon lepidus is one of the species that endured the climatic cycles of the Quaternary, which greatly influenced population evolutionary dynamics in the Iberian Peninsula. For this lizard, these phenomena resulted in three parapatric subspecies, nowadays distributed along an ecological cline. Along this cline, phenotypic differences in biometry and colour pattern arise, such that each phenotype occurs in association with a specific bioclimatic region of the species distribution. Coloration and body size can influence mate choice, crypsis and thermoregulation which can translate into dramatic effects on fitness, raising the question of whether we are looking into a speciation continuum, driven by ecology. Understanding speciation is a long-standing goal of Evolutionary Biology and researchers have been excited about the number and type of genetic alterations that underlie evolutionary change. Questions about the modification and origin of traits driven either by ecological factors, natural selection or genetic drift are currently being actively investigated, and Next-Generation-Sequencing brings new tools to the field. Looking to phenotype, genome and ecology, we will search for signatures of selection and divergence in the genome of these beautiful lizards and try to further understand their evolutionary future.

Telma has a degree in Biology in FCUL, and his currently working on her master thesis in Evolution and Development at cE3c. Evolutionary Biology attracted her for its interdisciplinary nature, and that has been her main career focus. A boot, bench and a (little bit of) byte biologist, she's excited with the uprising of Eco-Evo-Devo, since her main interests rely on multidisciplinary approaches to evolutionary questions, with interchange of various levels of biological knowledge.