Introgressive hybridization promoted by climate change: a case study on hares (*Lepus* spp.)

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Resumo

As alterações climáticas, incluindo as causadas pela atividade humana, impõem grandes desafios à biodiversidade, forçando as espécies a movimentarem-se e/ou a adaptarem-se a novos ambientes sob pena de extinção. As oscilações climáticas ocorrem naturalmente ao longo do tempo geológico causando flutuações cíclicas na distribuição das espécies, deixando vestígios na composição genética das populações naturais e moldando o seu potencial adaptativo, determinando também a sua capacidade para enfrentar mudanças ambientais. As atividades antropogénicas contemporâneas têm vindo a provocar um rápido aquecimento global e a destruição de habitats, ameaçando seriamente a persistência de populações naturais e espécies, e assim desafiando a sua capacidade de sobrevivência. Entre os impactos mais relevantes das mudanças climáticas estão as alterações na distribuição das espécies, sejam contrações ou fragmentações de espécies vulneráveis ou eventuais expansões de espécies favorecidas. Estas tendências demográficas contrastantes podem levar à substituição de populações de diferentes espécies e, quando essas espécies ainda são capazes de hibridar, podem promover introgressão da variação genética. A hibridação introgressiva pode ser uma grande ameaça para as populações naturais, introduzindo variação deletéria e/ou mal-adaptativa, mas pode também introduzir variação genética adaptativa e promover rápida adaptação às novas condições. Caracterizar essas potenciais trocas genéticas é, portanto, crucial para avaliar e prever o impacto que as alterações climáticas podem ter nas espécies que invadem e nas espécies que contraem a sua distribuição.

Neste trabalho inspecionamos as interações genéticas entre a lebre Europeia (*Lepus europaeus*), uma espécie clima temperado que tende a expandir pelas alterações climáticas, e a lebre da montanha (*Lepus timidus*), uma espécie ártica/boreal bem adaptada a ambientes frios, que tende a contrair a sua distribuição. O estudo foi desenvolvido em duas zonas de contacto entre estas espécies, na Suécia e Escócia, onde foi descrita uma dinâmica de invasão-contração entre as espécies com hibridação. Este estudo foi baseado num conjunto de amostras de ambas as espécies ao longo das suas distribuições europeias, incluindo populações alopátricas e simpátricas/parapátricas. Utilizando-se um método de representação reduzida, a sequenciação de fragmentos de DNA associado a sítios de restrição (RAD sequencing), para obter um grande conjunto de marcadores genómicos, analisámos a estrutura populacional e a diversidade genética, avaliámos os padrões de troca genética entre as espécies, descrevemos a história demográfica das populações e criámos modelos de

nicho ecológico para ambas as espécies em cada região de contacto. As análises da estrutura populacional (PCA e ADMIXTURE) sugerem que as populações de *L. timidus* são mais fragmentadas na sua área de distribuição do que as populações *de L. europaeus*, o que está de acordo com a história biogeográfica passada e recente de ambas as espécies. Em particular, as populações insulares de ambas as espécies (populações das Ilhas Faroé, Irlanda e Escócia referentes a *L. timidus*, e Escócia a *L. europaeus*) aparecem isoladas das populações continentais, o que é possivelmente um resultado do efeito fundador e isolamento genético. Ao explorar os padrões de hibridação em ambas as regiões de contacto, usando ADMIXTURE e D-statistics, encontrámos resultados por um lado semelhantes e, por outro, contrastantes. Em ambas as zonas de contacto, a análise de índice de híbridos detetou baixos níveis de introgressão, e indivíduos com mistura genética que correspondem a híbridos de gerações antigas, descendentes de retrocruzamentos. Deste modo, nenhuma evidência de mistura extensa em curso foi encontrada. Para além disso, foram encontradas diferenças na direção da introgressão nas diferentes zonas de contacto estudadas. Na Escócia, encontrámos evidências de introgressão bidirecional entre as espécies, enquanto na Suécia só se inferiu introgressão de *L. timidus* para *L. europaeus*. Relativamente aos perfis demográficos, estes revelaram histórias semelhantes para ambas as populações escocesas. Em oposição, na Suécia, enquanto *L. timidus* apresenta um tamanho populacional estável, *L. europaeus* apresentou um padrão decrescente coincidente com um *efeito gargalo*, que deverá resultar da sua introdução recente da Suécia. Em conjunto, os padrões contrastantes de introgressão e histórias demográficas encontrados em ambas as regiões de contacto, sugerem que a dinâmica demográfica é um dos principais determinantes dos padrões de introgressão, ao invés de um comportamento intrínseco das espécies. Assim, a hibridação durante a invasão da Suécia em direção a norte da distribuição de *L. timidus* por *L. europaeus* levou a uma introgressão preferencial em *L. europaeus*, um padrão compatível com introgressão durante uma substituição da distribuição das espécies. Em oposição, na Escócia, a ocorrência de introgressão em ambas as direções poderá ser mantida por um equilíbrio entre migração e seleção, um padrão compatível com a existência de uma zona híbrida possivelmente mais estável. As projeções baseadas na modelação de nicho climático das áreas parapátricas sugeriram que, em geral, a favorabilidade relativa de *L. europaeus* pode tender a aumentar no futuro, promovendo a continuação da sua invasão e substituição da *L. timidus* na Suécia, enquanto na Escócia, pode ocorrer mudanças no equilíbrio das interações das espécies com a invasão de *L. europaeus*. Além disso, embora os níveis de introgressão observados tenham sido bastante reduzidos, a partilha de variantes genéticas entre *L. timidus* e *L. europaeus* via

hibridação introgressiva ocorre tanto na Suécia quanto na Escócia. Esta troca de informação genética pode criar oportunidades para adaptação a partir das variantes introgredidas. De facto, entre as variantes alternativamente fixadas entre as populações parentais de ambas espécies, encontrámos duas que apresentam uma introgressão extensa de *L. europaeus* para *L. timidus* na Escócia, em contraste com os padrões de introgressão reduzidos encontrados nos restantes *loci*. Embora seja prematuro interpretar tal padrão como resultante de uma introgressão adaptativa, este resultado incentiva pesquisas futuras sobre essa possibilidade.

Em geral, este trabalho quantifica de forma robusta a magnitude da troca genética entre *L. timidus* e *L. europaeus* em duas áreas de contacto, sugerindo que a base demográfica das interações é um dos principais determinantes das trocas genéticas. Este trabalho fornece assim as bases para estudos futuros que pretendam entender as mudanças na taxa de introgressão ao longo do século passado, usando amostragem histórica, e para a implementação de simulações demográficas informadas que possam prever resultados futuros da hibridação, utilizando como guia as nossas inferências demográficas e projeções de distribuição futura.

Palavras-chave: Alterações climáticas, substituição de distribuições, demografia, genómica e hibridação introgressiva.

Abstract

Climate changes, including those caused by anthropogenic activities, pose important challenges for biodiversity, forcing species to move, adapt to new environments or go extinct. Climate oscillations occurred naturally throughout the geological timescale causing cyclical fluctuations on species ranges, leaving traces on the genetic composition of extant natural populations, and ultimately shaping their adaptive potential to face environmental changes. Contemporaneous anthropogenic activities are causing rapid global warming and habitat destruction, posing serious threats to the survival of natural populations and whole species and challenging their capacity to survive. Among the most striking impacts of climate change are the important shifts of the distribution of species, either retractions or fragmentations of vulnerable species or eventual expansions of favoured ones. These opposing demographic trends may lead to the replacement of populations from different species and, when these species are still able to hybridize, may cause introgression of genetic variation. Introgressive hybridization can be a major threat to natural populations, introducing deleterious and/or maladaptive variation, but may also introduce adaptive genetic variation and promote rapid adaptation to the new conditions. Characterizing these potential genetic exchanges is therefore crucial to assess and predict the impact of climate change in invading and retracting species.

In this work, we inspected the genetic interactions between the European brown hare (*Lepus europaeus*), an expanding temperate species, and the mountain hare (*Lepus timidus*), a retracting artic/boreal species well adapted to cold environments. The study was developed in two contact zones, in Sweden and Scotland, where invasion-retraction dynamics between the species with hybridization has been described. This study was based on a dataset of samples from both species across its European distributions, comprising allopatric and sympatric/parapatric populations. By using a reduced-representation method (RAD sequencing) to obtain a large set of genomic markers, we analysed population structure and genetic diversity, inspected patterns of genetic exchange, described the demographic history of the populations, and carried out ecological niche modelling for both species in each contact region.

Analysis of population structure (PCA and ADMIXTURE) suggested that the populations of *L. timidus* are more fragmented across their distribution range than the populations of *L. europaeus*, which is in line with the past and recent biogeographic history of both species. In particular, island populations of both species (Faroe, Ireland and Scotland referring to *L. timidus* and Scotland to *L. europaeus*) appear isolated from the continental

populations, which is likely the result of a founder effect and genetic isolation. When exploring the hybridization patterns across both contact regions using ADMIXTURE and D-statistics, we found both similar and contrasting genetic patterns. In both contact zones, the hybrid index detected low levels of introgression, and admixed individuals corresponding to late generation hybrids, descendant of backcrosses. No evidence of extensive ongoing admixture was thus found. Yet, differences in the directionality of introgression were found in the studied contact zones. In Scotland, we found evidence of bidirectional introgression between the species whereas in Sweden we could only infer introgression from *L. timidus* towards *L. europaeus*. Additionally, long-term demographic profiles revealed similar histories for both Scottish populations. In Sweden, whereas *L. timidus* exhibited a stable population size, *L. europaeus* displayed a decreasing pattern coincident with a bottleneck resulting from the colonization of Sweden. Altogether, the contrasting patterns of introgression and demographic histories, found across both contact regions, suggest that the demographic dynamics are a major determinant of introgression patterns, rather than intrinsic behaviour of the species. In Sweden, hybridization occurred during the northwards invasion of the range of *L. timidus* by *L. europaeus* in Sweden, leading to preferential introgression into *L. europaeus*, a pattern expected when hybridization occurs during a range replacement. In Scotland, the inferred pattern of introgression in both directions suggests a possibly more stable hybrid zone in Scotland, with introgression maintained by a balance of migration and selection. Projections based on climatic niche modelling of the parapatric areas suggested overall that the relative favorability of *L. europaeus* will tend to increase in the future, which may lead to the continuation of the invasion and replacement dynamics in Sweden, and to a change of the balance of species interactions towards an invasion of *L. europaeus* in Scotland. Further, even though introgression levels were found to be rather limited, sharing of genetic variants via introgressive hybridization occurs both in Sweden and Scotland between *L. timidus* and *L. europaeus*. This may create opportunities for adaptation from standing introgressed variation. Indeed, among the genetic variants alternatively fixed between parental population of the species, we found that two show extensive introgression from *L. europaeus* to *L. timidus* in Scotland, contrary to the patterns of limited introgression found at the remaining loci. While it is premature to interpret such pattern as resulting from adaptive introgression, this result encourages future research on the matter.

Altogether, this work provided a robust quantification of the magnitude of genetic exchange between *L. timidus* and *L. europaeus* in two contact areas, suggesting that the demographic underpinning of the interactions is a major determinant of genetic

exchanges. It sets the ground for future research to understand changes of introgression rate over the past century using historical sampling, and for the implementation of guided demographic simulations to predict future outcomes of hybridization, guided by our demographic inferences and future range projections.

Keywords: Climate change, range replacement, demography, genomics, introgressive hybridization.

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List of Abbreviations

AFR – France

BART - Bayesian Additive Regression Tree

bp – base-pair

C1orf68 – Chromosome 1 open reading frame 68

CAT – captivity

CRNN – Cornulin

ddRAD-seq - Double-digest restriction site-associated DNA sequencing

DNA – deoxyribonucleic acid

EEMS - Estimated Effective Migration Surfaces

ERG1 – Early Growth Response 1

ERG2 – Early Growth Response 2

F - inbreeding coefficient

FAR – Faroe Island

FIN – Finland

F_{ST} – fixation index

GBIF - Global Biodiversity Information Facilities

GER – Germany

HET – Heterozygous

HOM – Homozygous

HWE – Hardy-Weinberg Equilibrium

IRL – Ireland

IREC- Instituto de Investigación en Recursos Cinegéticos

ITA – Italy

kb – kilo base-pair

KOL - Kolyma River Basin

LAS – Austria

LD – Linkage Disequilibrium

LER – *Lepus europaeus*

LTM – *Lepus timidus*

MAF - Minor allele frequency

MAG - Magdan

mtDNA - mitochondrial DNA

NAB1 - NGFI-A Binding Protein 1

NEMP2 - Nuclear Envelope Integral Membrane Protein 2

NOR – Norway

P1 – population 1

P2 – population 2

PCA – Principal Components Analysis

PCR – Polymerase Chain Reaction

PRI – Primorsky territory

PYR – Pyrenees

qPCR – Quantitative Polymerase Chain Reaction

RAD – Restriction-site-associated DNA

SCO – Scotland

SE - standard errors

SFS – site frequency spectrum

SLZ – Salzburg

SNP - single nucleotide polymorphism

SSH1 – Snowshoe hare

SWE – Sweden

SWI – Switzerland

URA - Urals

1. Introduction

Climate changes have a large influence on biodiversity, forcing species to change their ranges, adapt to new conditions or risk extinction [1]. Natural climatic oscillations have occurred repeatedly along geological timescales but became faster nowadays as a result of anthropogenic activities [2]. Therefore, past and current climatic fluctuations, along with anthropogenic-induced changes, have been shaping species intraspecific genetic structure, as well as the history of interaction between closely related species, frequently leading to hybridization when the interacting species are not completely reproductively isolated [3]. Hybridization leading to exchanges of genetic information between species can have harmful effects, contributing to disrupt local adaptation [4]. Yet, since adaptation driven by *de novo* mutations is a slow process and is often dependent on extant standing genetic variation, the gain of genetic diversity from hybridization may also contribute to new adaptation and allow the persistence of the affected populations [5]. Characterizing genetic diversity of species affected by environmental changes and quantifying levels of introgression when these changes cause replacement between hybridizing species is, therefore, a crucial first step to start dissecting the complex dynamics between the putative maladaptive and adaptive nature of introgression.

1.1. The impact of environmental changes on natural populations

The current distribution of natural populations and patterns of genetic diversity are an intricate result of the evolution of species, which was strongly shaped by environmental changes. Natural climatic oscillations during the Pleistocene are important determinants of the existing genetic diversity, population structure and adaptations of natural populations [6]. In addition, rapid environmental changes caused by human activities, are impacting biodiversity at an unprecedented rate, and impose pressures that can mimic those at deeper evolutionary timescales, such as range changes and extinctions [2]. Yet anthropogenic climate changes are several orders of magnitude faster than the natural climatic oscillations and understanding how these differences affect species and whether adaptation can occur at a similar fast pace is one major endeavour of biodiversity research today.

1.1.1. Pleistocene climatic oscillations and its impact on species distribution

Current patterns of genetic diversity of species across their natural ranges are a result of their evolutionary history, which is intimately related with changes in environment that have modelled biogeographical patterns through time. The inference of common phylogeographic patterns across coexisting species (comparative phylogeography), coupled with the understanding of paleoclimatic changes, has been showing the key role that climate fluctuations have played shaping current patterns of genetic diversity within and across species [6]. During the past ~2.5 million years global climate has oscillated greatly, leading to the recent three major ice ages of the Quaternary, during the Pleistocene [7, 8]. This epoch was mainly characterized by glacial periods, during which polar ice sheets would cover massive extensions of the northern hemisphere, and by temperate periods (interglacial), characterized by the rise of temperature and retraction of ice sheets [8]. These repeated global cooling and warming of the climate led to considerable changes in species distribution but, across the globe, these climate shifts has different magnitudes due to regional variation on topography, latitude, and ocean currents. High latitudes were covered with ice sheets and permafrost, and all living forms on temperate and tropical regions were compressed towards the equator [8]. Tropical forests were reduced, and deserts increased due to heightened aridity. The mountains accumulated ice which led to a significant reduction of the sea level (-120m) [8]. Parts of land as Bering Strait got uncovered and acted as bridges [9]. Blocks of mountains like Alps and Andes, and waters as Mediterranean, served as a barrier to species dispersal [1]. In general, some species went locally extinct over large part of its distribution, some were forced to move to new locations, and some survived in refugia and expanded later [1]. As an example, mid-latitude central Asia during the Pleistocene, an arid and deglaciated region due to the low precipitation levels at that time, served as refugia to some species, which had the opportunity to expand its demographic range during glacial periods [10]. Also, coastal temperate species faced conditions to expand its distribution with the emergence of coastal lowlands during Pleistocene ice ages [11]. In contrast, the cold and dry conditions at the tropics, reducing tropical forests and expanding savannahs, displaced species distributions and caused their contraction into refugia [12]. Hence, species range shifts were specific from the local geography and climate, but they were major and repeated events.

In addition, species' responses to climate oscillations was individual and widely dependent on their traits, environmental tolerances, ability to disperse and to adapt to new habitat conditions [13, 14]. In fact, it has been described that populations from distinct temperate species expanded from diverse southern refugees at the end of the last glacial age, suggesting that species have responded in distinctive ways to new climate conditions [15]. Similarly, it is known that during interglacial periods some cold-adapted species persisted in polar (high latitude) refugia while others were accommodated at lower latitudes, in southern refugia [16]. In any case, the species that have been exposed to Pleistocene climatic oscillations, tend to display signatures of demographic growth and/or decline depending on the period and adaptive traits of the organism [10]. Repeated recolonizations, during and after the glacial periods, were frequently accompanied by founding events, leading to the loss of alleles and increased homozygosity [17]. In fact, many studies have shown that in northern regions of the American and European continents, organisms have lower genetic variation (numbers of species, subspecific divisions and allelic diversity) than southern regions, where ice-age refugia were located [7, 18, 19]. Therefore, climate oscillations induced range changes and left a clear trace on species genomes which reflects in the intraspecific genetic structure that we observe today.

1.1.2. Range replacements induced by climatic changes

Even tough past climatic oscillations had a major impact in the species evolutionary history, the current emissions caused by human activities are responsible for more rapid climate shifts. Anthropogenic climate changes refer to long-term shifts in mean temperatures (increase), rainfall and general weather patterns [20, 21]. The effects of these changes can be observed on the distributions of species as well as in the decreasing levels of biodiversity [21-23]. Several modelling studies that have been carried out predict severe outcomes for biodiversity overall, including a loss of more than half of a species' range for 49% of all insect species, 44% of plants, and 26% of vertebrates [24, 25]. Large-scale shifts in ecosystems are also expected, such as in South America, where many rainforests may convert into savanna or grasslands [25]. Nevertheless, in addition to macro predictions, it is also important to understand the impact of these human induced changes within species. The survival of species through rapid climate changes can depend on the levels of exposure and their capacity to adapt: they might stay relatively untouched if the new conditions fit within their habitat tolerances

and shifts in biotic interactions do not influence them, if they manage to adapt to climate in their current range or if their habitat range shift to track climate conditions [2].

Geographic range changes is a well-reported biologic impact induced by climate changes [26]. Climate warming usually forces species to move upwards (either higher latitudes or altitudes) to track thermal suitable conditions and colonize regions within their metabolic temperature tolerances [27, 28]. In most cases, species establishment is expected to be driven by populations located at the upper periphery of its range [28]. The leading edge of a shifting range will carry only part of the original gene pool (founder effect) inducing overall reductions in genetic diversity and genetic population structure, with regions of low genetic variation alternated with sharp gradients in allele frequencies of specific loci (regions of newly invaded territory) [29]. Therefore, range shifts (including expansions and contractions) modify the distribution of genetic diversity across the range of species [30].

When species move at the same rate and direction, their ranges and interactions would naturally adjust in space and would not increase overlap or interaction pressure [31]. However, species do not experience climate changes equally and so, different responses and shift patterns are expected. For instance, on the one hand, populations of organisms well adapted to cold environments may tend to migrate northward and, in montane environments, upwards contracting its distribution [16]. In fact, changes are greatly felt at high latitudes and altitudes due to the decrease in snow-cover extent and duration, pushing snow-dependent species to higher elevations. On the other hand, temperate species might show little response under climate changes or migrate northward and expand their distribution (depending also on their dispersal abilities) [32]. A similar pattern is expected for specialized and not specialized organisms: the first are expected to be more affected by climate changes than the latter ones, for which fast weather changes can facilitate its expansion [33]. Such dissimilar demographic dynamics might result in species range invasions and overlaps, leading to competitive interactions between species with different characteristics. Sharing a distribution and competing for resources, might result in range replacements if one species exploits for food or shelter resources more effectively [34, 35]. As an example, cold tolerant species can deal better with stressful conditions but are poor competitors and so, may be replaced by more competitive (cold intolerant) species with warming [35]. Thus, the ability to adapt to the new environmental conditions and the intraspecific genetic diversity might be the difference between the persistence of a species or its extinction. Nevertheless, long-term effects of climate changes on species are still poorly understood since most of the

studies carried out so far are mainly projections and simulations, and the few empirical studies performed, often do not consider adaptation [29]. In addition, range shifts may promote hybridization between closely related species involved in an invasion-retraction dynamics, and consequently introgression [36].

1.1.2. Hybridization promoted by range replacements

Natural hybridization can be defined as the interbreeding between individuals/populations from two different lineages [37]. Often, these distinct populations were previously allopatric and meet in a novel overlapping region, resulting in a secondary contact and hybrid zones. This meeting might produce first generation hybrids (F1) and subsequently backcrosses and numerous generations of hybrids leading to introgression [37]. Introgression refers to the exchange and incorporation of genetic variants, from one divergent species into the gene pool of another, via hybridization and backcrossing [38]. However, there are barriers to gene flow, for instance, intrinsic boundaries that might limit assortative mating and/or formation of viable and fertile offspring [39]. Genomic regions associated with speciation phenotypes, i.e., responsible for maintaining reproductive isolation, would tend to remain impermeable to gene flow. These variations of evolutionary histories along the genome shows the semi-permeability of species boundaries, which results in differential introgression, with alleles at some loci being able to cross the boundary, whereas alleles at other loci are not [40]. Hybridization arenas are thus a natural laboratory that demonstrate the porous boundaries of species and how distinct lineages can persist under gene flow [41].

Studies focused on hybridization showed that the rates and extant of introgression vary either among genomic compartments of inheritance (organelle vs nuclear genome) or along the different regions of the same genome [42, 43]. The mitochondrial DNA (mtDNA) appears to be more prone to introgress than the nuclear genome, which often shows little or no nuclear introgression [44-46]. Yet, this may also represent the more frequent study of this single genomic marker, which facilitate detection of introgression. It has been shown that mtDNA introgression is frequently asymmetrical, occurring most of the times from native to colonizing species [44, 47]. The mtDNA maternally transmission associated with population size and unequal production of offspring have been suggested to explain this pattern [48]. In mammals, persistent and asymmetrical mtDNA introgression have been proposed to be connected to male-biased migrations and female philopatry [49]. Selection may also play a role in determining these frequent

mtDNA introgression patterns, but clear evidence for this implication is not widespread [50]. Regarding the nuclear genome, introgression tends to occur differentially across the genome, with alleles from some loci tending to introgress more than others. In principle, the alleles that confer global advantage, either due to the fitness increase of individuals in the alternative environment or positive interaction in the foreign genetic pool, will be easily introgressed [51]. In fact, evidences of adaptive introgression in natural animal populations are now numerous and spread across the whole tree of life, as in plants [52], hares [53], mice [54], mosquitoes [55] or fishes [56]. Alleles associated with reproductive isolation or under divergent directional selection will exhibit little introgression or none at all. Neutral regions are expected to introgress at varying extents, however, linkage to genes that contribute to local adaptation or reproductive isolation will limit its exchange [37, 39]. Therefore, hybridization can produce different patterns of introgression according to region of genome.

There are several types of hybrid zones, being the most frequent illustrated by a tension zone model [57]. In this situation, the frequencies of the various alleles or phenotypic traits forming clines across the contact region are a result of an equilibrium between the effects of dispersal (homogenization) and selection (diversification) [58]. This model is associated with stable hybrid zones, mainly characterized by narrow clines along the transect, concordant clines in genotypic markers and low abundance of hybrids [59]. Early studies of introgressive hybridization between species focused on hybrid zones, have showed that differential demographic dynamics between the hybridizing species also cause asymmetric and heterogeneous patterns of introgression [57]. Circumstances where a species invades the range of a retracting one (range invasion) and the species hybridize in the process have been shown to cause particular patterns of introgression. During range expansions, population sizes are smaller in the invasion front and therefore genetic drift is stronger, leading to more rapid loss or fixation of variants [60]. This process creates clines of allele frequencies in the direction of the expansion, where even rare alleles can become frequent in the invasion front, a process coined “allele surfing” [61]. If the invading species meets and hybridizes with a retracting one, introgressed variants can become frequent as the invasion progresses by a combination of drift and continued hybridization [62]. Such introgression signatures might also be diluted with migration from the resident individuals not affected by introgression, and so, the persistence of introgressed alleles will depend on the migration rates [60]. Hybridization during range replacements is predicted to lead to more frequent introgression from the resident to the invading species, and to produce clines of increased introgression frequencies in the invading species in the direction of the expansion [63]. Given the lower

effective population size of female-inherited mtDNA, such phenomenon may be more pronounced when analysing this genetic marker and explain in part the apparent tendency of mtDNA to introgress. This can be further enhanced by lower migration rates of mtDNA due to female philopatry, which decrease the influx of native haplotypes into the invasion front, and female biased assortative mating [64]. In some cases, throughout time populations on range replacements may eventually become demographically stable and a progressively diffusion of introgressed variants in both directions can be expected [63].

Interspecific gene flow can have numerous potential evolutionary causes and subsequent impacts. Introgression might be promoted by selection enhancing new genetic variation by creating combinations of alleles not present in either parents or by inserting combinations of favoured alleles from one population to another [65]. However, selection can also be a barrier, acting against hybrids and possible maladaptive variants introgressed [65]. In addition, introgression can be induced by species demographic dynamics and lead either to the loss of local adaptive variant and species collapse [66] or in opposition, facilitate colonization of new niches and reduce species range loss [67]. Particularly, in a context of range replacement, the patterns of introgression along the invading species will reflect a balance between the demographic processes of both interbreeding species, the purifying selection (against non-adaptive variants as selfish genetic elements) and selection of adapted genetic variants [65]. Therefore, from a conservation point of a view, hybridization and introgression may either be a major concern that leads to “pollution” of gene pools, or be a source of evolutionary novelty, allowing adaptation at faster rates than *de novo* mutation or standing variation would [5, 42].

1.2. Hares as a model study

Genus *Lepus* spp., which includes hares and jackrabbits, belongs to the order Lagomorpha. The genus currently comprises over 30 species and is distributed all over the world, occurring in contrasting types of habitats, from tundra to deserts [68]. Hares have emerged as models to study the causes and consequences of interspecific hybridization, given the numerous situations of introgression detected among pairs of species, and throughout the genus evolutionary history [69]. Many of the reported cases of introgressive hybridization in the genus involve the mtDNA of mountain hare (*Lepus*

timidus) which is present in more than 10 species, across the Eurasia and North America [70, 71].

This work uses two species of *Lepus*, the mountain hare (*Lepus timidus*), and the European brown hare (*Lepus europaeus*), as model systems. Both species can be found across Eurasia (see figure 1) [72] and have wide distribution areas that are parapatric in many instances [73]. The mountain hare is a boreal-arctic species, well adapted to cold environments and high altitudes. Currently, it is distributed in northern Europe and Asia (high latitudes) but isolated populations can also be found in Ireland, Scotland, Sweden, Alps, among others [74]. Additionally, the distribution of the species is known to have extended further south during the Pleistocene, as attested by the paleontological record [16]. The brown hare is a temperate species that occurs in the majority of Europe, including a broad range of latitudes (from the South of Italy to Scandinavia) [74]. Its presence in northern Europe is a result of gradual expansions by translocations and, potentially, climate change since the 19th century. Brown hares are presently abundant in southern and central Sweden and across Scotland, occurring in sympatry/parapatric with the mountain hare [66]. Furthermore, the two species have been shown to hybridize across their contact zones [32, 75, 76]

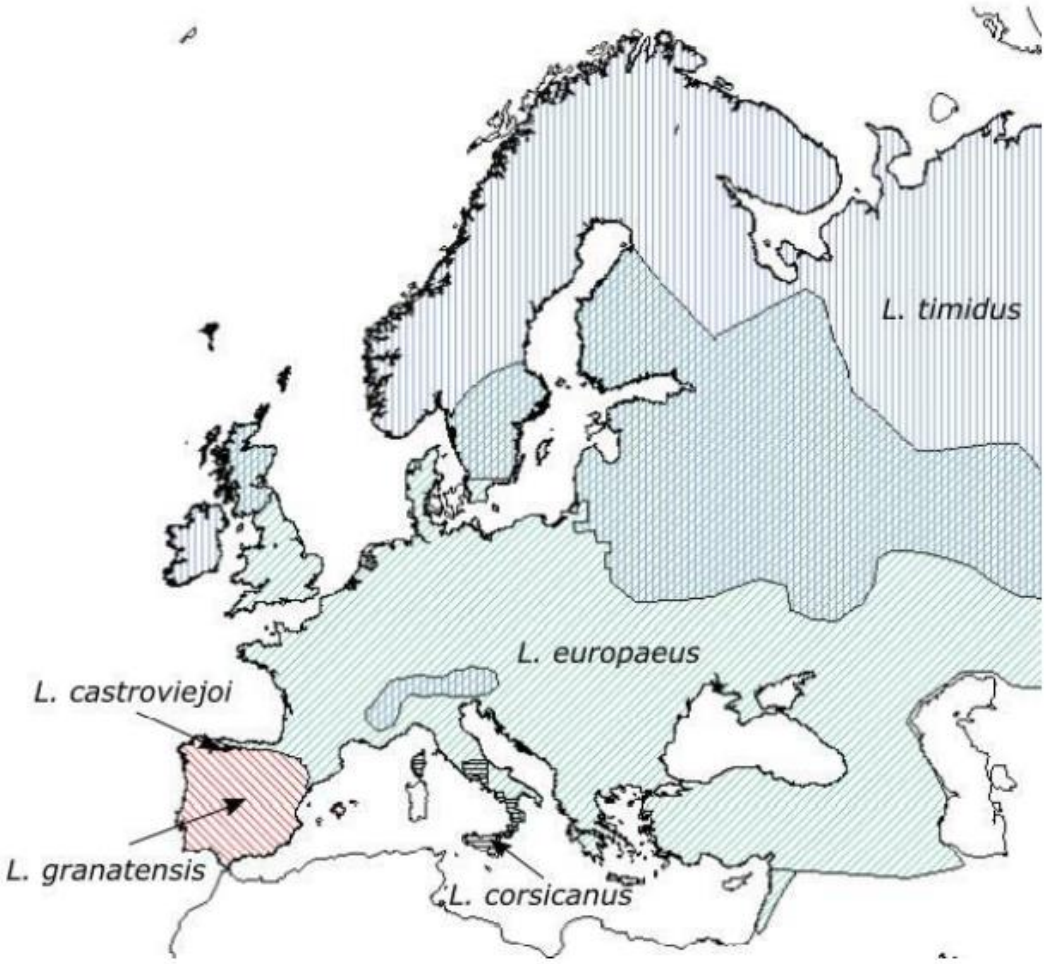

Figure 1: Geographic distribution of European hare species according to Mitchell-Jones et al. (1999) [77].

1.2.1. Contrasting phylogeographic histories of *L. timidus* and *L. europaeus*

Phylogeographic studies have suggested that *L. timidus* and *L. europaeus* had contrasting demographic histories from the Pleistocene to the present. *L. timidus*, an artic species, well adapted to cold conditions, have been suggested to have maintained a large area of distribution south of the ice-rim during the last glaciation, and to have moved northwards as the glacial ice melted, and in altitude in the Alps [16]. According to fossil records, *L. timidus* was present in south of Europe, including southern France and northern Iberian Peninsula [77] and fragmented into allopatric areas in Ireland, Scotland, and the Alps after the last glacial maximum [78]. Therefore, this cold-adapted species was the most common and widely distributed hare species in Europe during Pleistocene [77]. In contrast, *L. europaeus*, is a typical temperate species, and has been suggested

to have invaded western Europe post-glacially from the glacial refugium in the Balkans [79]. These contrasting range change dynamics have likely set the conditions for geographically and temporally moving ranges overlaps, leading to competition between these two species and eventually, hybridization [16]. After the Last Glacial Maximum, both species markedly changed their distributions. *L. timidus* colonized deglaciated areas and spread over northern Europe, to Scandinavia and British Isles [80]. *L. europaeus* also expanded its distribution, towards north and east, replacing *L. granatensis* (and eventually *L. timidus*) towards the Iberia across the Pyrenees, from where *L. timidus* disappeared at the end of the ice age [81]. As the sea rose, populations of *L. timidus* in England were separated from the Irish ones by the Irish Sea [82]. In Sweden, the oldest remains of *L. timidus* were established in the South and are more than 10 000 years old. These populations were probably originated from mountain hares that colonized Scandinavia along the land bridge that temporarily connected Sweden with Denmark [83].

After the glacial period, the formerly continuous distribution of mountain hare in Europe became restricted to few populations above 1300 m in the Alps, in remote forests in Poland, and in Ireland and Scottish Highlands [80]. In Scandinavia, although its current distribution is continuous towards Far East Russia, there are historical and recent evidence of fragmented populations in Southern and Central Sweden [80]. Nowadays this species is rare towards the south of Sweden [84] due to the northwards invasion of *L. europaeus*, which has been introduced in the south of the region in the 19th century [85]. In contrast, while *L. timidus* fragmented its range, the brown hare invaded western Europe, which may have been promoted by post-glacial climate warming and possibly associated with western civilizations and the development of the agricultural landscape [86].

1.2.2. Putative contrasting demographic dynamics promoted by recent climate change

Human-induced climate change is causing higher temperatures, change of precipitation patterns and more frequent extreme weather events, affecting species distributions, physiology and behaviour worldwide [87]. Alpine/artic *species as L. timidus,* are especially vulnerable to climate warming since they have specific adaptations to cold environments and likely lack the ability to adapt quickly enough to ongoing climate

changes [88, 89], such as physiological adaptations (e.g. control of the heat loss), behavioural (e.g. hibernation) and phenotypical (e.g. seasonal change in coloration) [90]. Most of these adaptations are a consequence of changes in the environment during winter months: hibernation is associated with decline of food supply and season moulting, from brown to white, refers to tracking the presence of snow [90]. However, the decrease in extent of winter season and snow coverage coupled with shifts in annual timing of species life history events leads to a mistiming with the environmental conditions [91]. One of the most direct and observable mismatches is the season coat colour: as the ice melt and the snow coverage reduces, the seasonal moulting and changing pelage colour expressed by *L. timidus* might result in a mismatch between pelage colour and background coloration. The disruption of camouflage increases vulnerability to potential predators [92, 93].

In addition, the range of *L. timidus* tends to be forced to move towards higher altitude and latitude regions. However, due to the inherent conic shape of mountains, the suitable area available will progressively become thinner [94]. This is predicted to lead to habitat fragmentation (distance between mountains-top habitats), reduced gene flow between populations, inbreeding within isolated populations and further reduction of genetic diversity [95]. Latitudinally, as the species go northwards, the occupied area is also expected to decrease along with the snow-cover, and stay confined in the northern regions of Europe. This type of range shift has been suggested to cause reductions of population sizes and genetic diversity within populations similarly to what occurred during Pleistocene [8].

On the other hand, fast weather changes can favour non-specialist species, as *L. europaeus*, and promote their expansion, impacting the relative demographic dynamics of the species in the contact zones with *L. timidus* [96]. Along with the climatic conditions, the progressively absence of mountain hare from many regions of its distribution may be a result of the interaction with *L. europaeus* and consequent competitive exclusion [80]. Analysing the distribution of both species it is possible to realise that in places where the species co-occur, *L. timidus* tends to be restricted to high altitudes and deep forests [97]. In fact, when comparing niches of *L. timidus* in allopatric and sympatric situations, it was found that they tend to occupy denser forests and occur farther away from open fields in the former context [98]. In keeping, studies indicate that when in contact with *L. europaeus*, *L timidus* is likely to retract, while the *L. europaeus* expands into *L. timidus* territory and replaces it [80].

The range shifts of these two interbreeding species have resulted in sympatric distributions and contact regions across Europe [75, 99]. In particular, the contact zones between *L. timidus* and *L. europaeus* in Scotland and Sweden have been shown to display the invasion-retraction dynamics between the species [80, 100]. Although *L. timidus* is a native species in both regions, *L. europaeus* has been introduced, at different timeframes, in southern Sweden and Scotland. The origin of *L. europaeus* in Britain/Scotland is still a matter of debate, with scientists suggesting different timings, from the Bronze age (1700–700 BC) to the Roman period (100-400 AD) [101]. However, the fact that it was introduced by humans appears to be consensual. In Sweden, is known that after *L. europaeus* introduction, the species has spread throughout south and central Sweden and into eastern Norway [80]. As an open landscape specialist, its rapid expansion was probably favoured by forest clearings and the subsequent development of agriculture [101].

1.2.3. Asymmetrical hybridization between *Lepus* species

The recurrent gene flow among *Lepus* species has been studied for long. Hybrids are easily promoted in captivity and frequently spotted wherever the two species occur in sympatry [80]. Historical interactions have been reported and the former cases of hybridization between *Lepus* species are thought to have occurred during Pleistocene [64]. Several studies have showed that, in the Iberian Peninsula, the mtDNA of *L. timidus* (a species no longer present in Iberia), can be found in populations of three species: *L. castroviejoi* (broom hare), *L. granatensis* (Iberian hare) and *L. europaeus*. In fact, the frequencies of the introgressed mtDNA in brown hare reached quasi-fixation [64, 71]. More recently, additional cases of introgression have been pointed out in Finland, Alps, and Russia [32, 75, 76, 99, 102].

A study performed in Finland, a contact region created by a natural expansion of *L. europaeus* in the 19th century and a posterior introduction in 20th century, described a strong preference of mtDNA being transferred from *L. timidus* to *L. europaeus* although little levels of introgression were detected in the opposite direction. In addition, the frequency of introgression was found to be higher on the northern expansion front of their distribution range [32]. In the Alps, a region where both species naturally occur, it has been shown that *L. timidus* individuals harbour mitochondrial DNA from *L. europaeus* [99]. However the identification of these individuals was based on a priori pelage and skull traits rather than on a genetic identification and therefore, the species identity of

these individuals may be uncertain [76]. In Russia, a contact region originated by the natural expansion of *L. europaeus* in the 20th century, three individuals were found to present nuclear (microsatellites) and mitochondrial DNA introgression in both directions [75]. Particularly in Sweden, a preferential introgression from *L. timidus* to *L. europaeus* was inferred, both in nuclear and mitochondrial DNA, exhibiting a high frequency of introgression in sympatric regions, especially on the leading edge of brown hare range expansion, and marginal in allopatric situations [102]. Another study found mtDNA introgression exclusively towards *L. europaeus,* displaying higher levels in current areas of sympatry than in areas of former species sympatry [103]. In Scotland, to our knowledge there are no reports from the last century studying the interaction between *L. timidus* and *L. europaeus*. The only study found that refers hybridization is from 1970 and reports that both species do not seem to interact and hybrids obtained in captivity are mainly sterile (1 in 3000 is fertile) [104].

Even though these regions refer to different types of contact regions (natural and semi-natural), they are all a consequence of range expansions. In general, there is evidence of small levels of hybridization which has preferentially *L. timidus* as the donor and *L. europaeus* as the receiver. Bidirectional introgression was found in Russia and Finland, but it was marginal. This asymmetric pattern of introgression appears to be very frequent among this species and could be explained by sex-biased mating (hybridization between female mountain hares and male brown hares occurs spontaneously during captive breeding but not vice versa) and/or demographic dynamics of populations (recent range expansion of *L. europaeus*) [65]. The higher frequencies of introgression inferred in areas of sympatry have been suggested to be related to genomic incompatibilities and reduced fitness of hybrids leading gradually to erase signatures of introgression in allopatry (when there is no continuous interspecific gene flow) [103]. However, this difference of frequencies of introgression may be a result of higher/lower levels of hybridization. Importantly, studies so far have used a limited sampling of the genome, which hampers detailed quantifications of introgression.

1.2.4. Use of reduced representation and genomics to infer species phytogeography history

The emergence of genomics allowed taking a step forward in our understanding of the population genetics and evolutionary processes of wild organisms. In the past, the methods used to identify various genetic markers involved exhaustive and expensive

laboratory work, and more thorough analyses were restricted to model organisms [105]. By joining technologies and genomic concepts with population genetics, the onset of genomics was revolutionary to many areas [106, 107], including evolutionary biology [108]. The modern genomic techniques, including next generation sequencing, whole genome scans and gene-expression pattern analysis allowed the transition from using few neutral markers to genome-wide estimates of functional genetic variation [109]. By comparing the results obtained from tens of microsatellites with results obtained from thousands of genome-wide SNPs in the same set of samples, is possible to realize how biased the perception has been on genetic variation in general and on the relationship with population size in particular [109]. In addition, genomics improved deduction regarding populations demography and evolutionary history. Unlike methods based on a few genetic markers, genomics enables the separation between locus-specific effects (as selection and introgression) from genome-wide effects (as drift and gene flow), which is imperative since the signature of demography and evolutionary history of populations are similarly spread across neutral loci along the genome, whereas loci under selection will often show “outlier” patterns [110, 111].

Reduced representation sequencing has increased over the years due to its ability to study the genetic variation without the need of reference genomes and absence of prior knowledge, at an affordable cost [112]. In particular, restriction-site-associated DNA (RAD), which combines digestion-based techniques with high-throughput sequencing, can produce a large number of single nucleotide polymorphism (SNP) throughout the entire species genome [113]. Along with reducing genome complexity, the use of SNPs for population genetic studies have been shown to improve the resolution of phylogenetic groups, genetic differences among populations as well as to disentangle the neutral and non-neutral processes as drivers of population structure and genetic diversity [114]. Hence, by analysing various loci and their heterogeneity, scientists are able to infer evolutionary processes, relationships, and demographic histories more reliably.

1.3. Main goals

Rapid climatic changes are promoting range expansions and possibly increasing hybridization rates and subsequently introgression between closely related species [115, 116]. In the future, under climate change, an aggravation of the range replacements may occur, which may lead to higher frequencies of introgressed DNA than presently. The changes over the last century could have left traces of this tendency. This thesis

analyses the impact of introgressive hybridization during species range replacements that will be possibly exacerbated by climate change, in the genetic integrity of the interacting species *L. europaeus* and *L. timidus* in two areas of contact, Sweden and Scotland. For this purpose, our main objectives were:

i) Describe the general patterns of genetic diversity and genetic structure of *L. europaeus* and *L. timidus* across its European distribution;

ii) Focus on two replicates (Sweden and Scotland) of presumable ongoing range replacements between the species, and describe their patterns of genetic diversity, genetic structure and demographic history;

iii) Characterize the patterns of hybridization during the range replacements of species with contrasting demographic trends (amount and directionality);

iv) Make predictions of the future demographic trends of *L. europaeus* and *L. timidus* under scenarios of climate change.

2. Methods

2.1. Sampling and data collection

Within the framework of this thesis, a sample set was put together through several international collaborations along with samples from the CIBIO biobank. The sample set comprised samples collected from 1980 to 2000, for a total of 360 samples collected around Europe (Table 1), originally identified as *L. europaeus* (N=113) or *L. timidus* (N=247). The 113 *L. europaeus* samples were originally collected in Germany (N=10), Scotland (N=40), Austria (N=10), France (N=8) and Sweden (45). The 247 samples were originally collected in Ireland (N=20), Finland (N=15), Scotland (N=123), Faroe Islands (N=20), Russia (N=20), Norway (N=3), Sweden (N=26), France (N=5), Italy (N=6), Austria (N=3) and Switzerland (N=6) (Table 1). Samples were collected from organ tissues, blood or muscle and stored individually in ethanol or frozen at -80 °C. The populations in the sample set were classified as parentals, if sampled away from contact regions, while those from Scotland and Sweden were considered contact regions. Austria, Germany, Pyrenees and Russia were considered parental populations in the following analysis, as only one of the focal species inhabits these regions (Austria, Germany and Pyrenees for *L. europaeus* and Russia for *L. timidus*).

Table 1: Species and geographic location of the samples collected in this study.

Species	Population	Abbreviation	Sample size	Total
Lepus europaeus	North Germany	GER	10	113
	Scotland	SCO	40	
	Lassée, Austria	LAS	10	
	Pyrenees	PYR	8	
	Sweden	SWE	45	
Lepus timidus	France	AFR	5	247
	Ireland	IRL	20	
	Scotland	SCO	123	
	Faroe Islands	FAR	20	
	Finland	FIN	15	
	Italy	ITA	6	
	Kolyma river basin, Russia	KOL	4	
	Magdan city, Russia	MAG	3	
	Norway	NOR	3	
	Primorsky territory,Russia	PRI	3	
	Salzburg	SLZ	3	
	Sweden	SWE	26	
	Switzerland	SWI	6	
	Urals, Russia	URA	10	
				360

2.2. DNA Extraction, RAD-Sequencing and Quality control

Genomic DNA was extracted from each sample using the EasySpin Genomic DNA tissue kit (Citomed) and quantified with Qubit® 2.0 fluorometer (Invitrogen). Then, by following a protocol adapted from Peterson et al. (2012) [117], double-digest restriction site-associated DNA sequencing (ddRAD-seq) libraries were prepared. For each individual,

genomic DNA were digested with SbfI and MspI restriction enzymes, purified, quantified, and ligated to barcodes. Samples were pooled based on DNA intactness assessed with gel electrophoresis and DNA concentrations measured with Qubit. Adapters of indexed Illumina sequencing were added by PCR enrichment of ligated fragments with Phusion High-Fidelity DNA Polymerase. After quantification by qPCR, libraries were sequenced on several lanes of an Illumina HiSeq 2500 platform. Each lane contained pools in equimolar ratios, which allowed the sequencing of the same information more than once and various samples at the same time.

The quality of raw Illumina reads was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then demultiplexed and cleaned using the program process-radtags included in the Stacks 2.4 pipeline [118]. The resulting reads were mapped to a hare pseudo-reference [65] using bwa-mem with default settings [119]. The pseudo-reference was built based on the European rabbit (*Oryctolagus cuniculus*) reference genome, which was modified by the replacement of fixed differences to a group of three hare species (*L. americanus*, *L. granatensis* and *L. timidus*) [69]. The European rabbit was selected as reference due to its high-quality chromosome-level assembly and annotation [120] and its relatedness to hares (11.8 million years divergence) [121]. After, SAMtools v1.4 [7] was used to merge and sort reads from different flow cells [122], and read groups were assigned with Picard v1.140 (http://broadinstitute.github.io/picard/). Finally, insertion-deletions (indels) were identified and reads were locally realigned using the RealignerTargetCreator and IndelRealigner functions from GATK v3.4.46 [123].

2.3. SNP calling and Filtering

Initially, ref_map.pl from Stacks 2.4 [118] was run for to assemble loci and estimate population summary statistics for each species, following the filters: 'gstacks: --min-mapq 20 --max-clipped 0.2' and 'populations: -p 1 -r 0.5'. RAD tags containing less than 10 single nucleotide polymorphisms (SNPs) were retained and samples with the final mean coverage per RAD locus lower than 4X were excluded. Multi-sample SNP/genotype calling was carried out using the commands mpileup, call and filter from bcftools v1.4 [124, 125]. Having in mind subsequent analysis, the call was made for three different types of dataset partitions: (a) for the complete dataset (including both species), (b) by species (*L. europaeus* and *L. timidus* separately), and (c) for targeted geographic regions

(Sweden and Scotland). These different datasets allowed us to identify distinct sets of SNPs.

The genotypes from each partition were then filtered using a minimum individual coverage (FMT/DP) of 10 and a minimum genotype quality (FMT/GQ) of 20. All sites failing any of the filtering criteria were coded as missing data. Also, to produce an informative dataset while further minimizing genotyping error, minor allele frequency (MAF) variants occurring only once (singletons) were excluded. The positions were then tested for Hardy Weinberg (HW) equilibrium (*p*-value ≤ 0.05) in the different SNP sets using the function --hwe in PLINK 2.0 [126], in order to understand i) whether sampled populations deviated from HW expectations, and ii) to identify wrongly inferred genotypes due to sequencing or mapping errors. Given that populations across the sampled species are naturally structured, which would cause the inference of deviations to HW equilibrium, tests were done separately in groups of samples representing geographic sampling localities, as proxies of genetic populations. Only sites repeatedly showing deviation of HW proportions across populations were removed from the dataset. Positions were also tested for linkage disequilibrium (LD), to ensure independence across sites, by using --r^2 function in PLINK 2.0 [126] to quantify pairwise LD between all pairs of SNPs with a window size of 50 and a step size of 10. To evaluate which value of r^2 to use, a LD decay with distance was performed by fitting a nonlinear regression curve using the nls package in R [127]. For datasets including both species, these were instead pruned with a physical distance criterion (according to the graph of LD decay), using the flag --bp-space in PLINK 2.0 [126]. Finally, variants with missing data larger than 30% and samples with less than 50% of the positions genotyped were removed.

2.4. Mitochondrial DNA typing

The mitochondrial DNA lineage was determined for all samples from the contact regions (Scotland and Sweden) by amplifying a 669 bp cytochrome *b* fragment using polymerase chain reaction (PCR) and specific primers [128]. The PCR products were then digested with the restriction enzyme AluI and run over a 2% agarose gel electrophoresis to inspect the restriction fragment lengths and respective genotypes. For Scottish samples, we used primers LCYTBF [129] and LCYTBR [128], which after digestion result in three fragments of 394, 181 and 95 bp for *Lepus europaeus* and four fragments of 300, 235, 71 and 63 bp for *Lepus timidus*. For the Swedish samples (more degraded DNA) we used primers Lmtof1 and Lmtnr1 [130] which, after digestion, resulted in two fragments

(of 51 and 145 bp) and in three fragments (of 50, 60 and 100 bp) for *Lepus europaeus* and *Lepus timidus*, respectively

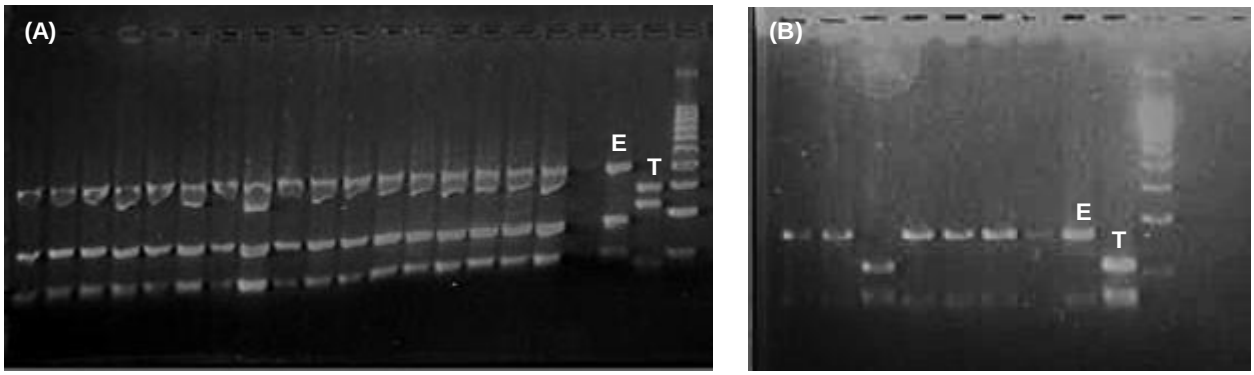

Figure 2: RFLP example of results for the Scottish samples (A) and for the Swedish ones (B). The letters "E" and "T" refer to the expected RFLP pattern of *L. europaeus* and *L. timidus*, respectively.

2.5. Genetic diversity, population structure and demographic profiles

Genetic diversity was assessed for all populations of each species by estimating the observed heterozygosity and the inbreeding coefficient (F) using --sample-counts and --het, respectively, in PLINK 2.0 [126]. Genetic differentiation was quantified by estimating F_{ST} [131] between all pair of populations and within each focal region in BCFtools [124].

An unsupervised population structure analysis was performed using the non-parametric principal component analysis (PCA), as implemented in PLINK 2.0 [126]. Additionally, we ran the software Admixture v.1.3 [132] to perform an ancestral clustering analysis on our dataset and different K numbers of populations were tested depending on the dataset. The best K value was identified according to the estimation of the cross-validation error [132]. The analyses were run both within species and for both species in each focal region (Scotland and Sweden) and based on LD pruned bi-allelic SNPs. PCA and admixture results were plotted with R v.4.2.0 using default functions [127] and with Distruct v.1.1 [133], respectively.

In addition, we explored the spatial structure of both species in each contact region, also based on LD pruned bi-allelic SNPs, using Estimating Effective Migration Surfaces (EEMS) [134], a spatially visualizing approach that correlates effective migration rates to expected genetic dissimilarities. By considering a stepping-stone migration model, EEMS identifies regions where genetic similarity decays quicker than expected for a given geographic distance (low effective migration) and regions where genetic similarity

decays more slowly than expected for a given geographic distance (high effective migration). The method utilizes a triangular grid that covers the studied area. The grid is built using two user-defined parameters, a list of coordinates referring to each sample, to delimitate the geographic area where gene flow will be modelled, and the number of demes, where a deme refers to a vertex in the grid. Then, genomic data from a given sampling location is scattered to the nearest deme, and the model uses the deme locations to estimate and map effective migration surfaces. We also estimated a matrix of genetic dissimilarities between all pairs of individuals of each species with the bed2cliffs method implemented in the EEMS package. For all the analyses we specified a total of 600 demes and performed 5 independent runs with 1×10^6 iterations, a thinning interval of 9999 iterations and $1x10^5$ iterations as burn-in. Finally, to follow the software documentation, migration and diversity parameters were adjusted to model acceptance rates between 10–40%.

To examine the demographic history of *L. europaeus* and *L. timidus*, ANGSD was used to perform multi-sample genotype likelihood estimations, followed by the realSFS module to generate unfolded site frequency spectrum (SFS) [135], based on the bam files of each sample. The historical population size changes were then estimated using Stairway Plot 2.1 [136], a non-parametric method that has proved applicable to reduced representation sequence data and that allows the inference of past population demographic events as expansions, retractions, splits and admixtures. Stairway plots were estimated for each species, in Scotland and Sweden, by assuming a mutation rate of 2.8×10^{-9} mutations per site per generation [137], and a generation time of two years [138]. In addition, to discern region-specific and species-wide demographic profile and inspect potential similarities, we explored the demographic history of parental populations: Lassée (Austria) and Urals (Russia) for *L. europaeus* and *L. timidus*, respectively.

2.6. Genetic relationships among and within *L. europaeus* and *L. timidus*

To address historical relationships and infer potential recent or ancient introgression among the several populations, we used TreeMix v.1.13 [139]. The analysis was performed based on the dataset containing both *L. europaeus* and *L. timidus*. To prepare the input for TreeMix, for each population the allelic frequency and count was estimated at bi-allelic SNPs at least 20K apart by using --freq and --within in PLINK 2.0 [126]. Then,

stratified allele counts output from PLINK were converted into TreeMix format using the plink2treemix.py script included in the software release. Subsequently, we ran the TreeMix model accounting for linkage disequilibrium, by grouping sites in blocks of 100 SNPs (-k 100) and using snowshoe hare (*Lepus americanus*) as the outgroup (root). Standard errors (-SE) and bootstrap values (-bootstrap) were used to assess the confidence in the topology of the inferred tree. In addition, we used the flag -noss to switch off the small sample size correction. After the inference of the best tree topology following a maximum likelihood approach, migration events were added (-m) to investigate the increase/decrease in the likelihood of the TreeMix model following each addition of a migration event. The resulting maximum-likelihood trees and respective residuals matrices were built using TreeMix R script plotting functions.

2.7. Levels and patterns of genetic admixture

In order to evaluate levels and patterns of genetic admixture between *L. timidus* and *L. europaeus*, datasets containing only diagnostic SNPs were used. Given that a strict alternative fixation of allelic variants reduced the collection of retained SNPs substantially, we used a relaxed criterion, considering diagnostic sites those with frequencies ≥ 0.8 in one species and ≤ 0.2 in the other species. A strict alternative fixed sites between the species was also retained.

The Dsuite toolkit [140] was used to test gene flow among the different populations by calculating genome-wide *D*-statistics (ABBA-BABA test) [141, 142] and f4-ratio [143] for all possible combinations, using *Lepus americanus* as the outgroup (O). This analysis was based on a complete dataset (including *L. europaeus* and *L. timidus*), comprising diagnostic SNPs at least 20K apart. Assuming a scenario with three populations possibly related and an additional population as outgroup (((P1,P2),P3),O) and assigning the ancestral allele as A and derived as B, it is predicted that under a scenario without gene flow, the ABBA and BABA patterns should display equal frequencies, due to incomplete lineage sorting [141, 142]. A significant deviation from that expectation is consistent with introgression between P3 and either P1 or P2. A Z-score value equal or greater than 3 was considered to be evidence of significant of gene flow.

Then, to investigate the pattern and direction of introgression between species in each contact region, the hybrid index was estimated using the R package introgress [144]. This analysis was based on datasets referring to each species and comprising positions

with strictly alternatively fixed variants. This index is a composite genotype scaled from 0 to 1, which quantifies the fraction of alleles derived from population 2 (either *L. timidus* or *L. europaeus*). As input, for each contact region, we used genotype data from admixed populations (Scottish and Swedish populations) and from parental populations (German, Pyrenees, and Russia). By measuring the introgression of shared ancestral alleles, it is possible to understand whether the introgression occurred from *L. europaeus* to *L. timidus*, from *L. timidus* to *L. europaeus* or in both directions and to quantify the proportion of allospecific ancestry per analysed individual. In addition, the genomic.clines function from the introgress R package was used to estimate the probability of observing homozygous and heterozygous genotypes as a function of hybrid index [145].

2.8. Inspection of genic content of regions of interest

Given that SNPs were detected with complete introgression in one of the species (see Results), the genomic regions containing these markers were inspected. First, a 2 Kb region containing each SNP of interest was extracted using the function 'getfasta' from bedtools. On the NCBI database (https://www.ncbi.nlm.nih.gov/), the obtained FASTA files were blasted against the rabbit annotated genome (*Oryctolagus cuniculus*). The results from BLAST concerning homologue regions in the rabbit provided information regarding the chromosome and genomic region where the SNPs of interest might be located. Then, we used the ENSEMBL browser (https://www.ensembl.org/) to identify the genes present in this region within a 20kb window.

2.9. Ecological niche modelling

Spatial distribution models for *L. timidus* and *L. europaeus* were built in collaboration with Pelayo Acevedo and Sonia Calvo (Instituto de Investigación en Recursos Cinegéticos IREC (UCLM-CSIC-JCCM), Universidad de Castilla-La Mancha (UCLM)). The presence and absence of *L. timidus* and *L. europaeus* are recorded in the European atlas (50x50grid; Mitchell-Jones et al., 1999) [146]. Moreover, many records are also registered in the Global Biodiversity Information Facilities (GBIF), a live platform where new registers are updated more easily than the European atlas. Data from both species were downloaded using the rgbif package [147] applying a spatial mask of Europe and a filter to use data from uncertainty in spatial location lower than 2000. Then the European atlas presence for each species was updated with the information downloaded

from the GBIF, that is, if a grid showed absence of a species but GBIF information gave presence in that grid, the no presence grid was transformed into presence.

To model the drivers of the probability of presence for each species we used Bayesian Additive Regression Tree (BART) from embarcadero R package (CITA), in which climatic variables and latitude/longitude were used as explicative variables (Table 2) [148]. The BART model developed was retuned using *retune* from the same package to slightly improve the predictive capacity of the model.

Table 2: Climatic variables used to model i) the spatial pattern of wild ruminant abundance and (ii) distribution based on hunting yield and occurrence data, respectively.

Code	***Variable description***	***Code***	***Variable description***
BIO1	Annual mean temperature	BIO12	Annual precipitation
BIO2	Mean diurnal range (mean of monthly (max temp - min temp))	BIO13	Precipitation of wettest month
BIO3	Isothermality (BIO2/BIO7) (x 100)	BIO14	Precipitation of driest month
BIO4	Temperature seasonality (SD x 100)	BIO15	Precipitation seasonality (coefficient of variation)
BIO5	Max temperature of warmest month	BIO16	Precipitation of wettest quarter
BIO6	Min temperature of coldest month	BIO17	Precipitation of driest quarter
BIO7	Temperature annual range (BIO5-BIO6)	BIO18	Precipitation of Warmest Quarter
BIO8	Mean temperature of the Wettest Quarter	BIO19	Precipitation of Coldest Quarter
BIO9	Mean temperature of the Driest Quarter	Latitude	Scaled X coordinate of the centroid of the unit area
BIO10	Mean temperature of warmest quarter	Longitude	Scaled Y coordinate of the centroid of the unit area
BIO11	Mean temperature of coldest quarter		

To assess how future climate change scenarios are expected to affect the favourability of each species across the landscape, we modelled the present climatic niche of *L. timidus* and *L. europaeus* and then projected the model for the future (2061-2080) under two different climate change scenarios: the SSP2-4.5, characterized by an intermediate GG emissions: CO2 emissions around current levels until 2050, then falling but not reaching net zero by 2100, and SSP3-7.0 scenario characterized by high GHG emissions: CO2 emissions double by 2100 [149]. Model result values were transformed from probability into favorability considering the prevalence of each species [150]. Model predictions were projected into a 10x10km grid.

After, the intersection of both species was calculated using sharedFav function from fuzzySim package (CITA), which consists of calculating the minimum value of favorability between both species for each grid. Then, the minimum value is divided in ten classes and the mean favorability value for each species is calculated according to those classes. Species interaction was calculated for all model scenarios (past, current and future climatic conditions). This exploration will allow to predict future changes in the species distributions and parapatry dynamics due to climate change.

2.10. Capture design

To perform further temporal analysis (to compare patterns of genetic diversity, introgression and demography between modern and historical samples) a historical dataset will be used in the future. Samples (skin patches or toe pads) from Swedish populations were collected from the Swedish Museum of Natural History, comprising 22 samples from *L. europaeus* and 123 from *L. timidus*. These samples, corresponding to a time period between the late 19th century and 1940, predictably represent the beginning of the range replacement, and can be used to inspect changes in rate of introgression compared to the present. Given that RAD-Sequencing techniques cannot be used in historical, degraded, DNA, a hybridization capture experiment was designed, selecting SNPs with two criteria: i) variable SNPs within species to assess changes in genetic diversity and structure patterns (polymorphic SNPs), and ii) alternatively fixed or nearly fixed sites between the species (diagnostic SNPs). The list of diagnostic SNPs, created following the same procedure detailed above for the analysis of the patterns of genetic admixture, was created based on the complete dataset (*L. europaeus* and *L. timidus*) comprising all populations except the populations from the contact regions (Scotland and Sweden). The list of polymorphic SNPs was firstly created from two datasets (*L. europaeus* and *L. timidus*) from which we extracted data only from Scottish and Swedish regions. Then, the previous selected diagnostic positions were removed from each dataset and random polymorphic independent sites were selected. The resulting polymorphic SNPs within species were compared between Scotland and Sweden (to maximize the inclusion of informative sites for both populations in the capture). Then, to complete a set of 10k positions for the final targeted capture design, a set of polymorphic positions was randomly chosen from the population dataset per region/species (Scotland, *L. europaeus*; Scotland, *L. timidus*; Sweden, *L. europaeus*; Sweden, *L. timidus*). Overall, seven lists were created comprising 10K SNPs (Table 3)

that passed the quality control procedures from the manufacturer and baits were produced by Arbor Bioscience® (USA).

Table 3: Types of lists and respective number of SNPs used for the capture design.

Type of list	**Number of SNPs**
Diagnostic SNPs	767
Polymorphic *L. europaeus* SNPs	2107
Polymorphic *L. timidus* SNPs	2291
Random SNPs- Scotland, *L. europaeus*	1209
Random SNPs- Scotland, *L. timidus*	1208
Random SNPs- Sweden, *L. europaeus*	1209
Random SNPs- Sweden, *L. timidus*	1209

3. Results

3.1. Sequencing statistics and SNP dataset

We produced RAD-sequencing data for 360 hare specimens, comprising 5 sampled populations of *Lepus europaeus* and 14 populations of *Lepus timidus*. Sequencing efforts resulted in a mean number of RADtags per samples of 103685 and in an average coverage per RADtag per sample of 27.445 X (Table 4). Processed reads were mapped onto a pseudo-reference genome of *Lepus spp.* [65]. To accomplish the research aims of this thesis, we built 9 different datasets comprising species and focal population datasets (Table 4).

Table 4: Summary of the coverage statistics for each dataset resulting from ref_map.pl program, containing the number of individuals, average coverage, and the minimum and maximum values

Dataset	Number of individuals	Average coverage	Minimum value	Maximum value
L. europaeus* and *L. timidus	360	27.445	1.255	109.192
L. europaeus	113	28.145	1.777	109.192
L. timidus	247	27.125	1.255	106.879
Scotland	163	29.853	1.899	55.530
Scotland, *L. europaeus*	40	34.028	8.478	52.928
Scotland, *L. timidus*	123	28.497	1.899	55.530
Sweden	72	21.952	1.379	106.879
Sweden, *L. europaeus*	45	20.979	1.777	92.954
Sweden, *L. timidus*	26	23.636	1.379	106.879

The SNP call carried out for each dataset was first filtered to minimum allele frequency and the singletons were removed. The Hardy-Weinberg filter only detected 17 positions with consistent deviations across populations, which were eliminated. The pattern of linkage disequilibrium (LD) decay within *L. europaeus* and *L. timidus*, quantified based

on 85481 and 169811 SNPs respectively, was similar. LD plots exhibited a rapid decay with r^2 reaching a more stable value by around 1000 kb distance (Figure S1 and S2-Appendix I). Below r^2 values of 0.5 (strong LD), the linkage does not show significant fluctuations, nevertheless we chose a stricter threshold of LD (r^2=0.2). This filter removed several linked positions particularly in the dataset of *L. europaeus* + *L. timidus* and in the dataset of Scotland for both species (Table S1-Appendix I). In addition, samples with more than 50% of SNPs missing were removed. See the Methods for details regarding the filtering criteria. .This resulted in a final dataset partitioned in 9 distinct datasets (as detailed in Table 5). The geographic distribution of samples contributing to the final dataset is shown in Figure 3

Table 5: Description of the number of individuals and positions, for each dataset, before and after the filtering process, including minimum allele frequency, Hardy Weinberg equilibrium, linkage disequilibrium and removal of individuals containing values of missing data greater than 50%):

Dataset	**Number of individuals**		**Number of positions**	
	Before filtering	After filtering	Before filtering	After filtering
L. europaeus* and *L. timidus	360	305	802604	11938
L. europaeus	113	93	669238	24584
L. timidus	247	212	773587	61149
Scotland	163	155	415870	17202
Scotland, *L. europaeus*	40	38	415870	17202
Scotland, *L. timidus*	123	113	415870	17202
Sweden	72	53	575201	9977
Sweden, *L. europaeus*	45	34	575201	9977
Sweden, *L. timidus*	26	19	575201	9977

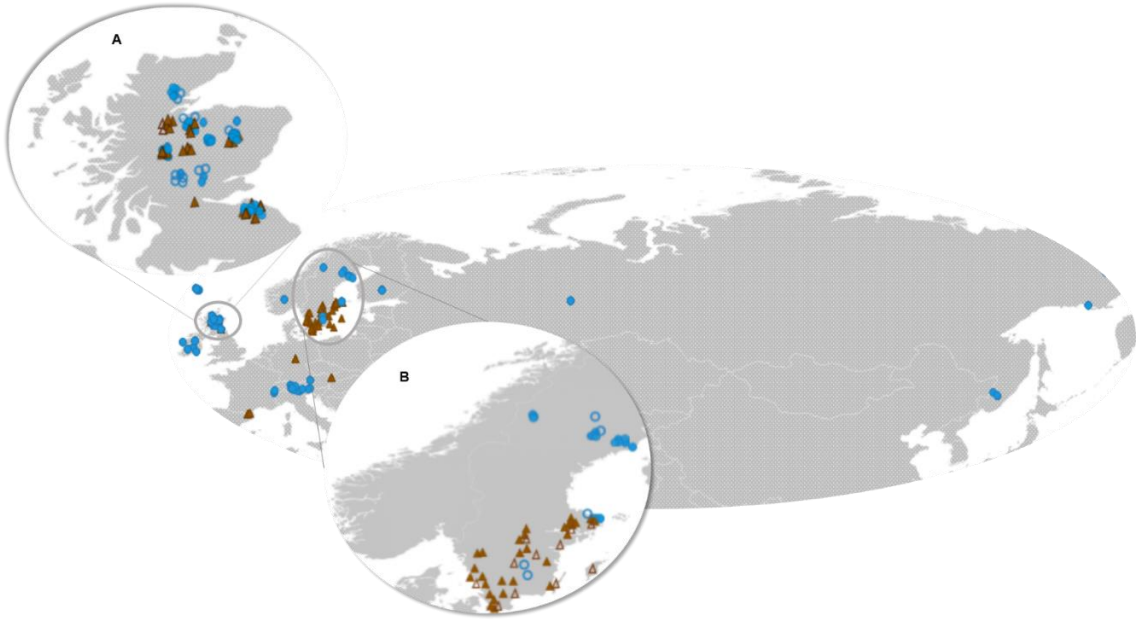

Figure 3: Geographical distribution of all sampled individuals corresponding to *L. timidus* (blue circle) and *L. europaeus* (brown triangle). The map on the background refers to all the samples and the smaller maps to each contact region, Scotland (A) and Sweden (B). The filled and unfilled triangles/circles concern the samples that were kept after the filtering and the samples that were excluded, respectively.

3.2. Broad genetic characterization of *L. europaeus* and *L. timidus* across their range

First, intraspecific analyses were performed using two datasets (one per species) comprising 24,584 and 61,149 SNPs for *L. europaeus* and *L. timidus* respectively. These datasets were used to understand genetic diversity and population structure across the range of both species.

3.2.1. Population structure and genetic diversity of *L. europaeus*

The PCA suggested the existence of two main genetic clusters in *L. europaeus*, with some variance inside each group (Figure 4A). The first principal component (PC1; 9.22% of explained genetic variance) separated the Scottish cluster from all the other populations and the second principal component (PC2; 2.29% of explained genetic variance) split these latter populations. Additional PCA plots are shown in Figure S3 (Appendix II). The analysis of ancestry proportions was conducted with ADMIXTURE

v.1.3 and the convergence of the estimates across the independent runs for each K was only achieved for a K value of 2 (Figure 4B). For K=2, one of the clusters included only individuals from Scotland (SCO), whereas the other corresponded to individuals from Sweden (SWE), Germany (GER), Austria (LAS), and Pyrenees (PYR). Yet, there were minor signals of genetic admixture in the German and Pyrenees populations. This is congruent with PCA results which show that, for PC1, the Pyrenees population is closer to Scottish population than any other continental population. Results for higher values of K are shown in Figure S5 (Appendix II). K=2 was estimated as the best partition according to the cross-validation error (Figure S4- Appendix II). Thus, both analyses suggest that the population of Scotland has a higher genetic distinctiveness in relation to continental populations.

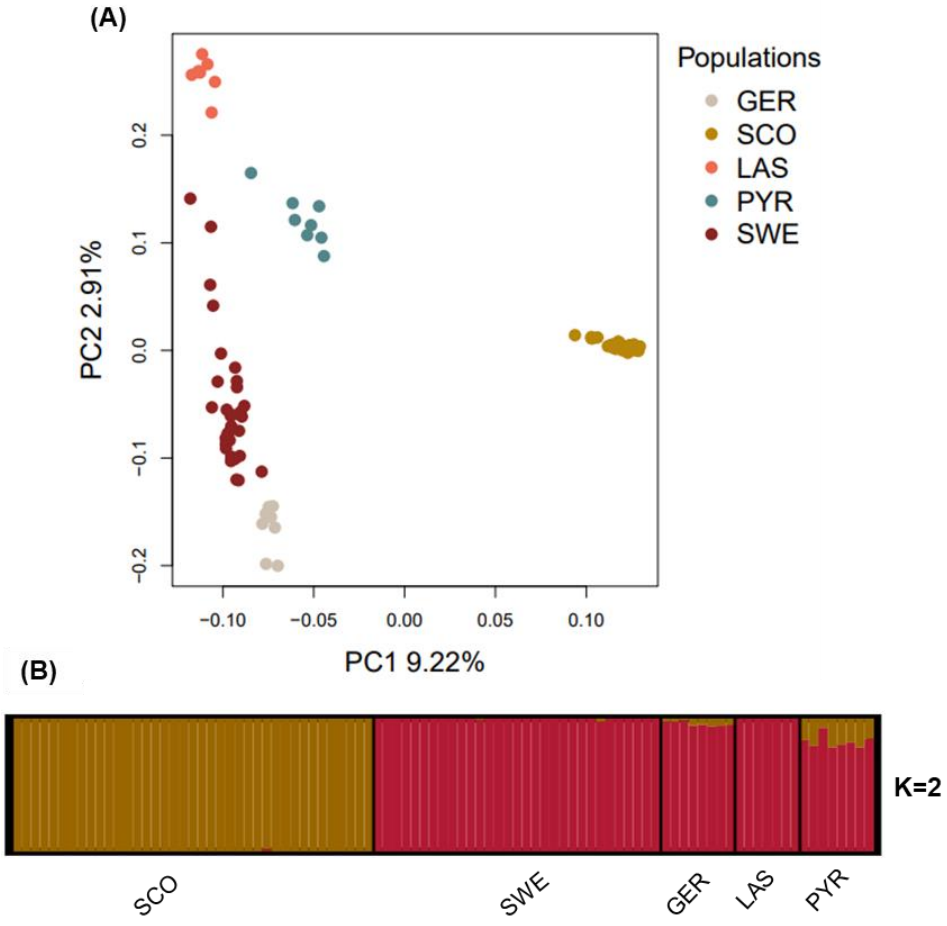

Figure 4: Analysis of population structure for *L. europaeus* with (A) PCA plot containing (B) ADMIXTURE plot of the best supported K (K=2). The analysis includes all the populations of *L. europaeus*: GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

The results of F_{ST} (Figure 5) showed that the highest value of differentiation refers to the pair of SCO-LAS (0.21274) and SCO-GER (0.17578), two parental populations with no evidence of admixture. This value is lower for the pair SCO-PYR (0.16595), an expected result since the clustering analysis (Figure 4) has shown shared genetic diversity between PYR and SCO. The values of differentiation between the Swedish population and the remaining ones (parentals and SCO) are the lowest, ranging from 0.019318 (SWE-GER) to 0.10094 (SWE-SCO). The values of differentiation between the Scottish population and the other populations were the highest values observed (from 0.10094 to 0.21274) whereas the rest of the comparisons where significantly lower (from 0.019318 to 0.098843). These results point the Scottish population as the most genetically differentiated among the *L. europaeus* populations included in this dataset and it is in line with the previous population structure analysis.

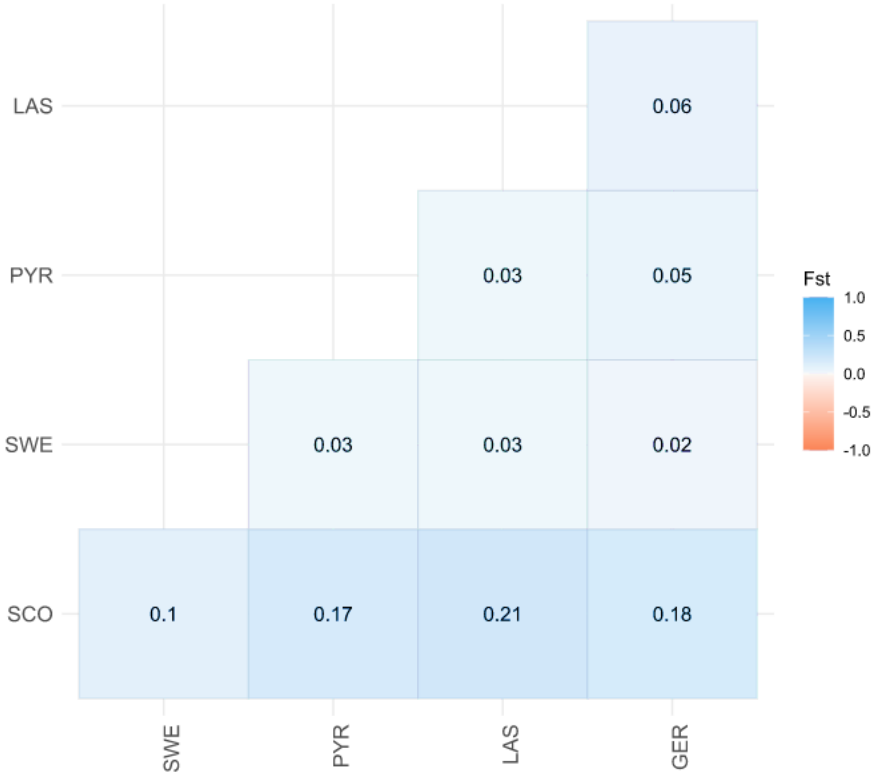

Figure 5: Plot of pairwise values of F_{ST} (averaging across all positions) for all the populations of *L. europaeus*. The analysis includes all the populations from *L. europaeus*: GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

Regarding genetic diversity, the results from the heterozygosity ratios (Table 6) were similar across the populations of *L. europaeus*, with the Scottish population presenting the highest value (1.088191) and the population from the Pyrenees the lowest (1.028328). Further, the Scottish population displayed the highest inbreeding coefficient

(F) (0.397872; see Table 7) whereas the Swedish population showed the lowest (0.001305). Apart from the German population, the parental populations showed excess of heterozygotes (negative values).

Table 6: Heterozygosity ratios (HET/HOM) across the populations of *L. europaeus* with GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

Species	Population	nNonRefHom	nHets	HET/HOM
LER	GER	3917.375	4039.75	1.031239
	SCO	2533.256	2756.667	1.088191
	LAS	3806.286	4019.714	1.056073
	PYR	3965.25	3997	1.008007
	SWE	4023.097	4137.065	1.028328

Table 7: Inbreeding coefficient (F) across the populations of *L. europaeus* with GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

Species	Population	F
LER	GER	0.071084
	SCO	0.397872
	LAS	-0.03042
	PYR	-0.00591
	SWE	0.001305

3.2.2. Population structure and genetic diversity of *L. timidus*

The results from PCA suggested the existence of four independent groups, with little variance inside each (Figure 6A). The first principal component (PC1; 7.98% of explained genetic variance) split the population from Scotland from all other populations, whereas the second principal component (PC2; 3.78% of explained genetic variance) isolates the Irish population (IRL) from the rest. Additional PCA plots are shown in Figure S6 (Appendix II). Regarding the results from ADMIXTURE (Figure 6B), for K=4, the four clusters formed referred to individuals from Faroe, Scotland, Ireland, and the remaining populations. For K=5, the value of K with the lowest cross-validation error (Figure S7-Appendix II), the only difference when compared to K=4 is the additional cluster

composed of individuals within the Scottish population, where there is a separation in two independent clusters. In addition, there are signs of shared variation in individuals from Fennoscandia (Finland, Norway, Sweden), as well as in individuals from Russia (KOL, MAG, URA, PRI) with the Irish population (results for higher values of K are shown in Figure S8 - Appendix II). For K=6 (Figure S8 - Appendix II), the populations from Alps (SLZ, SWI, AFR and ITA) formed an independent cluster.

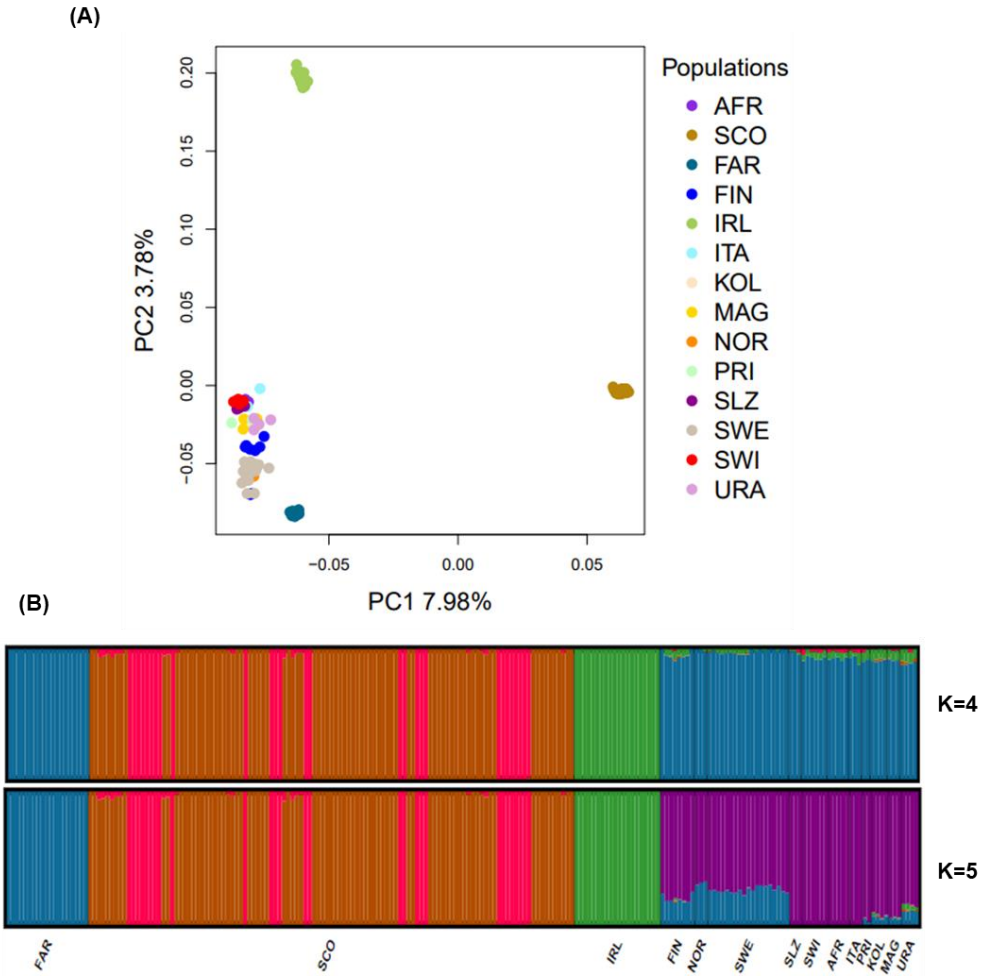

Figure 6: Analysis of population structure for *L. timidus* . (A) PCA plot (B) ADMIXTURE plot for K=4 and K=5 (best supported K). The analysis includes all the populations of *L. timidus*: FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy, PRI to Primorsky territory (Russia), KOL to Kolyma River Basin (Russia), MAG to Magdan (Russia) and URA to Urals (Russia). For the admixture ADMIXTURE plot, populations were grouped based on geographic proximity.

Since we did not detect signs of genetic structure among populations groups from Russia, in posterior analysis of genetic diversity all the populations from Russia (PRI,

MAG, URA and KOL) were concatenated in a single group, in order to increase the sample size. Regarding the F_{ST} results (Figure 7), the highest values were estimated between the Scottish population and the remaining populations (ranging from 0.19255 to 0.31642), whereas the other comparisons were relatively lower (ranging from 0.019318 to 0.098843). The Swedish and Scottish populations showed higher levels of differentiation from the parental populations than between each other.

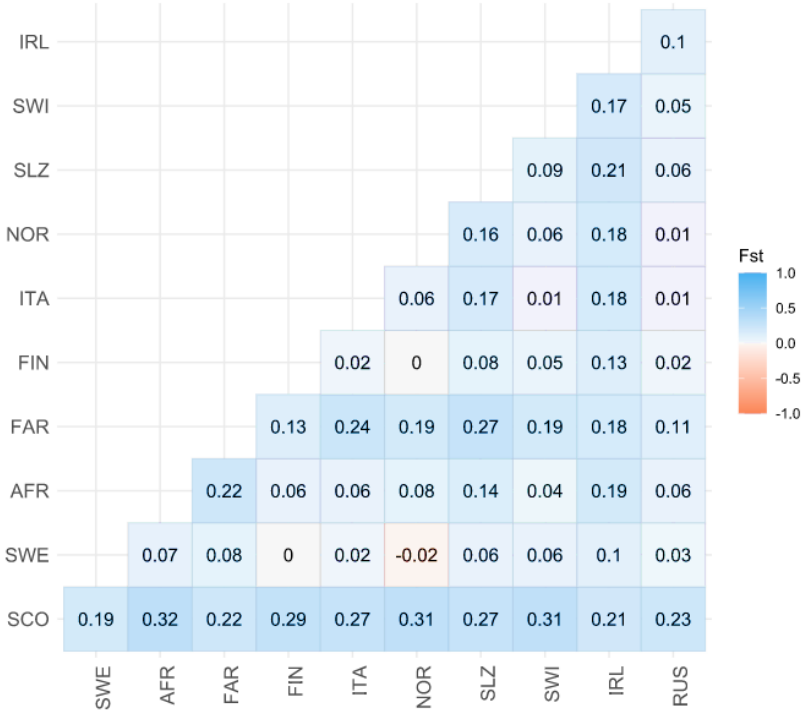

Figure 7: Plot of pairwise values of F_{ST} (averaging across all positions) for all the populations of *L. timidus*. The analysis includes all the populations of *L. timidus*: FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy, PRI to Primorsky territory (Russia), KOL to Kolyma River Basin (Russia), MAG to Magdan (Russia) and URA to Urals (Russia).

The results from the heterozygosity ratio (Table 8) showed similar values among populations, with the Irish population showing the highest value (1.264964) and the Scottish the lowest (1.054686), followed by the Swedish population (1.065587). Regarding the inbreeding coefficient (Table 9), the Scottish population displayed the highest value, while the Swedish population had no evidence of inbreeding. Most of the parental populations showed excess of heterozygotes as well, apart from the populations of Ireland, France, and Faroe.

Table 8: Heterozygosity ratios (HET/HOM) across the populations of *L. timidus* with FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy and RUS to Russia.

Species	Population	nNonRefHom	nHets	HET/HOM
LTM	AFR	6189.6	7413.8	1.1978
	IRL	4977.2	6355.9	1.2770
	SCO	4117.283	4342.442	1.0547
	FAR	4826.421	5962.316	1.2353
	FIN	6623.875	7057.8	1.0655
	ITA	5645.333	6268	1.1103
	NOR	6815.333	7476.333	1.0969
	RUS	26865.75	28862.08	1.0676
	SLZ	6931.667	7591	1.0951
	SWE	6609.947	7043.474	1.0655
	SWI	6992.5	7772.333	1.1115

Table 9: Inbreeding coefficient (F) across the populations of *L. timidus* with FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy and RUS to Russia.

Species	Population	F
LTM	AFR	0.082188
	IRL	0.258607
	SCO	0.380612
	FAR	0.276289
	FIN	-0.05837
	ITA	-0.02746
	NOR	-0.00346
	RUS	-0.06151
	SLZ	-0.05353
	SWE	-0.04665
	SWI	-0.02709

3.3. Genetic relationship between *L. europaeus* and *L. timidus*

To gain deeper insights into the genetic relationship among populations within and between both species, the drift relationships across populations of both species was

inferred based on a total of 11938 putatively independent sites by using the graph-based model implemented in TreeMix v.1.13. In order to increase the population sample size and since there are no signs of population structure among populations, in this analysis we concatenated the populations from France (AFR), Switzerland (SWI), Italy (ITA) and Austria (LAS) in a single group (ALP). Simulations of 0 to 4 migrations events were ran and according to the matrix of residuals there was no improvement from m=3 onwards (Figure 8). The first migration event concerns the gene flow between the Austrian population (LAS) and the root (*L. americanus*) which might suggest an ancient genetic contribution for a non-sampled species. The two additional migration events refer to occurrence of gene flow between the populations from Pyrenees and Scotland of *L. europaeus*, and between the Scottish populations of *L. europaeus* and *L. timidus*. It is noteworthy that the population of *L. europaeus* from Scotland was placed closer to the populations of *L. timidus* in the tree, rather than grouping with the remaining populations of *L. europaeus*. Resulting trees from different migration scenarios (m=1 to m=4) as well as with sample size correction (-noss disabled), are shown in the Appendix III (Figure S9 and S10). It is also important to pinpoint congruence across trees, such as the Scottish and Irish populations from *L. timidus* composing a clade in additional tested models (see Appendix III- Figure S10A and S12A), the split between the Scottish populations of *L. europaeus* and the remaining populations of *L. europaeus* as well as the similar migration events (compare with Appendix III- Figure S9 to S12). These common features along with the low residuals standard error exhibited (3.6 SE), gives support to the relations inferred on the resulting maximum likelihood tree.

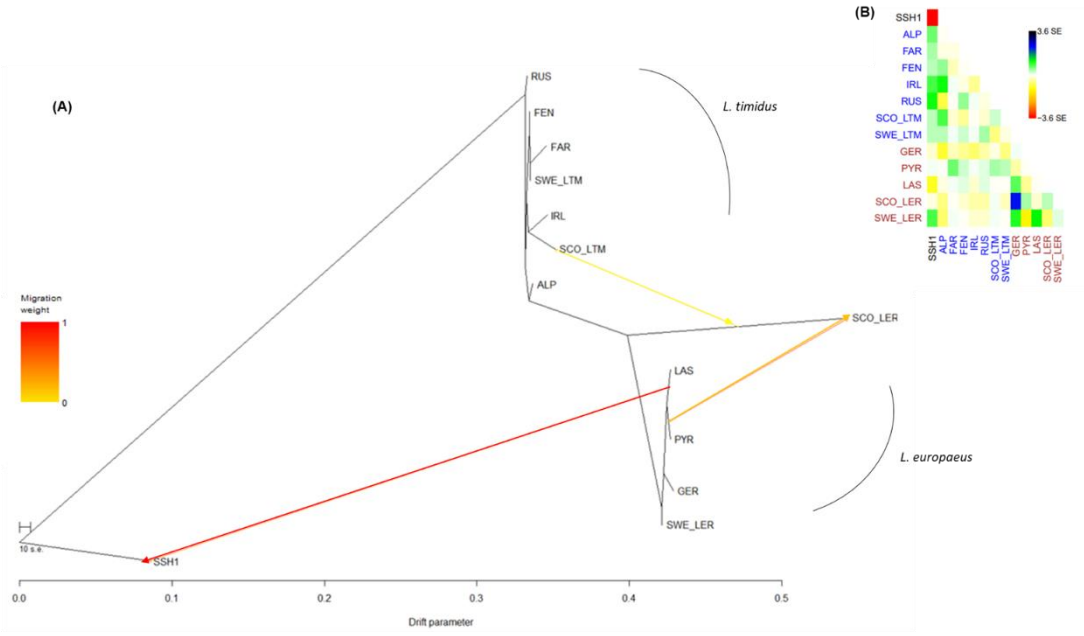

Figure 8: (A) TreeMix phylogram with three migration events and with sample size correction disabled (-noss). Migration

edge was coloured according to migration weight, the per cent ancestry received from the source population. Branch lengths are proportional to the evolutionary change (the drift parameter) and terminal nodes were labelled with population codes and colours; (B) The matrix of residuals refers to the fit of the model to the data.

3.4. Broad genetic characterization of *L. europaeus* and *L. timidus* across the contact regions

Focusing on the contact zones between *L. europaeus* and *L. timidus*, in Scotland and Sweden we inferred the population structure and demographic history of each species in each contact region. Next, interspecific analysis were carried to assess the levels of genetic exchange in Scotland and Sweden. The intraspecific analysis was based on four datasets (Scotland, *L. europaeus*; Scotland, *L. timidus*; Sweden, *L. europaeus*; Sweden, *L. timidus*) whereas interspecific ones rely on two datasets (Scotland and Sweden).

3.4.1. Broad genetic characterization of *L. timidus* and *L. europaeus* in Scotland

3.4.1.1. Population structure of Scottish populations

To investigate the population structure of *L. europaeus* and *L. timidus* in Scotland, we performed PCA and clustering analyses (with ADMIXTURE v.1.3). The spatial structure of both species was further explored with EEMS (Estimated Effective Migration Surfaces). A subset of linkage disequilibrium (LD) pruned 17202 biallelic single nucleotide polymorphisms (SNPs) was used.

For *L. europaeus,* the PCA (Figure 9A) suggested some fragmentation and the existence of small clusters, but both the first and second principal component analysis explained a small percentage of the global variance (6.47% and 4.98%, respectively). Additional PCA plots are shown in Figure S13 (Appendix IV). The results from ADMIXTURE (Figure 9B) points to K=1 as the best inferred K (Figure S15- Appendix IV), suggesting no genetic structure within the population.

For *L. timidus,* although both principal component analyses exhibit a small percentage of explained variance (5.46% and 1.75%, respectively), the first principal component (PC1) split the population into two main clusters potentially corresponding to a geographical division (Figure 9C). Additional PCA plots are shown in Figure S14

(Appendix IV). The analysis from ADMIXTURE, as K=2 the best inferred K (figure S15-Appendix IV), corroborates the existence of two main clusters (Figure 9D).

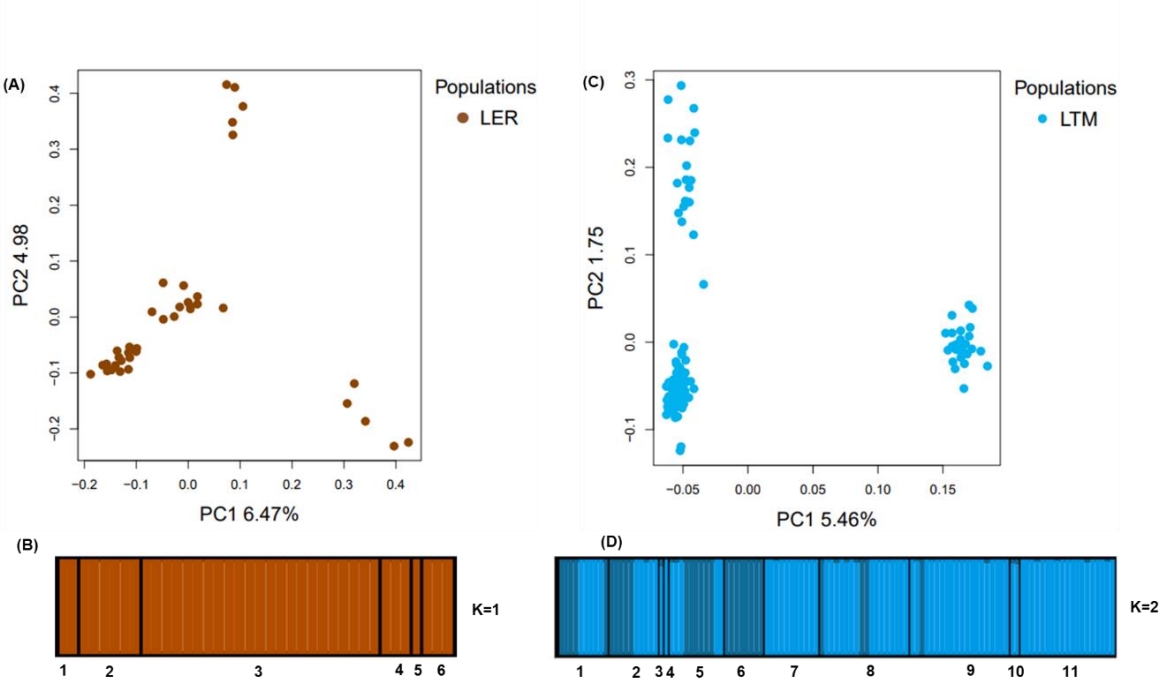

Figure 9: Analysis of population structure for Scottish populations. (A) PCA plot referring to *L. europaeus*. (B) ADMIXTURE plot for best supported K (K=1), containing six populations: 1-Lammermuirs, 2-Aberarder, 3-Moy, 4-Tomatin, 5-Cairngorm Mountains and 6-Strathdon. (C) PCA plot referring to *L. timidus*. (D) ADMIXTURE plot for best supported K (K=2), containing eleven populations: 1-Lammermuirs, 2-Lammermuir Hills, 3-Lynerbrack, 4-Garrogie, 5-Caingorm Mountains, 6-Strathdon, 7-Aberarder, 8-Moy, 9-Tomatin, 10-Edinglassie, 11-Bonar Bridge.

To understand the geographic distribution of the inferred clusters (displayed in figure 9D), we designed a pie chart plot containing the percentage of assignment to each cluster for the individuals of the eleven populations of *L. timidus* (Figure 10). The distribution of each cluster do not seem to be a result of geographic fragmentation. However, populations with 50% or higher number of individuals assigned to the dark blue cluster in Fig. 10 refers to populations located in regions with higher altitude (Lammermuirs, Lammermuir Hills, Cairngorm Mountains and Strathdon).

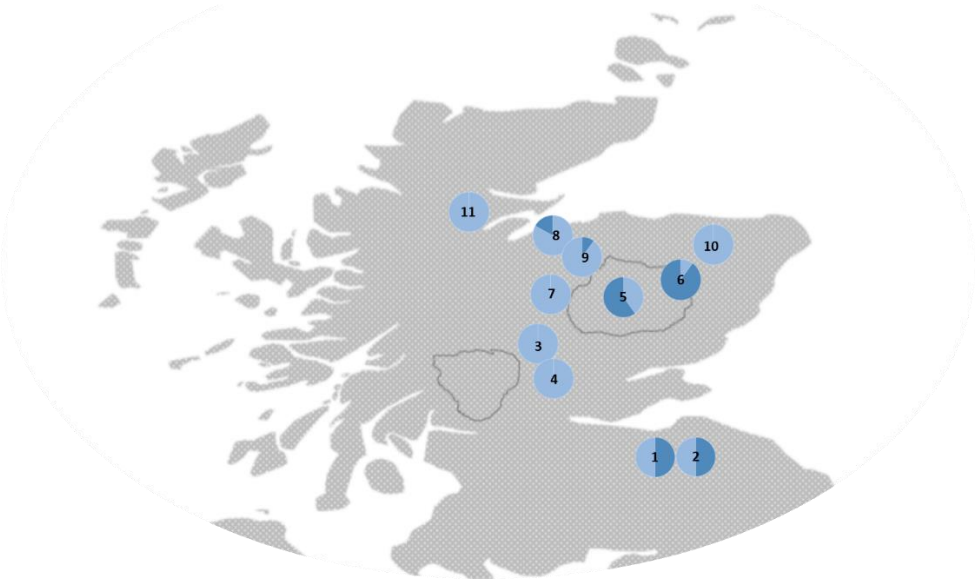

Figure 10: Pie charts containing the percentage of assignment to each cluster distributed on the map according to the geographic location of each population for *L. timidus*. The numbers within pie charts refer to the populations (1-Lammermuirs, 2-Lammermuir Hills, 3-Lynerbrack, 4-Garrogie, 5-Caingorm Mountains, 6-Strathdon, 7-Aberarder, 8-Moy, 9-Tomatin, 10-Edinglassie, 11-Bonar Bridge). The regions delimited in grey refers to Loch Lomond & The Trossachs National Park (on the left) and the Cairngorms National Park (on the right)

Then, we explored the genetic structure pattern by using the spatially visualizer EEMS. Regarding *L. europaeus* diagnostic plots for model fitting (Figure S16 – Appendix IV) showed that EEMS results present a very good fitting with the data concerning dissimilarities within demes ($r^2 = 0.85$) but a weaker fit was when considering dissimilarities between demes ($r^2 = 0.446$). In addition, the absence of correlation between geographic distance and genetic distance indicates that a model of isolation by distance cannot explain the population structure of *L. europaeus* observed in Scotland. Dissimilar patters were inferred in the North/Centre of Scotland and in the South (Figure 11A): there was evidence of greater population fragmentation in the North/Centre while in the South of Scotland, a region of high effective migration rates was inferred, suggesting high genetic similarity of the individuals within this region.

Regarding *L. timidus*, EEMS diagnostic plots for model fitting (Figure S17- Appendix IV) exhibited a very good fitting both with the data regarding dissimilarities within demes ($r^2 = 0.878$) and between demes ($r^2 = 0.787$). In addition, the lack of correlation between geographic distance and genetic distance suggests that the estimated population structure of *L. timidus* in Scotland cannot be explained by a model of isolation by distance. The results in Figure 11B indicate the existence of two regions with great effective migration rate, on the north and South of Scotland, separated by a barrier to gene flow extending from the east coast to the west coast of Scotland

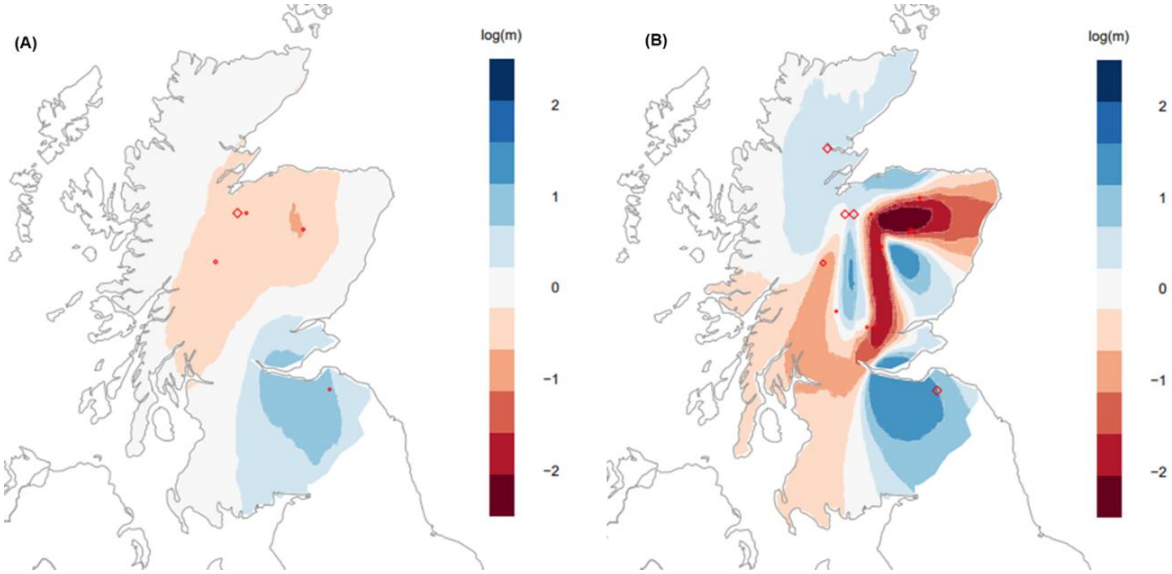

Figure 11: Effective Migration Maps estimated for the Scottish populations of (A) *L. europaeus* and (B) *L. timidus*. The plots were estimated with EEMS under a log10 scale and after mean centring. The blue indicates areas with an effective migration between populations above average (more genetic similarity) and the red regions with an effective migration below average (less genetic similarity).

3.4.1.2. Demographic history of Scottish populations

Since demographic factors are critical to understand species responses to climate oscillations, we investigated the long-term demographic profiles of *L. europaeus* and *L. timidus* in the contact zone in Scotland using stairway plots. The demographic history of parental populations was also explored for further comparisons. The plots (Figure 12) suggested broadly similar demographic histories in both species, detecting 1) a bottleneck ~100'000 years ago, 2) a recovery of the effective population size of the population, 3) another bottleneck ~20'000 years ago, and 4) great increase of effective population size resulting in the recent populations. These bottlenecks were similar in magnitude across populations. The Stairway plots concerning the parental populations (Figure S18- Appendix IV) suggest that, apart from the first bottleneck ~100'000 years ago (also detected here), both effective populations size have been declining until the present, a contrast pattern when compared to Scottish populations.

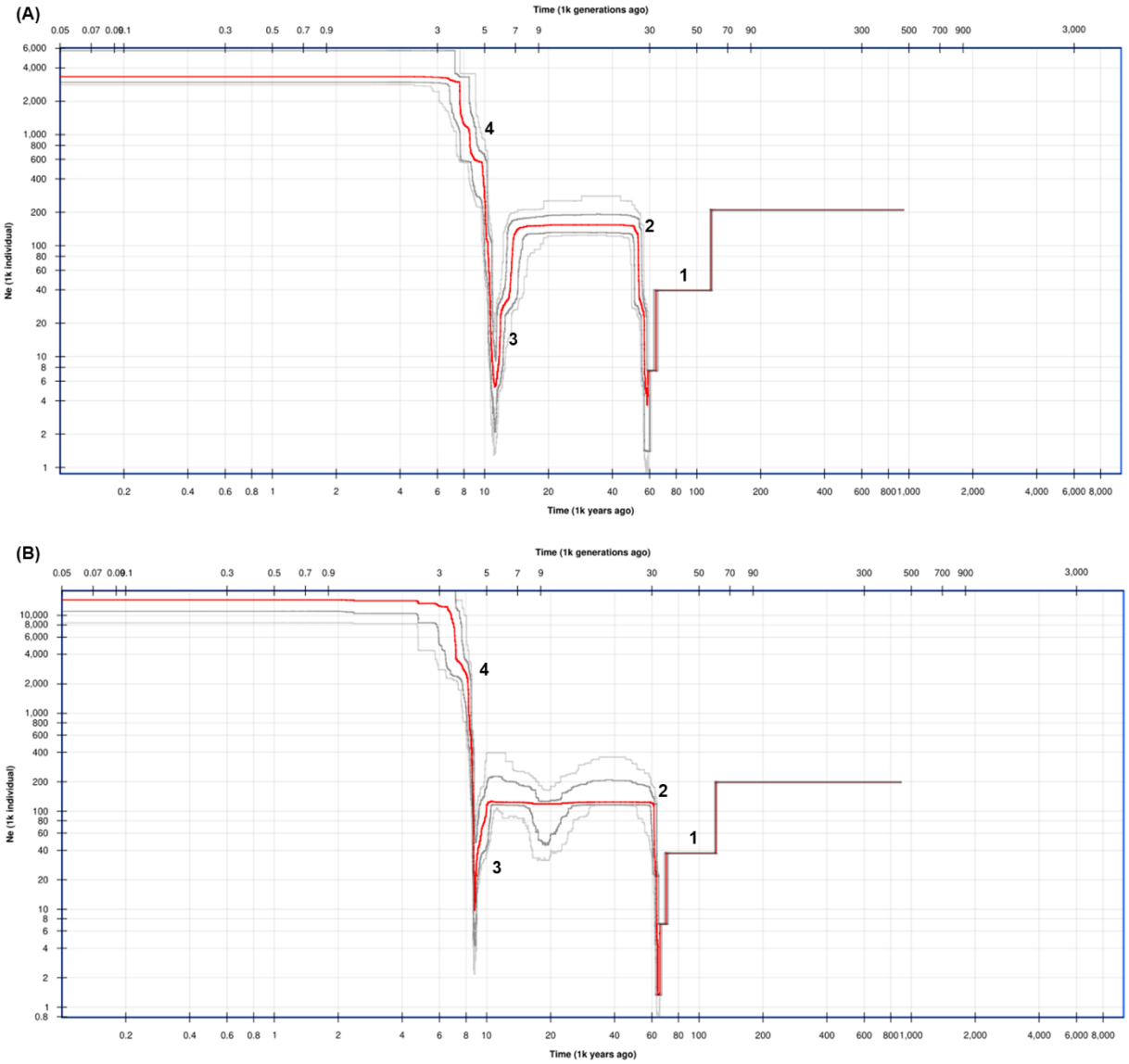

Figure 12: Stairway plot reconstructions of demographic history of *L. europaeus* (A) and *L. timidus* (B) in Scotland. The x-axis is the time in thousand years (lower scale bar) and in thousand generations (upper scale bar) before present. The y-axis is the effective population size in thousand individuals. The red line shows median of effective population size. The upper and lower thick and light gray lines show 75% and 95% confidence intervals, respectively.

3.4.1.3. Levels of genetic exchange across Scottish populations

MtDNA genotyping was performed to inspect the haplotypes harboured by the individuals of both species in Scotland. In Table 10, the results show low levels of introgression of mtDNA, with *L. timidus* displaying a higher percentage of individuals introgressed (7.5%) when compared to *L. europaeus* (2.6%).

Table 10: Number of individuals of *L. europaeus* and *L. timidus* showing mitochondrial DNA introgression in Scotland.

Region	Species	Introgressed	Non-introgressed
Scotland	*L. europaeus*	1	38
	L. timidus	9	111

Then, to investigate the patterns of introgression between *L. timidus* and *L. europaeus* in Scotland, PCAs and ancestral clustering analysis were conducted, followed by the estimation of gene flow using genome-wide *D*-statistics (ABBA-BABA test) and hybrid index. To do so, we used a dataset of 647 SNPs found to be alternatively fixed or nearly fixed between the parental population of the species (see Methods for a detailed description of the criteria).

The first principal component (PC1; 77.95% of explained genetic variance) strongly splits both species, with no evidence of individuals in an intermediate position (Figure 13A). Additional PCA plots are shown in Figure S19 (Appendix IV). The analysis of ancestry with ADMIXTURE (Figure 13B) shows that for K=2, each cluster refers to each species. In addition, there is marginal evidence of admixture, especially in *L. europaeus* from *L. timidus* (see Figure S21- Appendix IV for extra ancestral components (K=2 and K=3) and its respective cross-validation error support in Figure S20- Appendix IV).

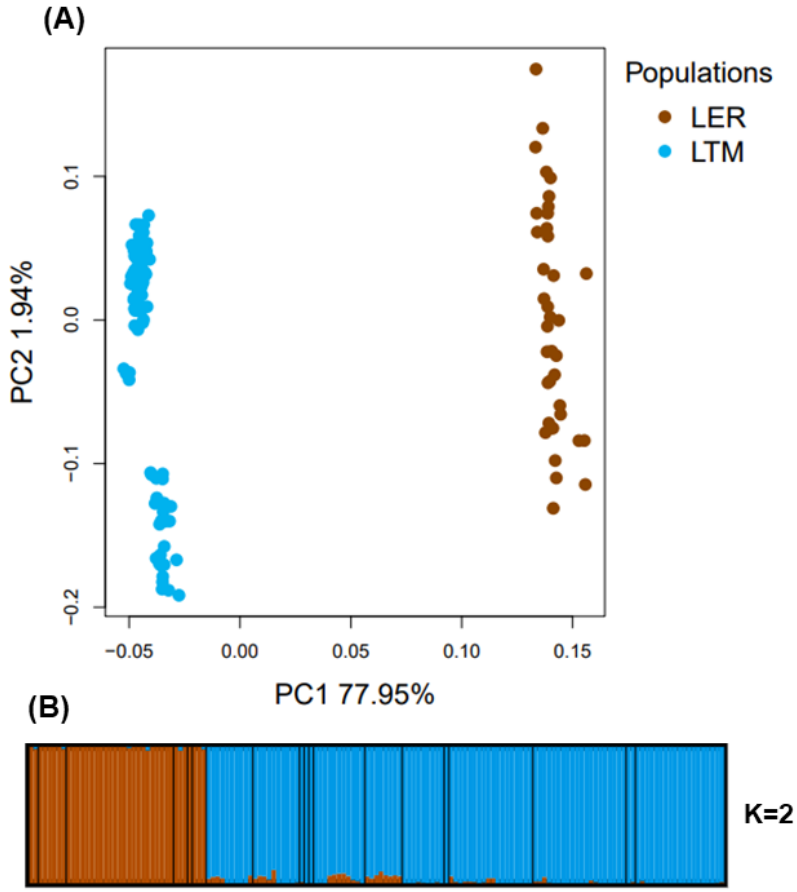

Figure 13: Analysis of genetic exchange for Scottish populations with PCA (A) and (B) ADMIXTURE plots containing *L. europaeus* (brown) and *L. timidus* (blue). The analysis includes six populations of *L. europaeus* (Lammermuirs, Aberarder, Moy, Tomatin, Cairngorm Mountains and Strathdon) and thirteen of *L. timidus* (Lammermuirs, Lammermuir Hills, Lynerbrack, Lochindorb Garrogie, Caingorm Mountains, Strathdon, Aberarder, Tulchan, Moy, Tomatin, Edinglassie, Bonar Bridge.

To better explore the signs of admixture suggested by the previous analysis, we conducted ABBA-BABA tests, assuming P1 as the parental populations, P2 as putative admixed populations, P3 as putative donor populations and O as outgroup (*Lepus americanus*). The results in Table 9 show a significant excess of shared derived alleles between the Scottish populations of *L. timidus* and *L. europaeus* (bold values). While the proportion of introgression is low (the f4-ratio varies between 4% and 7%), there is evidence of bidirectional introgression, i.e., from *L. timidus* towards *L. europaeus* and vice-versa. These results are in line with the pattern inferred in the ADMIXTURE plot (Figure 13B).

Table 11: Results from ABBA and BABA's test and f4-ratio tests between Scottish populations of *L. europaeus* (SCO_LER, brown background) and *L. timidus* (SCO_LTM, blue background), with significance values (p-value). The parental populations (brown background) of *L. europaeus* includes Germany (GER) and PYR (Pyrenees) whereas parental populations of *L. timidus* (blue background) comprise all the Russian populations (Kolyma River Basin (KOL), MAG to Magdan (MAG), Primorsky territory (PRI) and Urals (URA). Values of Z-score > 3 suggests significant excess of shared derived alleles because. The values in bold refers to Z-scores >3 with a respective p-value ≤ 0.05.

P1	P2	P3	Dstatistic	Z-score	p-value	f4-ratio	BBAA	ABBA	BABA
KOL	SCO_LTM	GER	0.556271	***10.2013***	0	0.052622	295.929	23.489	6.69727
MAG	SCO_LTM	GER	0.584137	***11.5104***	0	0.053558	294.206	23.1538	6.07825
PRI	SCO_LTM	GER	0.408657	***5.86957***	2.18E-09	0.042983	284.998	23.347	9.80088
URA	SCO_LTM	GER	0.561599	***11.5071***	0	0.052535	296.361	23.2302	6.52162
KOL	SCO_LTM	PYR	0.541614	***9.69256***	0	0.051058	293.711	22.7442	6.76281
MAG	SCO_LTM	PYR	0.570716	***12.6576***	0	0.052886	292.439	22.8611	6.24804
PRI	SCO_LTM	PYR	0.399298	***5.41483***	3.07E-08	0.041106	282.429	22.4101	9.62037
URA	SCO_LTM	PYR	0.557662	***11.523***	0	0.050839	294.167	22.5039	6.39056
GER	SCO_LER	KOL	0.43886	***12.3575***	0	0.070485	314.395	35.1765	13.7185
GER	SCO_LER	MAG	0.428649	***10.2057***	0	0.067283	313.191	34.625	13.8474
GER	SCO_LER	PRI	0.4271	***11.5949***	0	0.071516	309.612	34.3412	13.7861
GER	SCO_LER	URA	0.446364	***10.3229***	0	0.071048	314.231	35.5185	13.5957
PYR	SCO_LER	KOL	0.413612	***9.261***	0	0.064439	309.262	33.525	13.9067
PYR	SCO_LER	MAG	0.413189	***8.67316***	2.11E-18	0.063317	308.122	32.9798	13.6945
PYR	SCO_LER	PRI	0.393576	***8.751***	1.08E-18	0.065518	304.778	32.9491	14.338
PYR	SCO_LER	URA	0.425189	***8.67705***	2.01E-18	0.06678	309.26	34.0287	13.7246

Next, we applied another method to evaluate the amount and direction of introgression, by calculating the hybrid index, which quantifies the fraction of alleles derived from a donor population (either *L. europaeus* or *L. timidus*). Here, the analysis was based in 81 positions, a more restricted dataset of diagnostic SNPs only retaining strictly (100% frequency) alternatively fixed alleles. In keeping with the results from PCA and ADMIXTURE, the plots obtained (Figure 14A) suggest no presence of early generation hybrids and minor levels of introgression were detected in both directions (hybrid index ranging from 1 to 0.83 in *L. timidus* and from 0.17 to 0 in *L. europaeus*). Despite the low levels of introgression, two SNPs derived from *L. europaeus* exhibit particularly high levels of introgression in *L. timidus*. The plot correlating the hybrid index with the interspecific heterozygosity (Figure 14B) places the individuals referring to *L. europaeus* and *L. timidus* on the left and right side of the triangle, respectively, according to its hybrid index values. Both sides display similar levels of interspecific heterozygosity, likely reflecting the identical levels of introgression in each population. Also, the fact that most individuals are placed on the line of theoretical maximum values of heterozygosity,

especially the individuals of *L. timidus*, suggests that most part of the Scottish population are descendants of backcrosses.

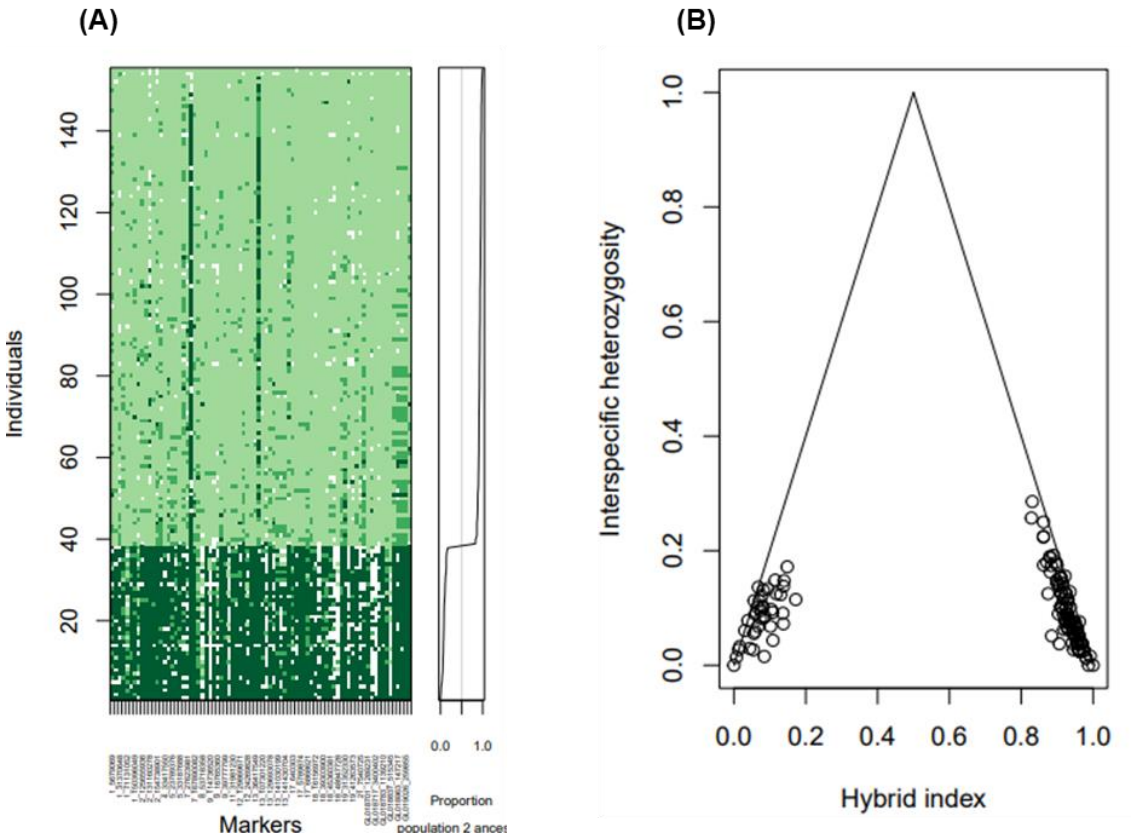

Figure 14: (A) Plot of hybrid index for the populations of Scotland: the light green refers to *L. timidus* genotypes, the dark green to *L. europaeus* genotypes, the intermediate green refers to an heterozygotic genotype and the white to missing data. Down the plot there are the 81 markers used and on the right is a graph referring to the proportion of alleles attributed to *L. timidus*. (B) Plot correlating interspecific heterozygosity with hybrid index, in which the lines correspond to theoretical maximum values of heterozygosity and individuals that fall in the maximum line are probably F1s or descendants of backcrosses.

Considering that fixed (or nearly fixed) introgressed genomic portions in *L. timidus* would present a much higher number of genotypes associated with the *L. europaeus* rather than either genotypes of *L. timidus* or heterozygous, two SNPs were found to be completely introgressed from *L. europaeus* into *L. timidus* in Scotland (Figure 14A). The two highly introgressed loci (Figure 15) are located in chromosome 7:131009004 and chromosome 13:39808821. Using a window of 40kb, we were not able to identify any gene in the annotated genome of the rabbit, which may be due to the incomplete annotation of the rabbit genome currently available. However, we searched for the flanking genes of the regions, considering that in the genome of the hares these may be located within the inspected window. Hence, in chromosome 7 we identified NEMP2, an active gene in nuclear envelope and NAB1 which acts as a transcriptional repressor for

zinc finger transcription factors EGR1 and EGR2. In chromosome 11 we detected C1orf68, a gene associated with keratinization with restricted expression in stomach adult and CRNN which is involved in the positive regulation of keratinocyte proliferation and is expressed in epidermis, esophagus epithelium and nails.

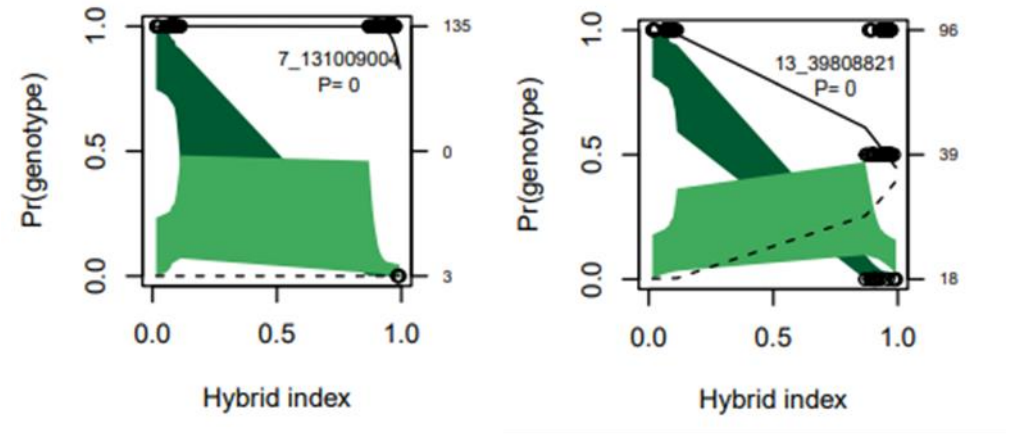

Figure 15: Genomic cline plots for two markers from Scottish populations. The name of each locus (7_131009004 and 13_39808821) and P-value from the analysis are given in each plot. Considering *L. europaeus* as P1 and *L. timidus* as P2, the solid-coloured regions represent the 95% confidence intervals for the P1/P1 (dark green) and P1/P2 (light green) genotypes. The solid line and dashed lines denote the genomic clines for the P1/P1 and P1/P2 genotypes respectively. Circles indicate the raw genotype data (P1/P1 on the top line, P1/P2 in the middle and P2/P2 on the bottom line), with counts of individuals on the right vertical axis. The hybrid index quantifies the fraction of alleles derived from *L. timidus*.

3.4.2. Broad genetic characterization of *L. europaeus* and *L. timidus* in Sweden

3.4.2.1. Population structure of Swedish hare populations

To investigate the population structure of *L. europaeus* and *L. timidus* in Sweden, a subset of LD-pruned 9977 biallelic single nucleotide polymorphisms (SNPs) was used. For both species the results from PCAs (Figure 16, A and C) shows no evidence of population structure and some dispersion among the Swedish population, since neither the first nor the second principal component (PC1: 4.54% of explained genetic variance; PC2: 3.89% of explained genetic variance) separates the individuals in clear groups. Additional PCA plots are shown in Figure S22 and S23 (Appendix IV). The ADMIXTURE plots (Figure 16, B and D) are in line with PCA results, as K=1 was the best inferred K (Figure S24- Appendix IV) for both species.

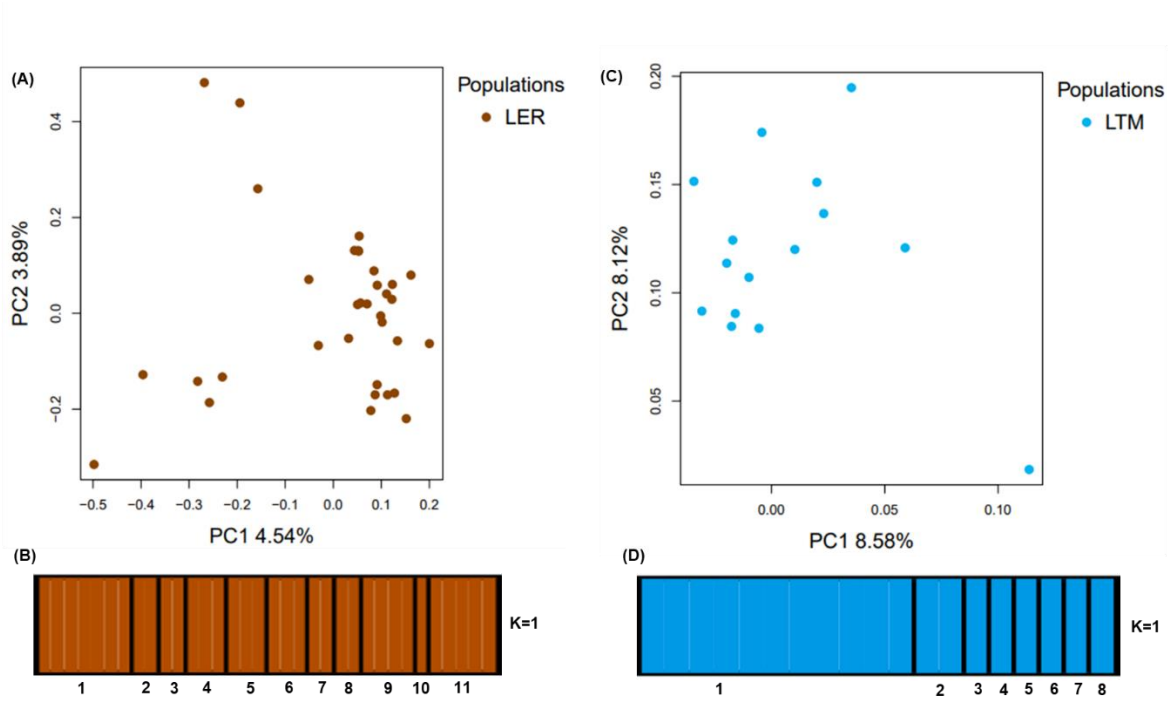

Figure 16: Analysis of population structure for Swedish populations. (A) PCA plot referring to L. europaeus. (B) ADMIXTURE plot for best supported K (K=1), containing eleven populations: 1-Uppsala, 2-Östergötland, 3-Stockholm, 4-Orebro, 5-Västra Götaland, 6-Halland, 7-Kronoberg, 8-Kalmar, 9-Skane, 10-Gotland and 11- Herräng. (C) PCA plot referring to L. timidus. (D) ADMIXTURE plot for best supported K (K=1), containing eight populations: 1-Herräng, 2-Tärnaby, 3-Kroksjö, 4-Umeä, 5-Hällnäs, 6-Lycksele, 7-Tavelsjö, 8-Sävar.

Then, we explored the migration pattern by using the spatially visualizer EEMS. Regarding *L. europaeus*, diagnostic plots for model fitting (Figure S26 – Appendix IV) showed that EEMS results present an excellent fitting both with the data regarding dissimilarities within demes ($r^2 = 0.987$) and between demes ($r^2 = 0.944$). In addition, the absence of correlation between geographic distance and genetic distance indicates that isolation by distance cannot explain the population structure of *L. europaeus* estimated in Sweden. The results in Figure 17 show the existence of a barrier to intraspecific gene flow (a region of higher genetic differentiation) on the west region of Sweden, evidence of a great population fragmentation which might correspond to a region of expansion of this species. On the contrary, there is a clear passageway along the Eastern Swedish coast, where populations might contact more frequently with each other thus being more genetically similar. Regarding *L. timidus*, the lower number of individuals available made it impossible to evaluate the population structure for the species in Sweden (Figure S25 and S27- Appendix IV).

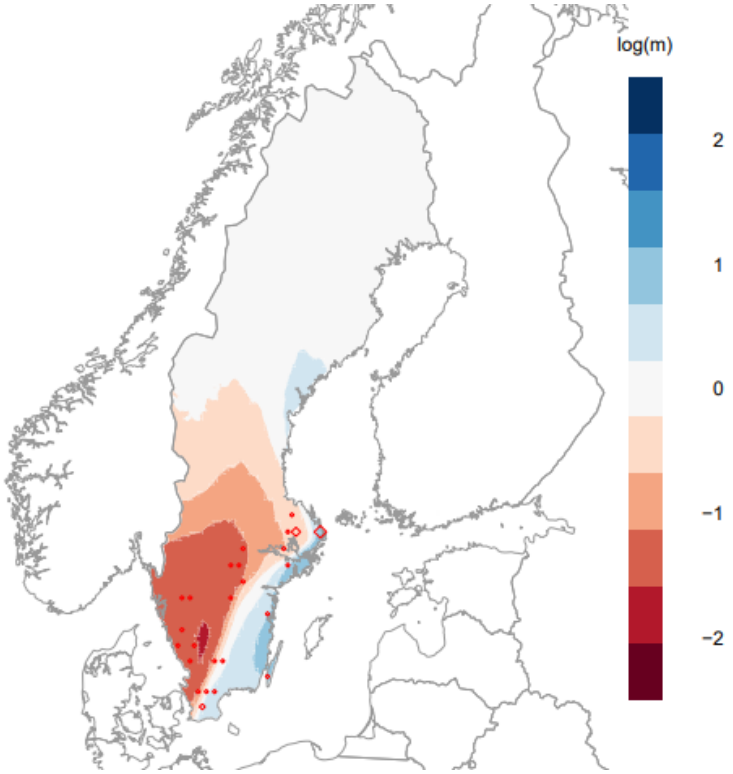

Figure 17: Effective Migration Maps estimated for the Swedish population of *L. europaeus*. The plots were estimated with EEMS under a log10 scale and after mean centring. The blue indicates areas with an effective migration between populations above average (more genetic similarity) and the red regions with an effective migration below average (less genetic similarity).

3.4.2.2. Demographic history of Swedish hare populations

Then we investigated the demographic history of *L. europaeus* and *L. timidus* in Sweden. Stairway plots results for both *L. europaeus* (Figure 18) and *L. timidus* (Figure 19) suggest 1) a bottleneck ~220'000 and 160'000 years ago and a 2) quick recovery of the effective population size of both populations, reaching values similar to those verified before the demographic event. The event might be the same but affecting the species in different periods, according to its distribution at that time. In *L. europaeus*, the analysis suggests 3) an additional bottleneck ~20'000 years ago, reducing its effective population size for one fifth and to recover but has been 4) suffering a decrease of population size until now.

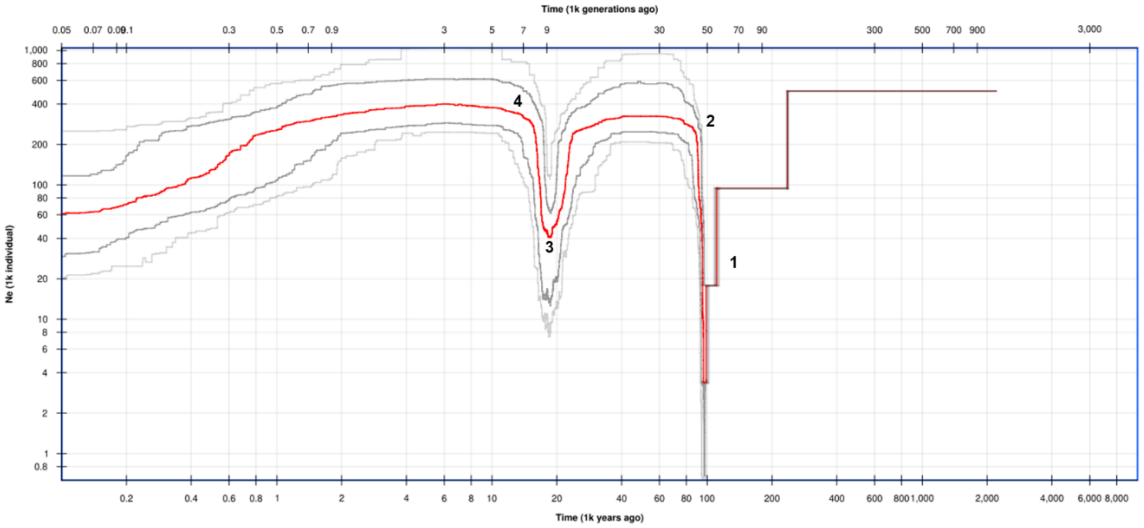

Figure 18: Stairway plot reconstructions of demographic history of *L. europaeus* in Sweden. The x-axis is the time in thousand years (lower scale bar) and in thousand generations (upper scale bar) before present. The y-axis is the effective population size in thousand individuals. The red line shows median of effective population size. The upper and lower thick and light gray lines show 75% and 95% confidence intervals, respectively.

For *L. timidus*, the stairway plot suggested 3) in Pleistocene ~40'000 years ago and 4) Holocene ~10'000 years ago. Since that last event, it suggested 5) an increasing population growth until it stabilized ~4'000 years ago, presenting now the higher effective population size of its demographic profile. The Stairway plot concerning the parental population of *L. europaeus* (Figure S18- Appendix IV) exhibit a similar pattern when compared to Sweden since its population size have been declining over the years, however, the Swedish population from *L. europaeus* have been progressively decreasing whereas the Austrian populations have passed through two recent events of bottleneck. The parental population of *L. timidus* display a different pattern from Sweden (Figure S18- Appendix IV) because apart from the first bottleneck (also detected here), its effective population size was suggested to have been declining while the Swedish population may have been stable with large effective population sizes for the last ~4'000 years.

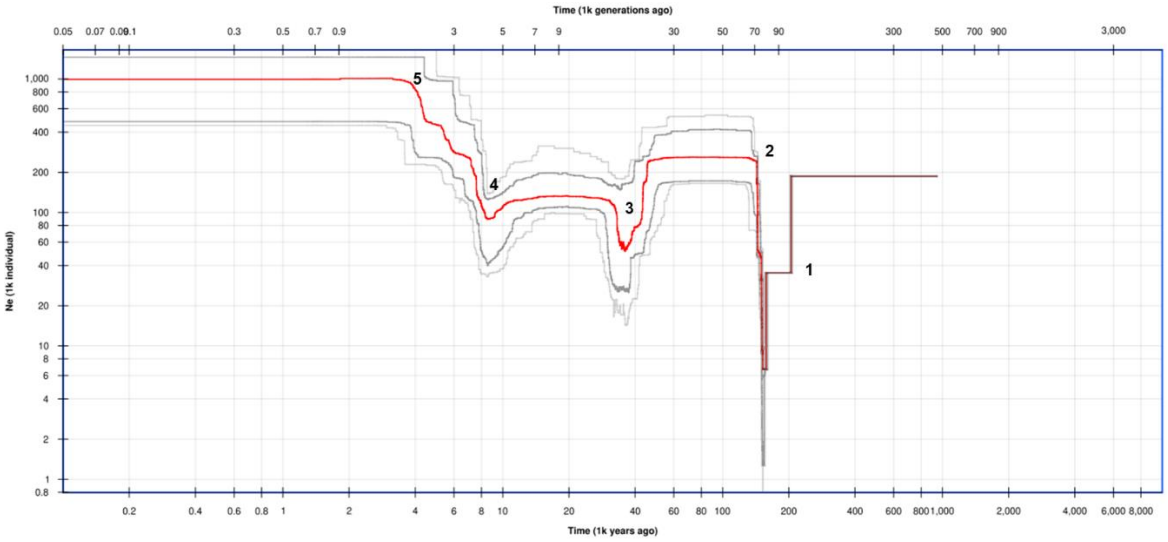

Figure 19: Stairway plot reconstructions of demographic history of *L. timidus* in Sweden. The x-axis is the time in thousand years (lower scale bar) and in thousand generations (upper scale bar) before present. The y-axis is the effective population size in thousand individuals. The red line shows median of effective population size. The upper and lower thick and light gray lines show 75% and 95% confidence intervals, respectively.

3.4.2.3. Levels of genetic exchange across Swedish populations

In table 12, the results show low levels of introgression of mtDNA, with the absence of introgressed specimens in *L. timidus* (0%) *and L. europaeus* presenting the higher percentage (11.6%).

Table 12: Number of individuals of *L. europaeus* and *L. timidus* showing mitochondrial DNA introgression in Sweden.

Region	Species	Introgressed	Non-introgressed
Sweden	*L. europaeus*	5	38
	L. timidus	0	23

Patterns of introgression between *L. timidus* and *L. europaeus* in Sweden were investigated based on a dataset of 193 diagnostic SNPs found to be alternatively fixed or nearly fixed between the parental population of the species. The first principal component (PC1; 85.3% of explained genetic variance) distinctly splits the species (Figure 20A). Additional PCA plots are shown in Figure S28 (Appendix IV). The analysis of ancestry with ADMIXTURE (Figure 20B), shows that for K=2, each cluster refers to

each species. In addition, there is minor evidence of admixture from *L. timidus* towards *L. europaeus* (see cross-validation errors support; Figure S29- Appendix IV).

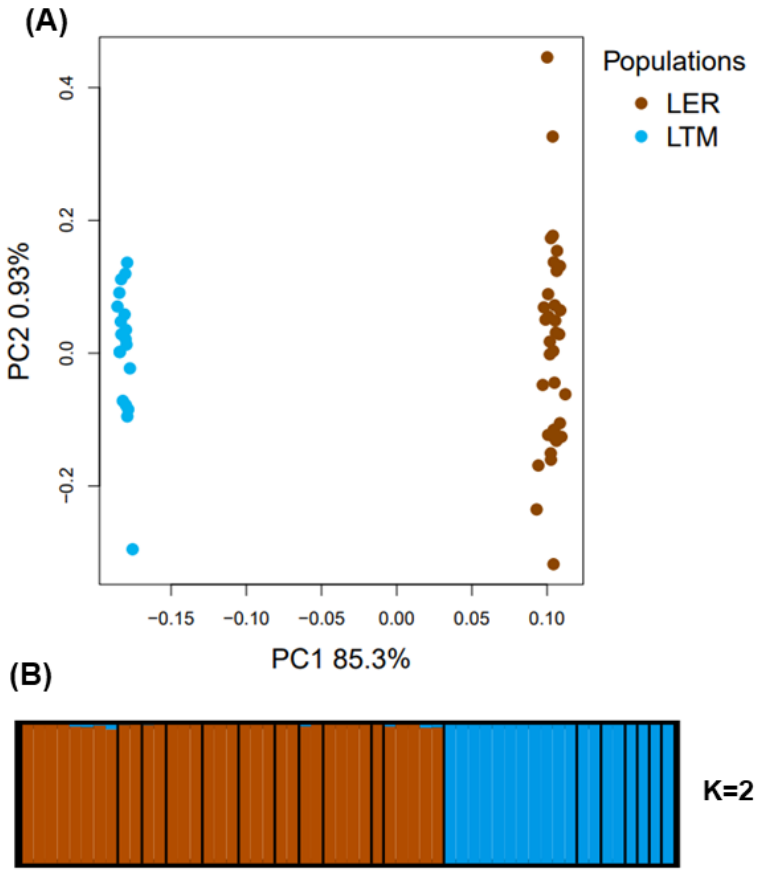

Figure 20: Analysis of genetic exchange for Swedish populations with PCA (A) and (B) ADMIXTURE plots containing *L. europaeus* (brown) and *L. timidus* (blue). The analysis includes eleven populations of *L. europaeus* (Uppsala, Östergötland, Stockholm, Orebro, Västra Götaland, Halland, Kronoberg, Kalmar, Skane, Gotland and Herräng) and seven of *L. timidus* (Herräng, Tämaby, Kroksjö, Umeå, Hällnäs, Lycksele, Tavelsjö, Sävar).

Next, we performed the D-statistics tests, and results (Table 13) showed a significant excess of shared derived alleles in the Swedish populations of *L. europaeus* (bold values) but the proportion of alleles derived from introgression is low (a f4-ratio around 2%). These minor levels of introgression from *L. timidus* towards *L. europaeus* are in line with the pattern inferred by ADMIXTURE (Figure 20B). Moreover, no evidence of introgression was found in the opposite direction, from *L. europaeus* to *L. timidus*.

Table 13: Results from ABBA and BABA's test and f4-ratio tests between Swedish populations of *L.europaeus* (SWE_LER, brown background) and *L. timidus* (SWE_LTM, blue background), with significance values (p-value). The parental populations (brown background) of *L.europaeus* includes Germany (GER) and PYR (Pyrenees) whereas parental populations of *L. timidus* (blue background) comprise all the Russian populations (Kolyma River Basin (KOL), MAG to Magdan (MAG), Primorsky territory (PRI) and Urals (URA). Values of Z-score > 3 suggests significant excess of shared derived alleles because. The values in bold refers to Z-scores >3 with a respective p-value ≤ 0.05.

P1	P2	P3	Dstatistic	Z-score	p-value	f4-ratio	BBAA	ABBA	BABA
GER	SUE_LER	KOL	0.247624	***6.12836***	4.44E-10	0.028667	317.792	22.0305	13.2854
GER	SUE_LER	MAG	0.248108	***6.02315***	8.55E-10	0.028636	316.423	22.1511	13.3444
GER	SUE_LER	PRI	0.241139	***5.9801***	1.11E-09	0.028737	312.958	21.5419	13.1712
GER	SUE_LER	URA	0.251865	***6.42159***	6.74E-11	0.029069	317.7	22.1465	13.2351
PYR	SUE_LER	KOL	0.204036	***7.00161***	1.27E-12	0.024375	312.851	22.0136	14.5528
PYR	SUE_LER	MAG	0.218164	***6.77739***	6.12E-12	0.025858	311.385	21.9717	14.1018
PYR	SUE_LER	PRI	0.191391	***7.09688***	6.38E-13	0.024949	308.233	21.7316	14.7494
PYR	SUE_LER	URA	0.21417	***7.06599***	7.97E-13	0.025644	312.876	22.2469	14.3985
KOL	SUE_LTM	GER	0.021026	0.191347	0.424127	0.000818	313.692	6.34048	6.07935
MAG	SUE_LTM	GER	0.050367	0.419544	0.337409	0.001879	311.835	6.24614	5.64711
URA	SUE_LTM	GER	0.013448	0.118075	0.453004	0.000505	313.075	6.08542	5.92392
MAG	SUE_LTM	PYR	0.055643	0.463806	0.321394	0.002108	309.869	6.28086	5.61873
URA	SUE_LTM	PYR	0.008006	0.075066	0.470081	0.000286	310.658	5.66003	5.57012

Next, we calculated the hybrid index for the individuals from Sweden, using a dataset of 18 fixed sites between the species. The plot obtained (Figure 20A) showed an absence of early generation hybrids and evidence of introgression from *L. timidus* to *L. europaeus* (hybrid index ranging from 0.83 to 1 in *L. europaeus)* but not in the opposite direction. These results corroborate the previous results with ADMIXTURE and ABBA-BABA test: low but asymmetric introgression. The plot correlating the hybrid index with the interspecific heterozygosity (Figure 21B) shows that all the individuals of *L. timidus* are disposed on the left corner with a respective hybrid index and interspecific heterozygosity of 0. Whereas the individuals of *L. europaeus* are placed along the right side of the triangle, assuming various values of hybrid index and interspecific heterozygosity. In addition, the fact that most individuals are placed on the line of theoretical maximum values of heterozygosity, suggests that most part of the Swedish population are descendants of backcrosses.

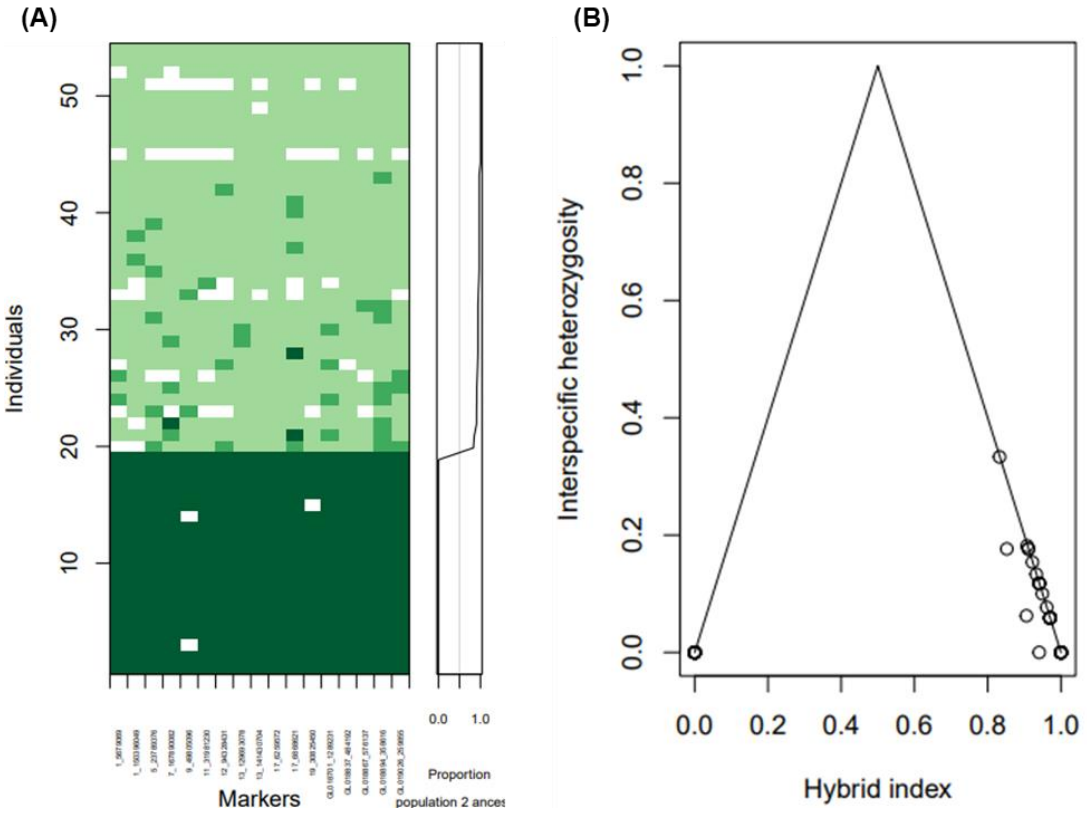

Figure 21: (A) Plot of hybrid index for the populations of Sweden: the light green refers to *L. europaeus* genotypes, the dark green to *L. timidus* genotypes, the intermediate green refers to an heterozygotic genotype and the white to missing data. Down the plot there are the 18 markers used and on the right is a graph referring to the proportion of alleles attributed to *L. europaeus*. (B) Plot correlating interspecific heterozygosity with hybrid index, in which the lines correspond to theoretical maximum values of heterozygosity and individuals that fall in the maximum line are probably F1s or descendants of backcrosses.

3.5. Ecological niche modelling

The distribution models of *L. timidus* suggest that the current climatic conditions (Figure 23b) are more favorable to the species than past ones (73-122 years ago) since past habitat favorability indicated worst conditions specially in Alps and United Kingdom, although favorability values in Fennoscandia remained high (Figure 22a). Moreover, under future climatic conditions (39-58 years from now) *L. timidus* may narrow its range in the Baltic countries and the Alps, but not many changes are expected in United Kingdom (Figure 24a and 25a).

The probable distribution of the *L. europaeus* showed that in the past, the habitat at lower latitudes was more favorable and that there should be a climatic limit from which distribution at high latitudes would be restricted (Figure 22b). The current climatic

conditions appear to have promoted a northwards shift of increasing favorability, a pattern that is predicted to surge in the future (Figure 23b, 24b, 25b).

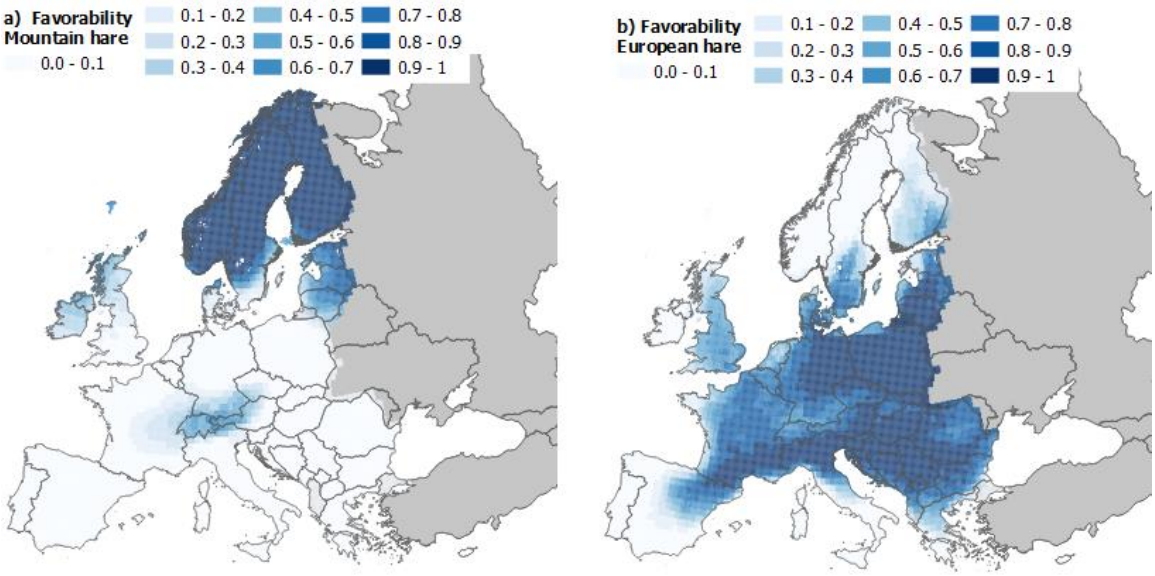

Figure 22: Projected model of the past favourability (1900-1949) in a 10x10km grid for (A) *L. timidus* and (B) *L. europaeus*. On the map, the darker and lighter blue correspond respectively to higher and lower levels of favourability.

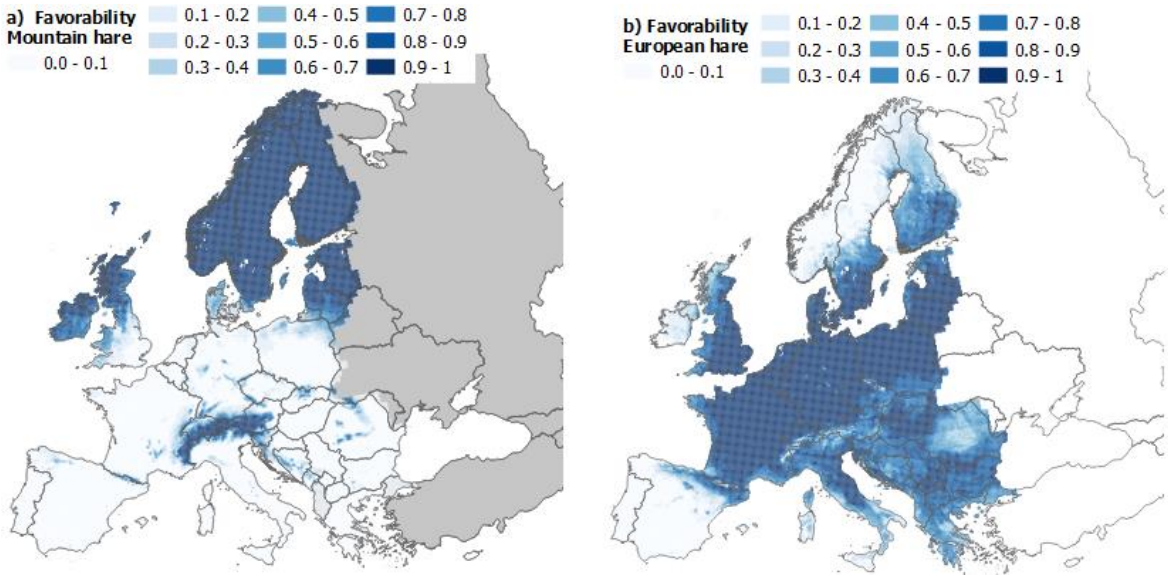

Figure 23: Projected model of the present favourability in a 10x10km grid for (A) *L. timidus* and (B) *L. europaeus*. On the map, the darker and lighter blue correspond respectively to higher and lower levels of favourability.

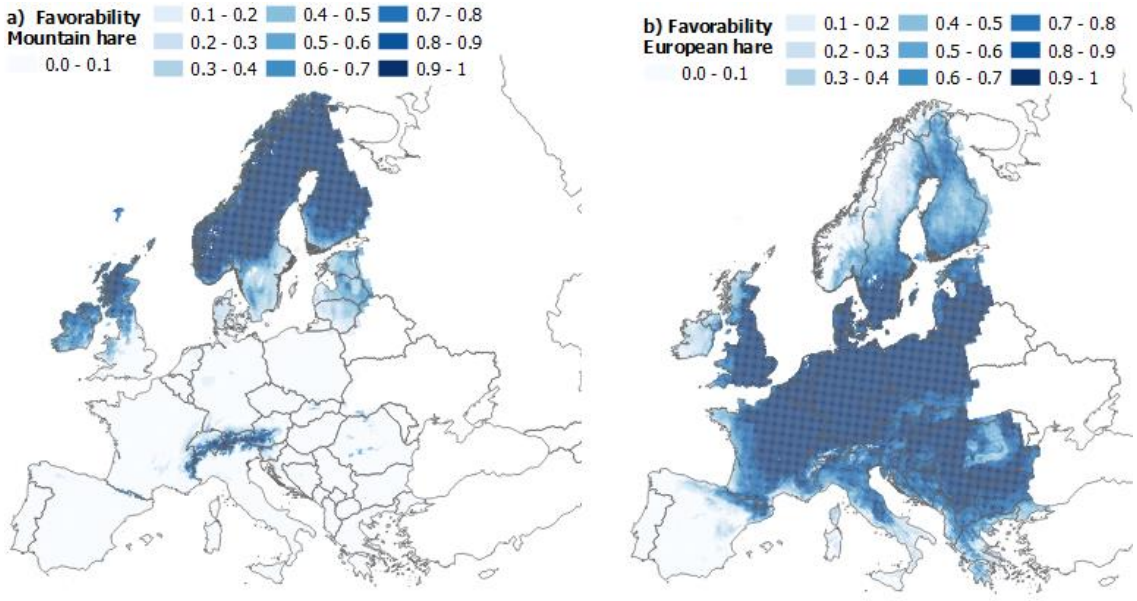

Figure 24: Projected model of the future favourability (2061-2080) under SPP2 4.5 climate change scenario in a 10x10km grid for (A) *L. timidus* and (B) *L. europaeus*. On the map, the darker and lighter blue correspond respectively to higher and lower levels of favourability.

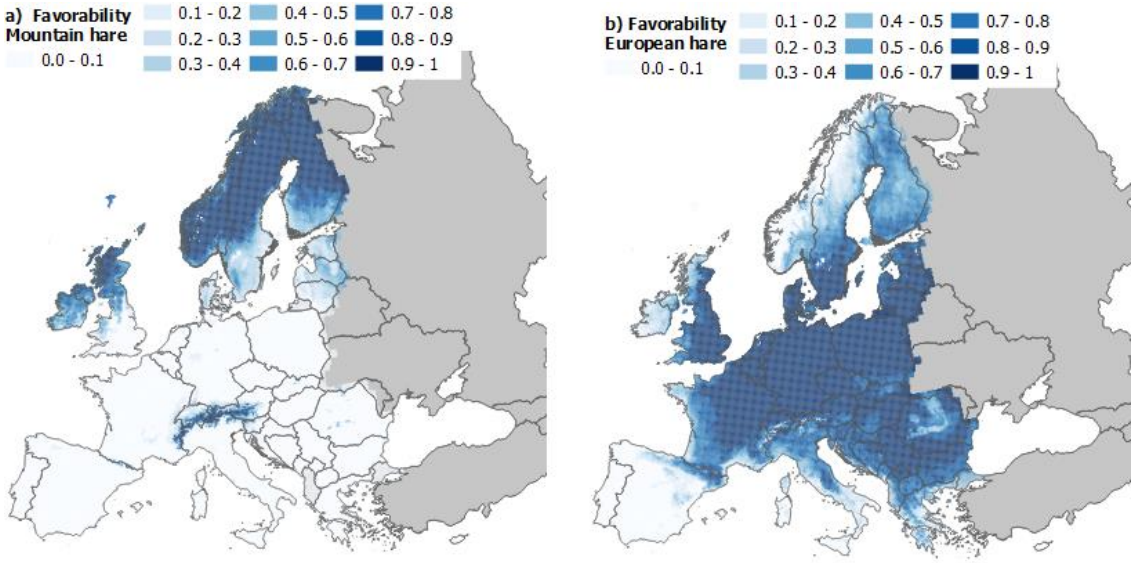

Figure 25: Projected model of the future favourability (2061-2080) under SPP3 7.0 climate change scenario in a 10x10km grid for (A) *L. timidus* and (B) *L. europaeus*. On the map, the darker and lighter blue correspond respectively to higher and lower levels of favourability.

The spatial pattern of interaction at past conditions (1900-1949; Figure 26) showed higher intensities of species overlapping at the Alps, and Baltic countries. Moreover, where both species overlapped, *L. europaeus* dominates over *L. timidus* in the United Kingdom and Ireland, but in Sweden *L. timidus* shows higher favorability.

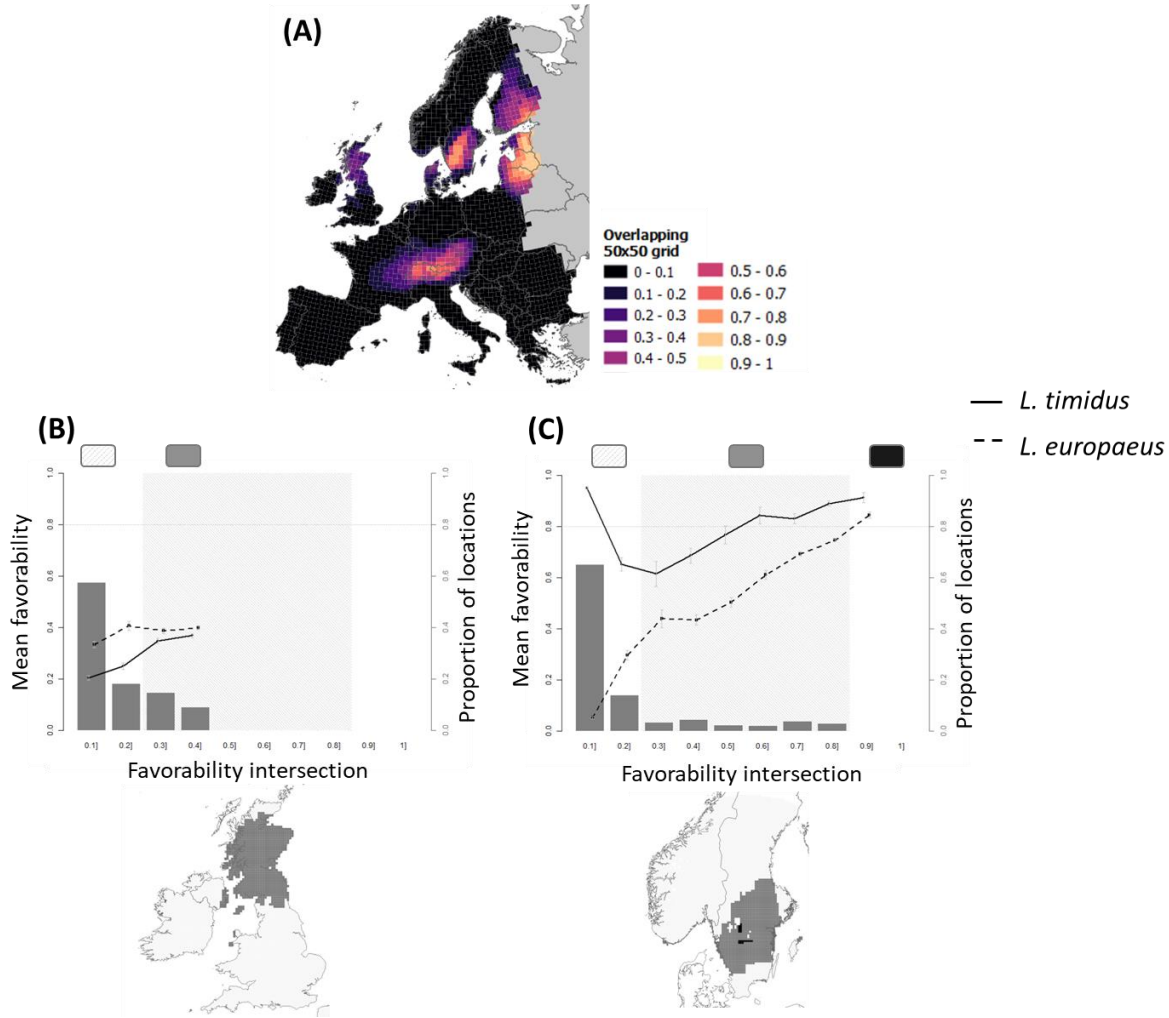

Figure 26: (A) Map referring to past overlapping patterns of both species (1909-1949) in Europe. Past species interaction of the species in United Kingdom (B) and Sweden (C). Above the graphic and on the map, the white colour refers to regions only favourable for one species, the grey colour to regions favourable for either of species and the black colour refers to regions favourable for both species. On the graph, the solid and dashed lines refer respectively to *L. timidus* and *L. europaeus*. The x-axis refers to the favourability intersection. The left and right y-axis are respectively the mean favourability and proportions of locations. The dashed yellow line marks the switch in the prevailed species.

The spatial pattern of interaction at present conditions indicated an increase in both the intensity of the overlap as well as the extension (Figure 27A). Furthermore, the pattern inferred currently for the United Kingdom and Ireland showed a shift when compared to the past projections, with *L. timidus* dominating the great majority of the overlapping region, except above 0.7 favorability values of the competence region where *L. europaeus* would slightly dominate (Figure 27B). Moreover, although *L. europaeus* has colonized southern Sweden, *L. timidus* prevails in regions where both species overlap (Figure 27C).

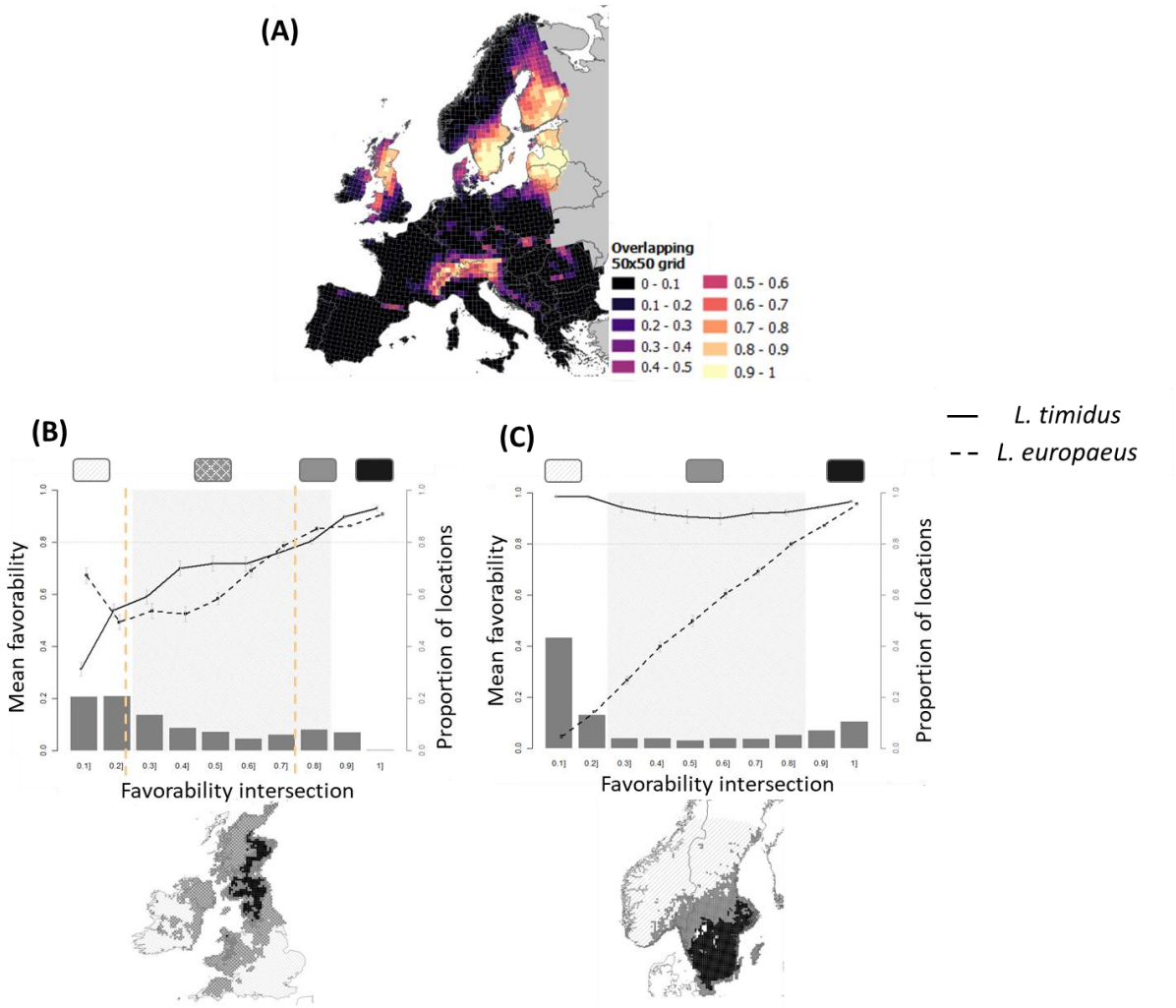

Figure 27: (A) Map referring to present overlapping patterns of both species in Europe. Present species interaction of the species in United Kingdom (B) and Sweden (C). Above the graphic and on the map, the white colour refers to regions only favourable for one species, the grey colour to regions favourable for either of species and the black colour refers to regions favourable for both species. On the graph, the solid and dashed lines refer respectively to *L. timidus* and *L. europaeus*. The x-axis refers to the favourability intersection. The left and right y-axis are respectively the mean favourability and proportions of locations. The dashed yellow line marks the switch in the prevailed species.

Climatic patterns under different future climate conditions produced very similar results, both showing a decrease in the intensity of the interaction between species in United Kingdom and Fennoscandia (Figure 28A and 29A). In addition, an increase on the extent of the interaction in Fennoscandia is suggested (Figure 28A and 29A). Furthermore, the observed interaction pattern in United Kingdom and Ireland showed that, at the competence region, the dominance of *L. europaeus* over *L. timidus* above middle favorability values increased (Figure 28B and 29B). Regarding Sweden, the pattern observed displayed a shift when compared to the previously ones observed (Figure 28C

and 29C). For the first time in Scandinavian countries, *L. europaeus* may dominate over *L. timidus* at low favorability values (below 0.5) in the competence region, however, *L. timidus* may still dominate at higher favorability values.

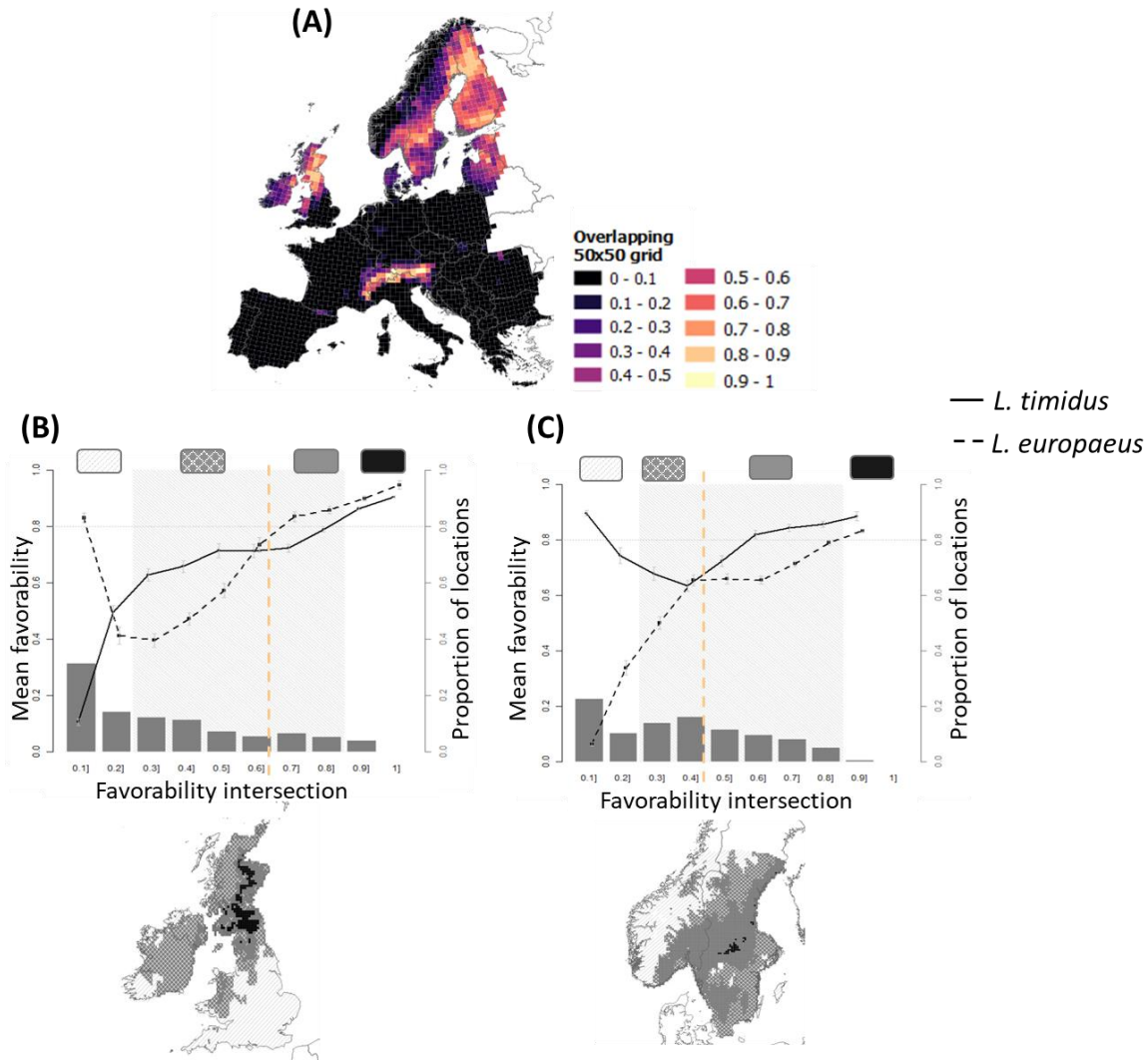

Figure 28: (A) Map referring to future overlapping patterns of both species (2061-2080), under SSP2 4.5 scenario in Europe. Future species overlapping in United Kingdom (B) and Sweden (C). Above the graphic and on the map, the white colour refers to regions only favourable for one species, the grey colour to regions favourable for either of species and the black colour refers to regions favourable for both species. On the graph, the solid and dashed lines refer respectively to *L. timidus* and *L. europaeus*. The x-axis refers to the favourability intersection. The left and right y-axis are respectively the mean favourability and proportions of locations. The dashed yellow line marks the switch in the prevailed species.

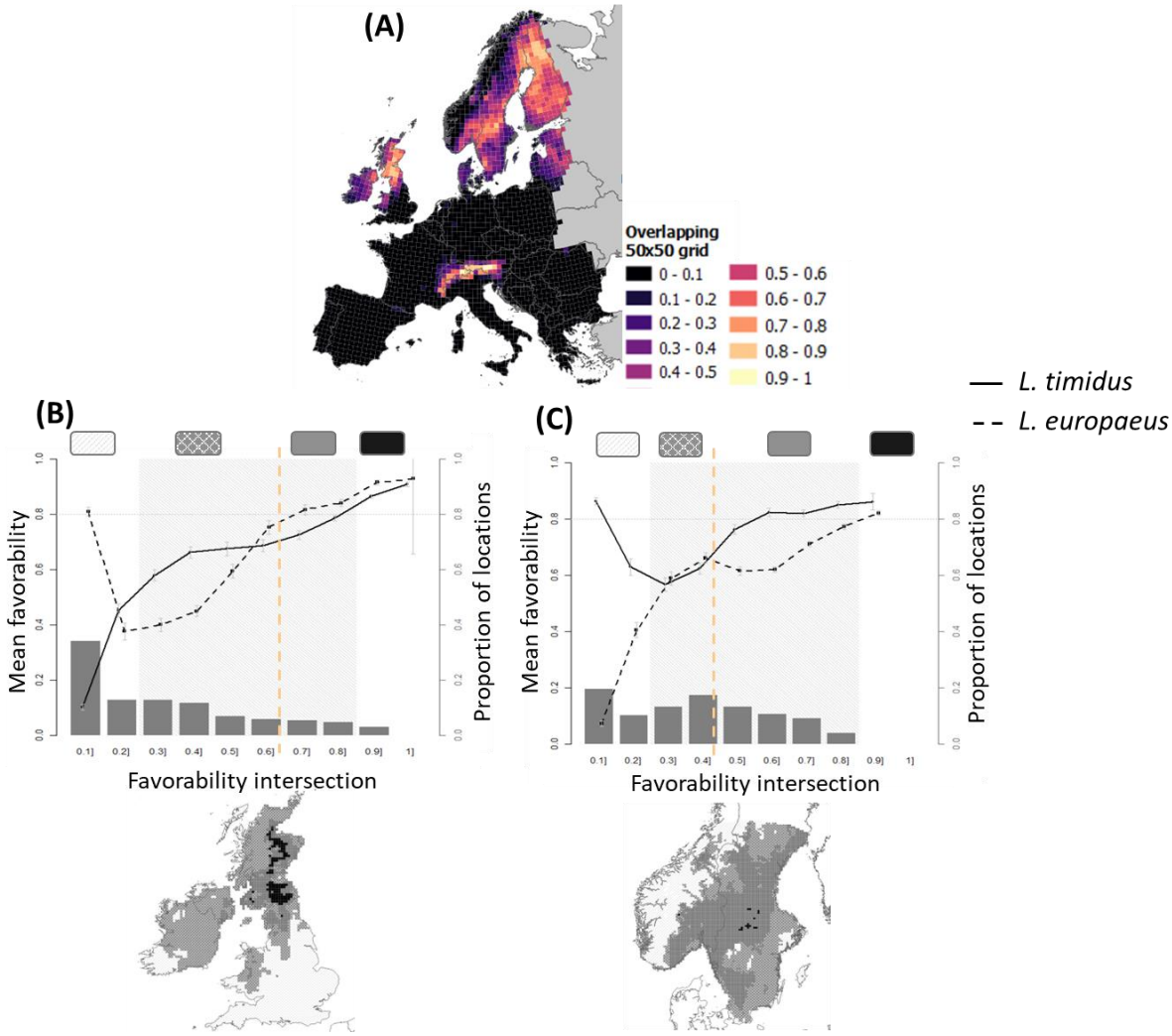

Figure 29: (A) Map referring to future overlapping patterns of both species (2061-2080), under SPP3 7.0 scenario in Europe. Future species overlapping in United Kingdom (B) and Sweden (C). Above the graphic and on the map, the white colour refers to regions only favourable for one species, the grey colour to regions favourable for either of species and the black colour refers to regions favourable for both species. On the graph, the solid and dashed lines refer respectively to *L. timidus* and *L. europaeus*. The x-axis refers to the favourability intersection. The left and right y-axis are respective the mean favourability and proportions of locations. The dashed yellow line marks the switch in the prevailed species.

4. Discussion

Environmental changes have an important impact on species' evolutionary history [6]. The distinct past biogeographic histories of *L. europaeus* and *L. timidus* resulted in the current patterns of genetic diversity and distribution of populations. Human-induced climate changes are affecting these species differently: *L. europaeus* tends to be expanding its range, while *L. timidus* is contracting [94, 96]. These contrasting demographic trends may promote species range replacement and, given that the species are known to hybridize, introgression between these species may have occurred in numerous contact regions across the Europe [64]. Here, we tackled these species' genetic interactions in Scotland and in Sweden, contact regions with suggested invasion-retraction dynamics. Although there are, to our knowledge, no studies focused on hybridization between the species in Scotland, in Sweden, asymmetrical introgression has been previously suggested [102], with more pronounced introgression from *L. timidus* towards *L. europaeus,* but these inferences were only based on mtDNA and few nuclear DNA markers. This pattern has been suggested to result from female biased assortative mating, with *L. timidus* females preferentially mating with *L. europaeus* males in a hybridization context [65]. If this hypothesis is valid, such patterns should prevail in any context of hybridization between the species. In other contexts of hybridization with *L. timidus*, a selective advantage of the *timidus* mtDNA type, a species well adapted to colder regions, has been hypothesized, though it has not been fully validated [128]. While genetic introgression may lead to the pollution of the gene pool of the affected species, which in the case of *L. timidus* and *L. europaeus* interactions could more seriously affect the retracting *L. timidus*, introgression may also be a source of adaptive variation [5].

In this work, we investigated the introgressive hybridization during range replacements promoted by climate changes, using genetic variation data obtained from a reduced-representation method: RAD sequencing. We explored the general patterns of genetic structure and diversity of *L. europaeus* and *L. timidus* across its European distribution and, particularly, in current contact regions. Next, we quantified the amount and direction of the genetic exchange between these species. Finally, we investigated the long-term demographic histories of both species in Scotland and Sweden as well as the likely population trajectories of the species in the future using ecological niche modelling. This work represents the most comprehensive analysis of genetic exchanges between these species in a unified framework across different contact zones.

4.1. General patterns of genetic diversity and genetic structure of the expanding *L. europaeus* and contracting *L. timidus*

The PCA and ADMIXTURE analyses for *L. europaeus*, revealed that the Scottish population appears more differentiated than the rest of the analysed population across the range of the species (Figure 4). Results from pairwise F_{ST} showed that while the remainder of the *L. europaeus* populations show moderate levels of differentiation among them (from 0.019318 to 0.098843), the values of differentiation for the Scottish population were amongst the highest values estimated (from 0.10094 to 0.21274; Figure 5). This genetic distinctiveness of the Scottish population in relation to its continental counterparts had been suggested in a previous study [16], likely resulting from genetic isolation. The isolation of this population and absence of inter-species gene flow may promote the genetic distinctiveness and divergence from the continental populations [151]. The values of genetic differentiation for the Swedish population (0.019318 to 0.10094; Figure 5) were also among the highest values estimated, but lower than those from Scotland. These lower levels of differentiation when compared to the other contact region, might be associated with the more recent colonization of Sweden by *L. europaeus*, caused by a recent human-mediated introduction [16]. In addition, the ADMIXTURE analysis for *L. europaeus* (Figure 4) uncovered substructure within the Scottish population, which will be discussed in subsequent sections. Sharing genetic ancestry was estimated between the populations of *L. europaeus* from Scotland and the Pyrenees, which is unexpected given the distribution areas these populations represent. This result was consistent across analyses (PCA, ADMIXTURE and TreeMix; see Figure 4 and 8), which could reflect some genetic similarity between the populations due to relatedness during the colonization process in western Europe, but other phenomena like human mediated translocations could also explain the pattern [152].

Regarding *L. timidus*, the population structure analysis showed the existence of four clusters isolating the populations from islands (Ireland, Scotland, Faroe) from the remaining ones located across the Europe (Figure 6). Regarding the pairwise F_{ST}, results showed also that the population from Scotland is the most genetically differentiated (0.19255 to 0.31642) whereas the remaining pairwise comparisons showed consistently lower values (0.019318 to 0.098843), including the Swedish population (Figure 7). Moreover, the Swedish and Scottish populations showed higher levels of differentiation in comparison with the remainder populations than between each other, which corroborates the hypotheses that current populations located in Great Britain and

Scandinavia may be part of the former ancestral population that colonized the north of Europe after the glaciation [83]. Overall, the higher values of pairwise differentiation and population structure exhibited by *L. timidus*, suggests that the populations of *L. timidus* are more fragmented across their distribution range than the populations of *L. europaeus*, which is consistent with a post-glacial expansion of *L. europaeus* towards western Europe, and the recent retreat to northern latitudes and higher altitudes by *L. timidus* accompanied by population fragmentation [16].

4.2. Dissimilar patterns of genetic diversity and genetic structure on an ongoing range replacement in Sweden and Scotland

After analysing the general patterns of each species, we inspected the contact regions in more detail. Heterozygosity ratio estimates were similar in both contact regions (Table 6 and 8), but the inbreeding coefficient was higher in Scotland (Table 7 and 9). In Scotland, *L. timidus* exhibited a lower inbreeding coefficient than *L. europaeus* which may be explained by an edge effect on the distribution of *L. europaeus* [153], while *L. timidus*, if retracting, may have not yet experienced serious bottlenecks and these signs are not yet seen in the genetic variation [30].

In Scotland, population structure analyses (PCA and ADMIXTURE) showed no genetic structure within the population of *L. europaeus* whereas the results from *L. timidus* pointed to the existence of two main clusters (Figure 9). These two groups could be related with the geographic distribution of the populations, since the second cluster is mainly comprised by individuals located in regions of high altitude (Cairngorm Mountains, Lammermuir Hills; see Figure 10). However, the remaining individuals are from lower latitudes of the distribution Tomatin, Moy (North of Scotland) and Lammermuirs (South of Scotland). Therefore, these clusters are not geographically constricted and may instead reflect recent re-admixture after fragmentation leading to the detected substructure.

We also evaluated the patterns of spatial structure and genetic heterogeneity within Scottish *L. europaeus* and *L. timidus*, applying the framework from EEMS. For *L. europaeus* (Figure 11A), and we found evidence of greater population fragmentation in northern regions of the distribution, which might correspond to its expansion front. Corridors of higher genetic similarity were inferred in southern Scotland, suggesting that the populations in this region might be connected. For *L. timidus* (Figure 11B), results for

the spatial structure show two regions of high genetic similarity, North and South of Cairngorm Mountains, separated by a barrier to gene flow extending from the east coast to the west coast of Scotland. This barrier may be associated to the Cairngorms National Park coupled with the Loch Lomond & The Trossachs National Park. As already discussed, mountains can work as physical barrier to the passage of species from one side to the other [154] and so, populations distributed North or South these mountains, will more likely interact with the neighbouring populations and display higher genetic similarity. On the other hand, the low genetic similarity displayed by the populations located within these mountains can be due to the conic shape of mountains populations which are expected to result in habitat fragmentation (distance between mountains-top habitats) and reduced gene flow between populations [94, 95]. This spatial pattern corroborates the existence of two potential population clusters within Scottish *L. timidus*.

Regarding Sweden, results for population structure (Figure 16) showed no evidence of genetic differentiation in populations of either species. EEMS analysis, for *L. europaeus* (Figure 17), shows a region of low gene flow in western regions of Sweden, which might be associated with a region of expansion of this species. Individuals in the northern regions (potential front of expansion) are more genetically similar in a South-North axis. This pattern is consistent with a South-North range expansion, reflecting a genetic gradient in which the group of individuals migrating are progressively more differentiated from the former group, with sectors of differentiation perpendicular to the direction of the invasion [61]. Also, the analysis suggested a corridor of gene flow along the Eastern Swedish coast, where populations might contact more frequently with each other thus being more genetically similar. This region of high genetic similarity might correspond to the area of introduction of *L. europaeus* in Sweden, where population have been established for the longest time [80]. Nevertheless, is important to pinpoint that these results were inferred based on few populations and so, to confirm the patterns observed, additional populations would be necessary.

4.3. Contrasting patterns of introgression in Scottish and Swedish hares

In Scotland, bidirectional introgression of mtDNA between *L. timidus* and *L. europaeus* was found (Table 10), with *L. timidus* exhibiting a higher percentage of introgressed individuals (7.5%) when compared to *L. europaeus* (2.6%). Introgression from *L. europaeus* to *L. timidus*, as well as reciprocal introgression between these species,

seems to be rare and it was only reported in Russia [75] and in Alps [99], both long-established contact areas. The genetic exchange between the Scottish populations of both species was also detected in the analysis of nuclear DNA markers. TreeMix results (Figure 8) inferred an event of gene flow between the Scottish population of *L. europaeus* and that of *L. timidus*, and both the ancestral clustering (Figure 13) and D-statistics analyses (Table 11) detected introgression in both directions, with a greater extent from *L. europaeus* to *L. timidus*. The hybrid index (Figure 14A) confirmed these results, inferring introgression as reciprocal but prevalent at very low levels. Also, the correlation of the hybrid index with the interspecific heterozygosity (Figure 14B) suggested that the admixed individuals are descendants of backcrosses in our dataset of Scottish individuals. Hence, the low levels of introgression observed along with the predominance of late generation hybrids are evidence of existing but limited admixture occurring between species.

In Sweden, we detected five *L. europaeus* harbouring mtDNA of *L. timidus* (11.6%) and no introgression at *L. timidus* individuals (Table 12). This result is concordant with most of previous studies showing preferential introgression from *L. timidus* to *L. europaeus* in Sweden and in the Iberian Peninsula [16, 102, 129]. The asymmetrical exchange between the Swedish populations of both species was also detected in the nuclear DNA markers. The analysis of ancestry regarding the individuals from Sweden (Figure 20) showed marginal signs of introgression from *L. timidus* towards *L. europaeus*, in concordance with the D-statistics (see Table 13). Also, the hybrid index estimates corroborate the low levels and asymmetrical introgression (Figure 21A).

Collectively, two major conclusions emerge from our inferences of introgression across the two contact zones. The first, common to the two areas of study, is that levels of introgression between the species are very low. We did not detect evidence of early generation hybrids, which would be indicative of more extensive ongoing admixture between the species. All individuals with allospecific genetic contribution are later generation hybrids, descendants of backcrosses with each of the parental species. Our reduced genomic representation approach does not allow distinguishing recent from more ancient introgression as the source of the secondary sharing of genetic variants. Two approaches can be used to clarify this issue. One is to analyse whole genome sequences of the admixed individuals and identify the length of the tracts of introgression. Recent introgression results in long tracts of allospecific ancestry, while for ancient introgression events these tracts are expected to be shorter, eroded by recombination with native haplotypes [65]. Another is to analyse historical samples from

these regions, to understand whether levels of introgression increased over the last century – suggesting recent increases in genetic exchanges – or if are comparable to the present – which would suggest prevalence of standing levels of introgression occurring since more profound time depths. In this work, probes for targeted enrichment was designed that will allow analysing this question in historical samples from the Swedish contact zone (see Methods). In any case, admixture between the species does not appear to be extensive nor ongoing, also suggesting that the species are partially reproductively isolated [155]. The second conclusion, which distinguishes the two areas under study, is that introgression is bidirectional in Scotland, but unidirectional in Sweden, from *L. europaeus* to *L. timidus*. Also, this pattern is common to the analysed genomic data and mtDNA. While previous works based on mtDNA suggested that the asymmetry in the introgression could result from *L. timidus* female-biased assortative mating [62], our results from Scotland suggests this is not a rule in the interactions between *L. timidus* and *L. europaeus*. Also, such phenomenon would be predicted to cause much more pronounced asymmetry in the direction of introgression at female-transmitted mtDNA than at nuclear DNA [73], but we found concordant patterns of introgression at both inheritance compartments in both contact zones. A more plausible explanation for our results is the demographic settings of the interspecific interactions. In Sweden, *L. europaeus* has been introduced in the southern part of the country in the 19th century, and has since then invaded northwards replacing the range of *L. timidus* [85]. Range replacement with hybridization has been shown to cause asymmetric patterns of introgression, from the resident (in this case *L. timidus*) to the invading species (here *L. europaeus*) which is what we estimated. The theoretical demographic model predicts that genetic drift can lead to increases in frequency of introgressed variants on the invasion front explaining [61]. Although such asymmetry could be more pronounced for mtDNA in female-philopatric species, due to increased drift at the invasion front and more reduced influx of native mtDNA from the range of the invading species, it is expected to also affect nuclear DNA [64], which is what we infer. In Scotland, our results of bidirectional introgression, suggest that these rapid invasion dynamics did not occur, or have not yet left traces in the genetic variation of the species. Our results suggest a more stable contact zone, possibly a tension zone, where gene flow between the species is governed by the combined effect of migration and selection against hybrids, with limited geographic movement [57, 58], even though we did not dectect evidences of ongoing gene flow. In sum, our results suggest that the demographic underpinning of the hybrid zones is a major determinant of the directionality and amount of introgression between the species. Understanding how the relative demography of the

species will evolve in the future, in response to the ongoing rapid environmental changes, is therefore key to predict how introgression will continue to impact the genomes of the interacting species.

Even though our work detected limited levels of introgression between *L. timidus* and *L. europaeus*, it showed that there is standing introgressed variation in both species in Scotland, and in *L europaeus* in Sweden. Given that adaptation based on new mutations is slow when compared to that based on standing pre-existent variation [42], standing introgressed variation may be source of rapid adaptation, if advantageous in the new environmental conditions [5]. Adaptive introgression or adaptation based on standing introgressed variation has been shown to be a major engine of population adaptation to environmental changes. For example, adaptive introgression was described to promote higher insecticide resistance in an African malaria mosquito [55]. Similarly, brown winter coats in snowshoe hares and grey winter coat in mountain hares (instead of the most predominant winter white coats in both species) are likely a result of introgressed variant from black-tailed jackrabbits and Iberian hares, respectively [53, 137]. Even though our reduced representation genomic sampling does not allow inferring selective sweeps or evidence of adaptive introgression, it is worth noting that among the 81 alternatively fixed sites between the parental population of *L. timidus* and *L. europaeus*, two were found to have extensively introgressed from *L. europaeus* to *L. timidus* in Scotland in the hybrid index analysis (chromosome 7, position 131009004 and chromosome 13, position 39808821; see Figure 15). The pattern in these two loci is in striking contrast with the limited levels of introgression shown at the other analysed SNPs. This would be an expected pattern if positive selection governed the introgression levels at these loci. We have explored the genic content of these genomic regions, to understand whether genes with potential relevant functions emerged. We found that in a window of 100 kb surrounding the focal SNPs, some genes are present. The genes detected in chromosome 7 are associated with a nuclear envelope function (NEMP2) and a transcriptional repressor for zinc finger transcription factors ERG1 and ERG2 (NAB1) whereas the ones found in chromosome 13 refer to keratinization (C1orf68 and CRNN). While any relation of these genes with adaptation is at this point tentative, these results encourage future research to detect evidence of selective sweeps, to understand how prevalent across the genome are regions showing extensive introgression, to characterize the introgression tracts showing such patterns, and more to efficiently identify sets of candidate genes that can potentially be involved in adaptive introgression. Such research requires the collection of full genome data from the analysed populations.

4.4. Predicting future demographic trends under future climate change scenarios

Our results based on the genetic variation of *L. timidus* and *L. europaeus* reflect past events, which have shaped the patterns of population diversity, structure, and amount and directionality of introgression between the species. This work underscored that the impact of introgression strongly depends on the demographic underpinnings of the interacting species, and thus that presumable changes in demography and invasion-retraction dynamics in the future will strongly determine the degree to which genomic admixture will (continue to) occur. To start predicting future demographic trends of *L. timidus* and *L. europaeus* in the areas of specie parapatric in Scotland and Sweden, we used spatial distribution modelling to project future range expectations under realistic predictions of climatic shifts. While a comprehensive understanding of the potential shift would require incorporating diverse parameters that could influence the future ranges of the species (e.g., land use), we have for the moment focused on climatic models and analyzed how these changes can influence the relative prevalence of the species in the coexisting areas.

Our results on the effect of climate change on *L. europaeus* and *L. timidus* distributions indicate changes in their relative favorability in sympatric areas between 2061-2080 according to two climatic scenarios for the future (Figure 24 and 25). When analyzing the distribution of the species separately, our models suggest that the favorability of *L. europaeus* strongly increased over the last century both in Sweden and Scotland (Figure 22 and 23). In the same period, these models also show an decrease in favorability for *L. timidus* in its distribution in both regions (Figure 22 and 23). Yet, in Sweden, it is known that *L. europaeus* invaded towards North after its introduction in southern Sweden replacing *L. timidus* [85], which suggests that the relative favorabilities of the species has favored *L. europaeus*. In that respect, the subsequent incorporation of both species in the same model allowed understanding whether we can predict changes in the relative favorabilities of the species in the areas where they overlap, from the present to the future under realistic climate change scenarios. In Sweden, our projections suggest an increase in the extent of the interactions between the species, and that *L. europaeus* may dominate over *L. timidus* at low favorability values (below 0.5), although *L. timidus* may still govern at higher favorability values (Figure 28C and 29C). In Scotland, our modelling suggests that the governance of *L. europaeus* over *L. timidus* above middle

favorability values will tend to increase (Figure 28B and 29B), being plausible that future worst climatic change scenarios promote an advantage of *L. europaeus* over *L. timidus*. In sum, while *L. timidus* is predicted to maintain higher relative favorability in some regions, both in Scotland and Sweden, our modeling predicts a general increase in the relative favorability of *L. europaeus* over *L. timidus*.

The results have important implications on the prediction of the relative dominance of the species in their overlapping range and the rate of hybridization in the future. While in Sweden the dynamics of range replacement with hybridization, which explains current patters of introgression [102], is expected to be maintained, in Scotland, the more stable dynamics of genetic exchange between the species we inferred for the past-present may change towards a tendency for *L. europaeus* to invade the range of *L. timidus*. This may lead to the progressive contraction of *L. timidus* towards the North, and an increase of introgression rates in the direction of *L. europaeus*. Further, it is important to stress that these models only considered the climatic envelope of the species. Other factors, like land use, may also play an important role in the invasion-retraction dynamics, and in this case could favor the more generalist *L. europaeus*. This could yet aggravate the invasion process. The design of more complex models by incorporating other parameters that influence the distribution of the species along with information onto geographic explicit genetic simulation models of hybridization, can in the future provide more robust quantitative assessments the impact of the demographic dynamics on genetic exchanges between the species in the future.

Importantly, these considerations take only into account the influence of the relative demographic dynamics of the species in patterns and rates of genetic exchanges. Yet, given the potential of introgression to fuel local adaptation, introgression may contribute for an adaptation of *L. timidus* to the changing environment and promote its resilience in the interactions with *L. europaeus*.

5. Conclusions and Future Prospects

Analysis of genetic exchange in both contact regions between *L. timidus* and *L. europaeus* detected admixed individuals corresponding to late generation hybrids (descendant of backcrosses) and low levels of introgression, suggesting absence of widespread ongoing admixture [103]. The contrasting patterns of introgression detected in the Scottish and Swedish contact zones suggested a strong influence of demography

in determining the introgression events. In Sweden, the asymmetric introgression detected is likely the result of a geographic range replacement, leading to gene flow from the native species into the invading species in its recently colonized areas [73]. In Scotland, the reciprocal gene flow between the focal species is rather more compatible with a more stable contact region, possibly a tension zone, in which bidirectional introgression is maintained by a balance of migration and selection [58]. Finally, the future projections under different climate scenarios indicate a general tendency for an increase in favourability of *L. europaeus* over *L. timidus* in the coexistent areas, which may maintain the dynamics in Sweden and change them in Scotland towards a range replacement.

Although our reduced representation approach allowed progressing in our understanding of genetic exchanges between *L. timidus* and *L. europaeus*, additional studies using a dataset of whole genome sequence data will allow to clarify some aspects that remained unanswered. For instance, the genomic regions containing the sites that we infer as highly introgressed from *L. europaeus* to *L. timidus* in Scotland may be fully inspected in whole genome analyses. Properly inferring the ancestry of tracts along the genome would also allow better understanding the timings of the introgression events, since there is a relationship between the length of the introgressed tracts and the time since the introgression event. This is because over time, backcrossing with the parental species leads to an erosion of the introgression tracts over time [156]. Whole genome population data would also allow exploring the prevalence and frequency of introgression tracts across the genome, infer additional high frequency introgression blocks as well as the genes contained in those regions, and determine whether the detected signals are compatible with selective processes.

Furthermore, sampling historical populations from Scotland and Sweden at Natural History Museums and analysing the genetic variation can elucidate whether changes of introgression rates occurred over the last century. By comparing the patterns of introgression in historical and modern samples, it would be possible to infer temporal allele frequency changes. Here, we have developed a hybridization capture assay that can be applied to analyse historical DNA. Finally, incorporating the information provided by i) past and present patterns and rates of introgression, ii) the relative demography of the species inferred from genomic variation and iii) predicted to the future under species distribution models in geographic explicit genetic simulations can provide a more detailed prediction of the impact of these dynamics in the genetic interactions between the species in the future.

6. References

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7. Appendices

Appendix I- Sequencing statistics and SNP dataset

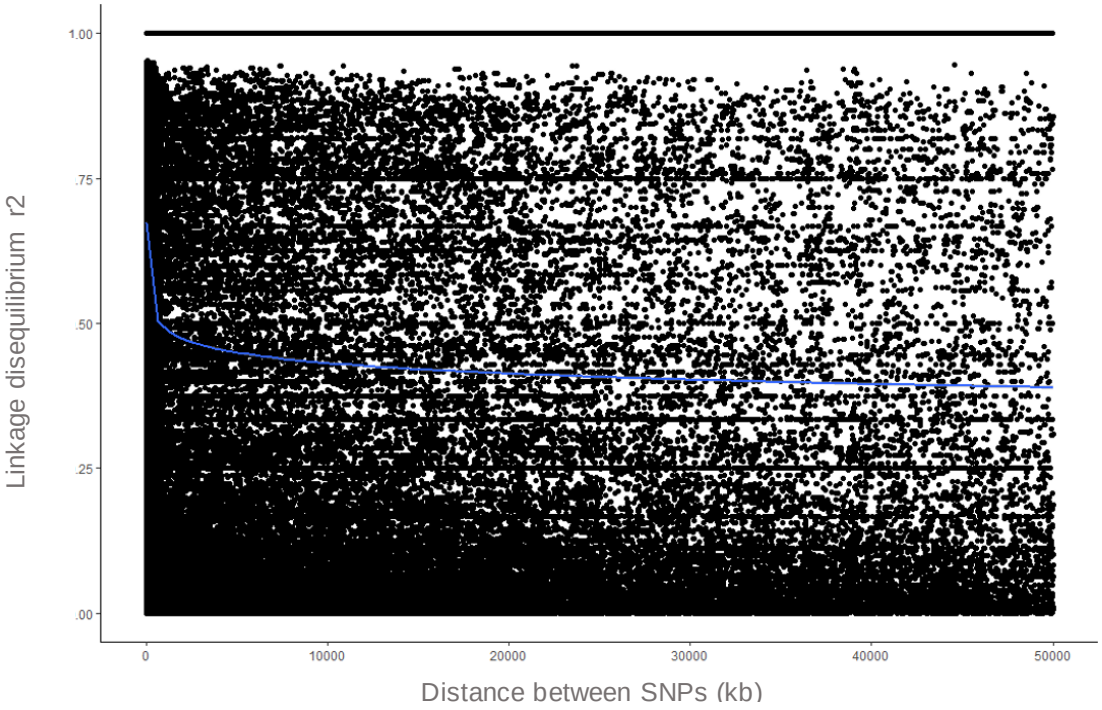

Figure S1: Plot of linkage disequilibrium (r^2) against physical distance between SNPs LD in *L. europaeus*. Black dots indicate observed pairwise LD values. The blue curve shows the expected decay of LD in the data estimated by nonlinear regression.

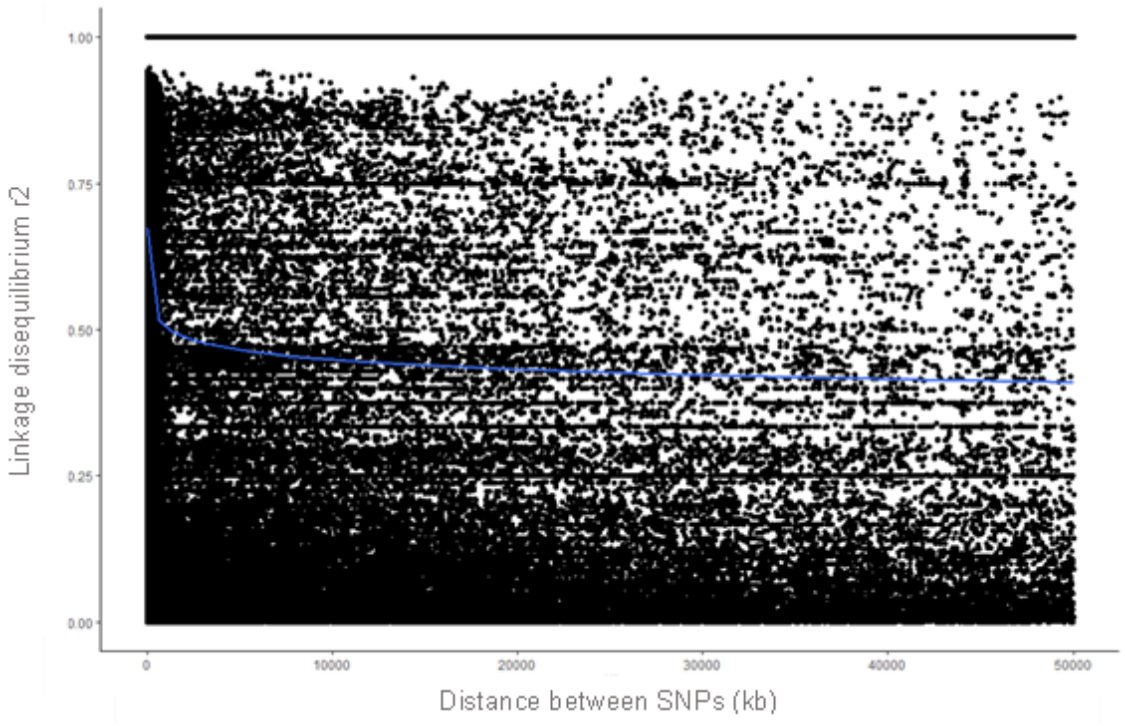

Figure S2: Plot of linkage disequilibrium (r^2) against physical distance between SNPs LD in *L. timidus*. Black dots indicate observed pairwise LD values. The blue curve shows the expected decay of LD in the data estimated by nonlinear regression.

Table S1: Number of positions of each dataset before and after filtering for linkage disequilibrium (LD) (50 window, 10 stepsize, r^2= 0.2). There was a great removal of positions after the filter, especially in the dataset of *L. europaeus* + *L. timidus* and in the dataset of Scotland (93% and 85% of positions removed, respectively).

Dataset	Number of positions	
	Before LD filter	After LD filter
L. europaeus + *L. timidus*	161175	11938
L. europaeus	85474	24584
L. timidus	169811	61149
Scotland	115237	17202
Scotland, *L. europaeus*	115237	17202
Scotland, *L. timidus*	115237	17202
Sweden	37914	9977
Sweden, *L. europaeus*	37914	9977
Sweden, *L. timidus*	37914	9977

Appendix II- Broad genetic characterization of *L. timidus* and *L. europaeus* across their range

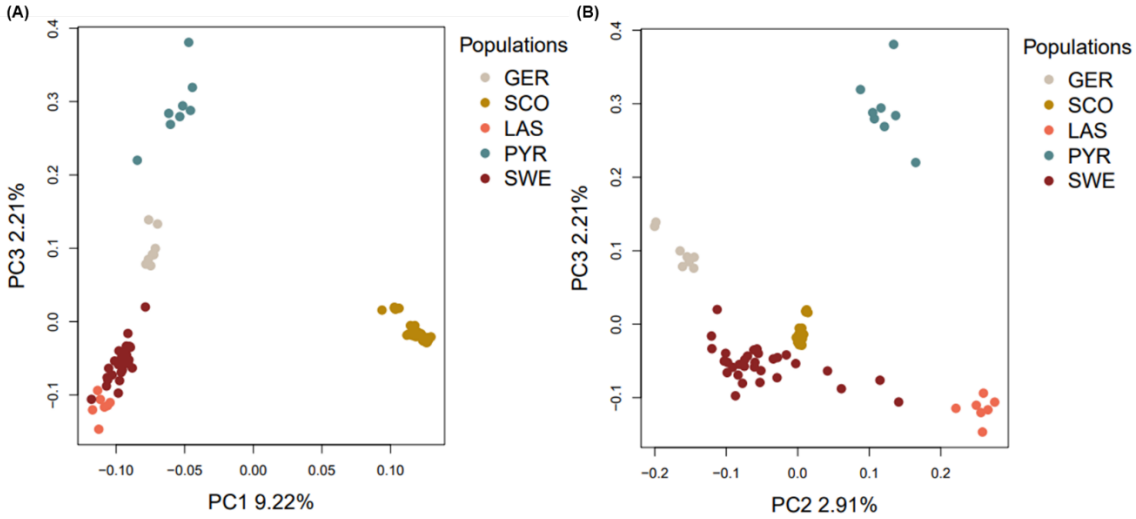

Figure S3: PCA plot *L. europaeus*. (A) PC1 vs PC3. (B) PC2 vs PC3. The analysis includes all the populations of *L. europaeus*: GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

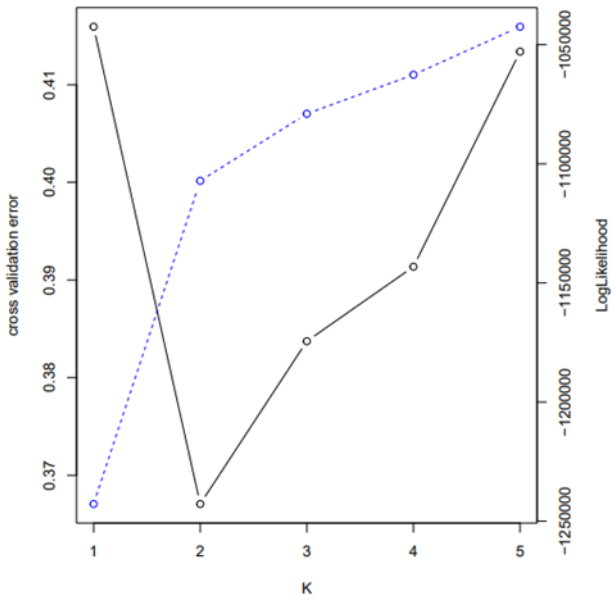

Figure S4: Cross validation error for *L. europaeus*, for each K value between 1 and 5. K = 2 was inferred as the best number of ancestral populations.

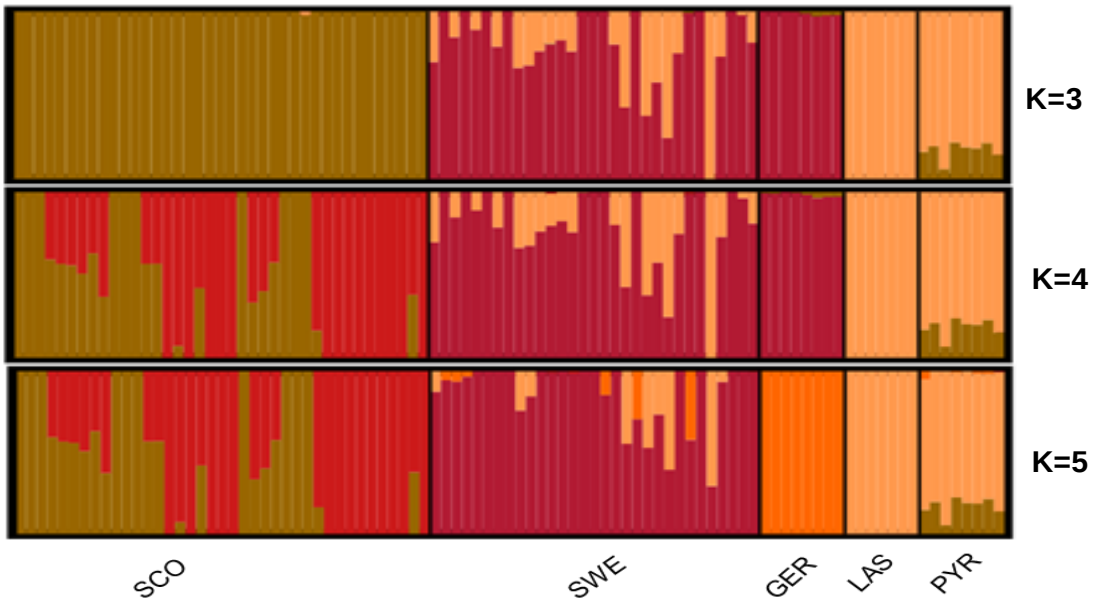

Figure S5: ADMIXTURE plots for K=4 to K=5. The analysis includes all the populations of *L. europaeus*: GER referring to Germany, SCO to Scotland, LAS to Lassée (Austria), PYR to Pyrenees and SWE to Sweden.

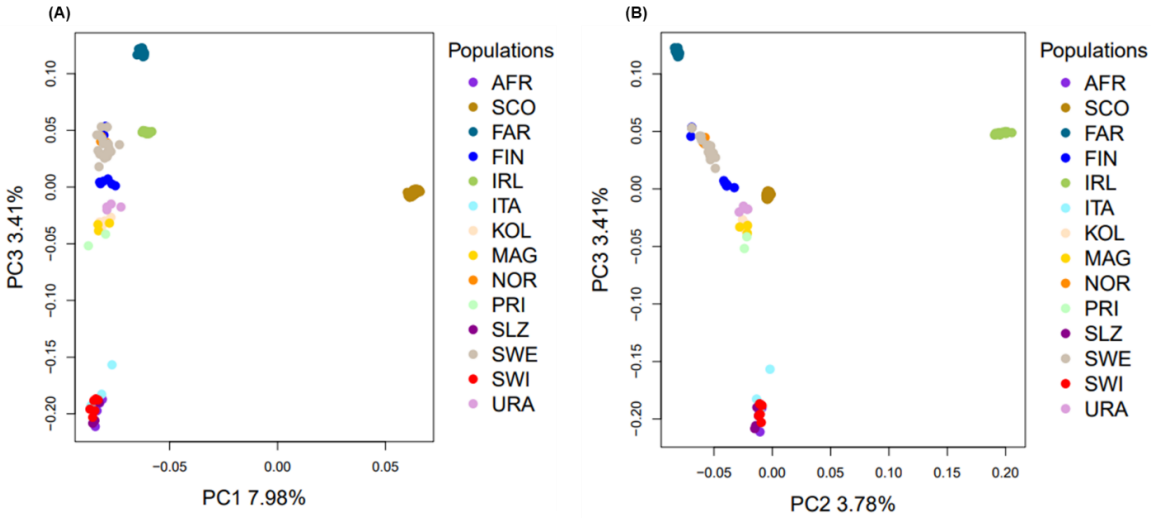

Figure S6: PCA plot *L. timidus*. (A) PC1 vs PC3. (B) PC2 vs PC3. The analysis includes all the populations of *L. timidus*: FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy, PRI to Primorsky territory (Russia), KOL to Kolyma River Basin (Russia), MAG to Magdan (Russia) and URA to Urals (Russia).

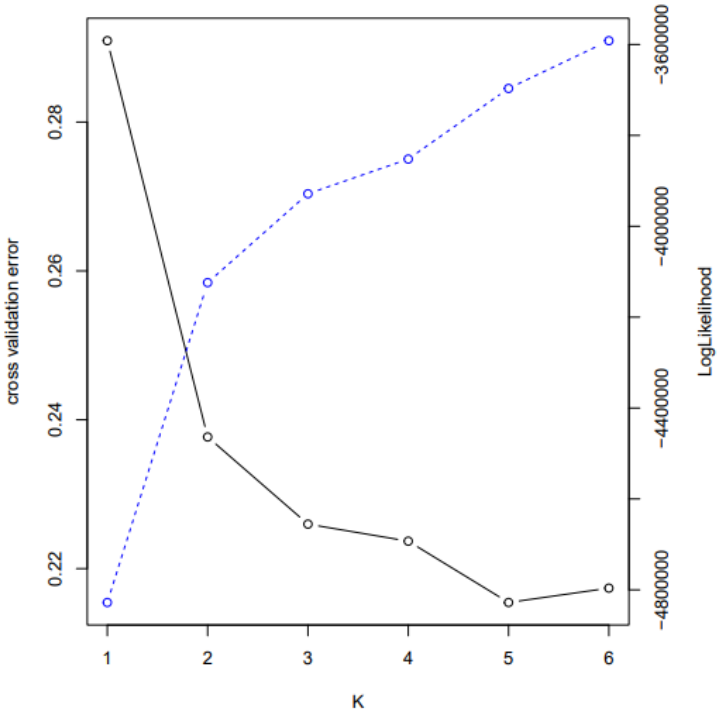

Figure S7: Cross validation error for *L. timidus*, for each K value between 1 and 5. K = 5 was inferred as the best number of ancestral populations.

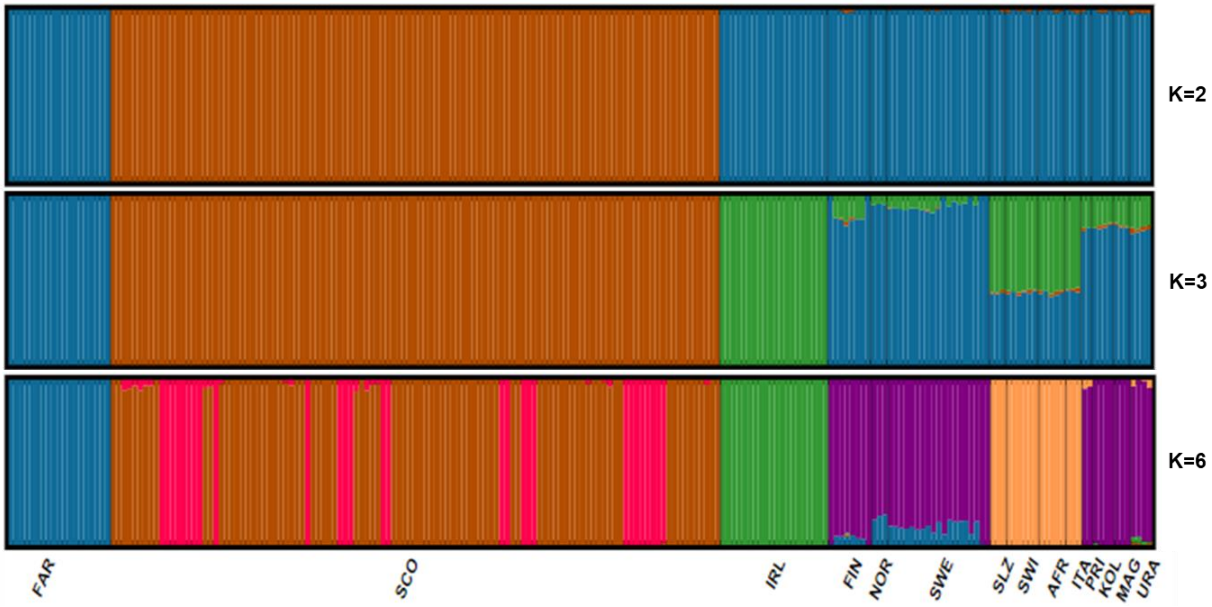

Figure S8: ADMIXTURE plots for K=2, K=3 and K=6. The analysis includes all the populations of *L. timidus*: FAR referring to Faroe Island, SCO to Scotland, IRL to Ireland, FIN to Finland, NOR to Norway, SWE to Sweden, SLZ to Salzburg (Austria), SWI to Switzerland, AFR to France, ITA to Italy, PRI to Primorsky territory (Russia), KOL to Kolyma River Basin (Russia), MAG to Magdan (Russia) and URA to Urals (Russia).

Appendix III- Genetic relationship between *L. europaeus* and *L. timidus*

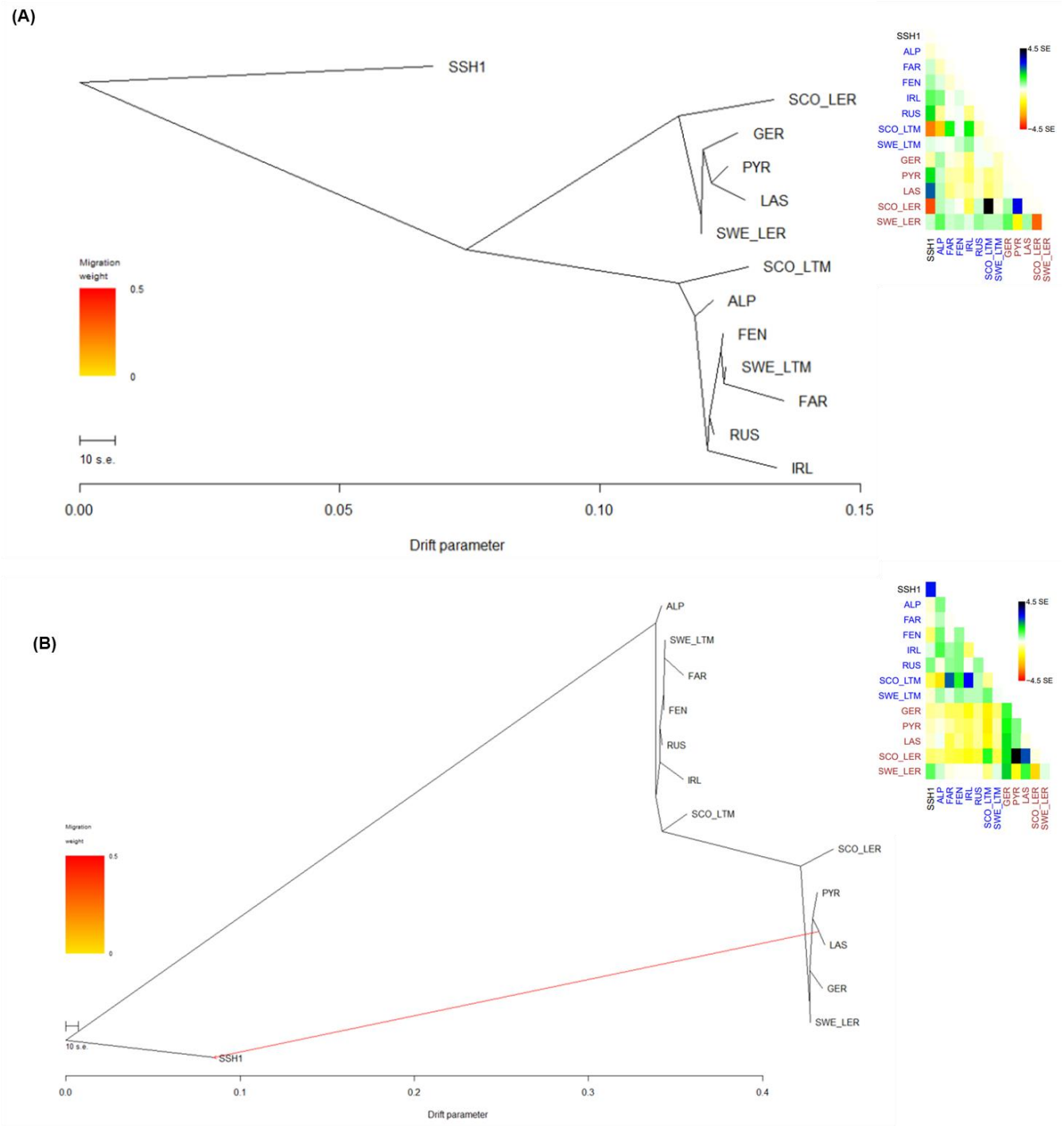

Figure S9: TreeMix phylogram with sample size correction disabled (-noss) with (A) zero migration events and (B) one migration event, and its matrix of residuals refers to the fit of the model to the data.

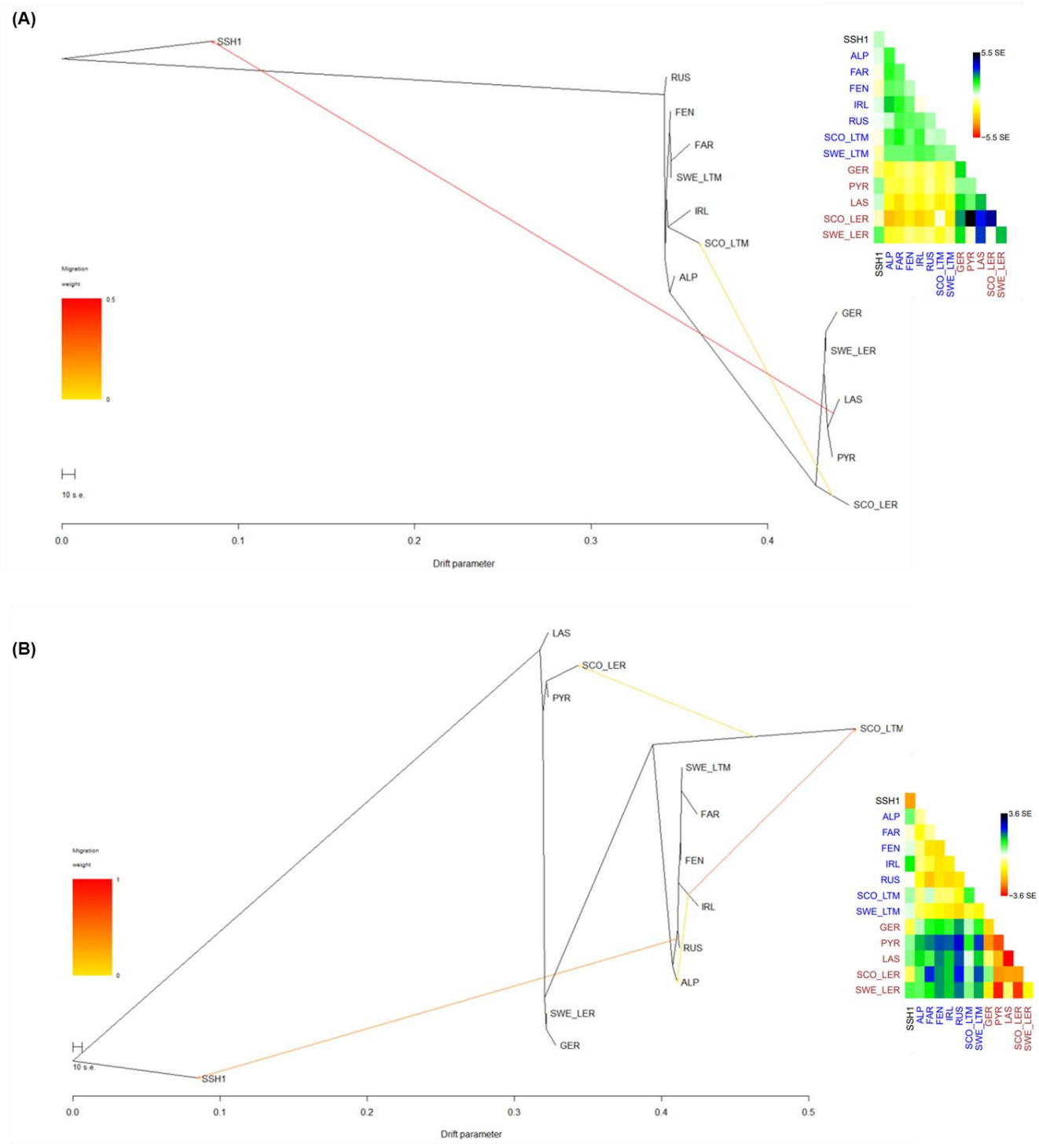

Figure S10: TreeMix phylogram with sample size correction disabled (-noss) with (A) two migration events and (B) four migration events, and its matrix of residuals refers to the fit of the model to the data.

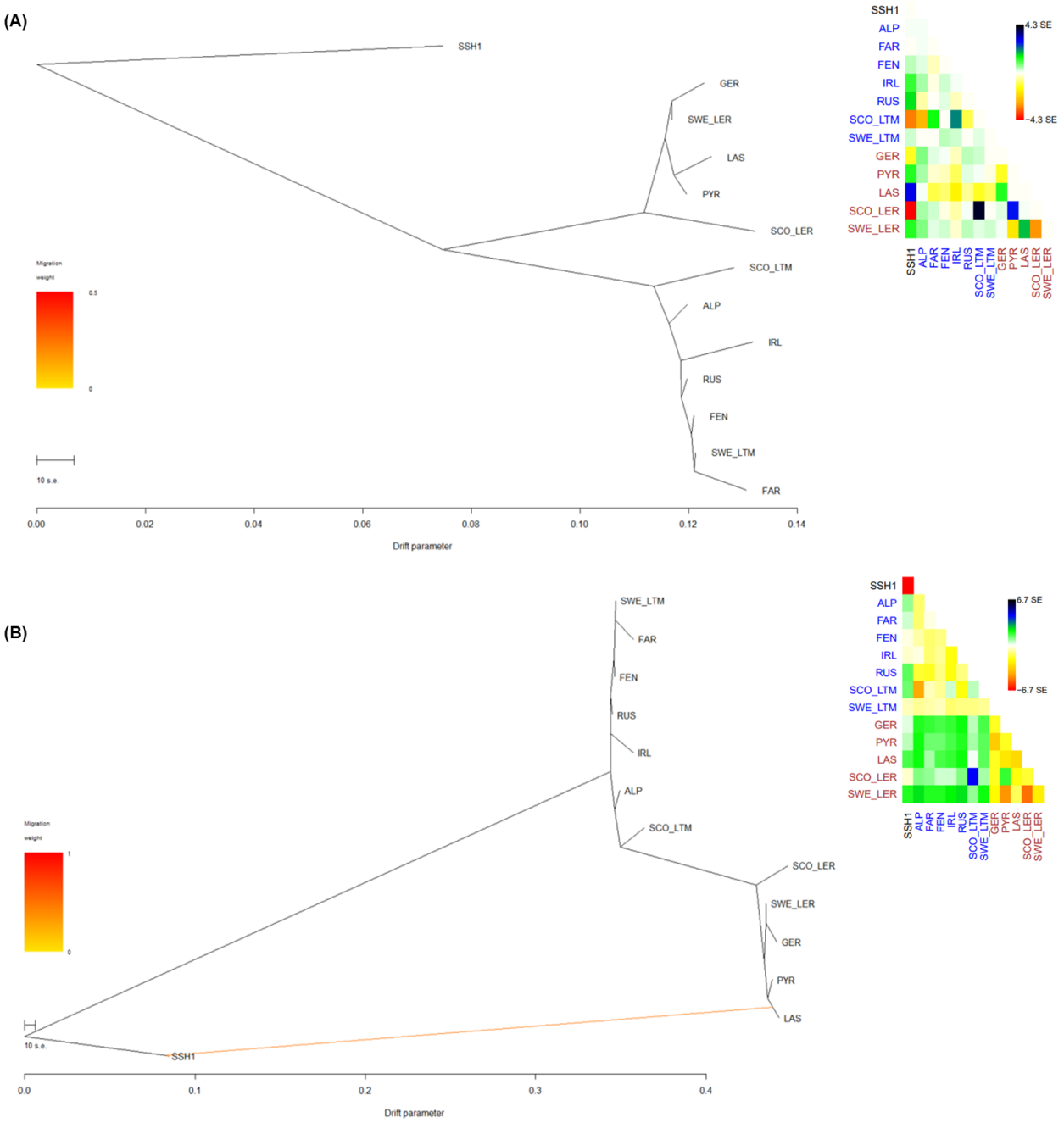

Figure S11: TreeMix phylogram with sample size correction with (A) zero migration events and (B) one migration events, and its matrix of residuals refers to the fit of the model to the data.

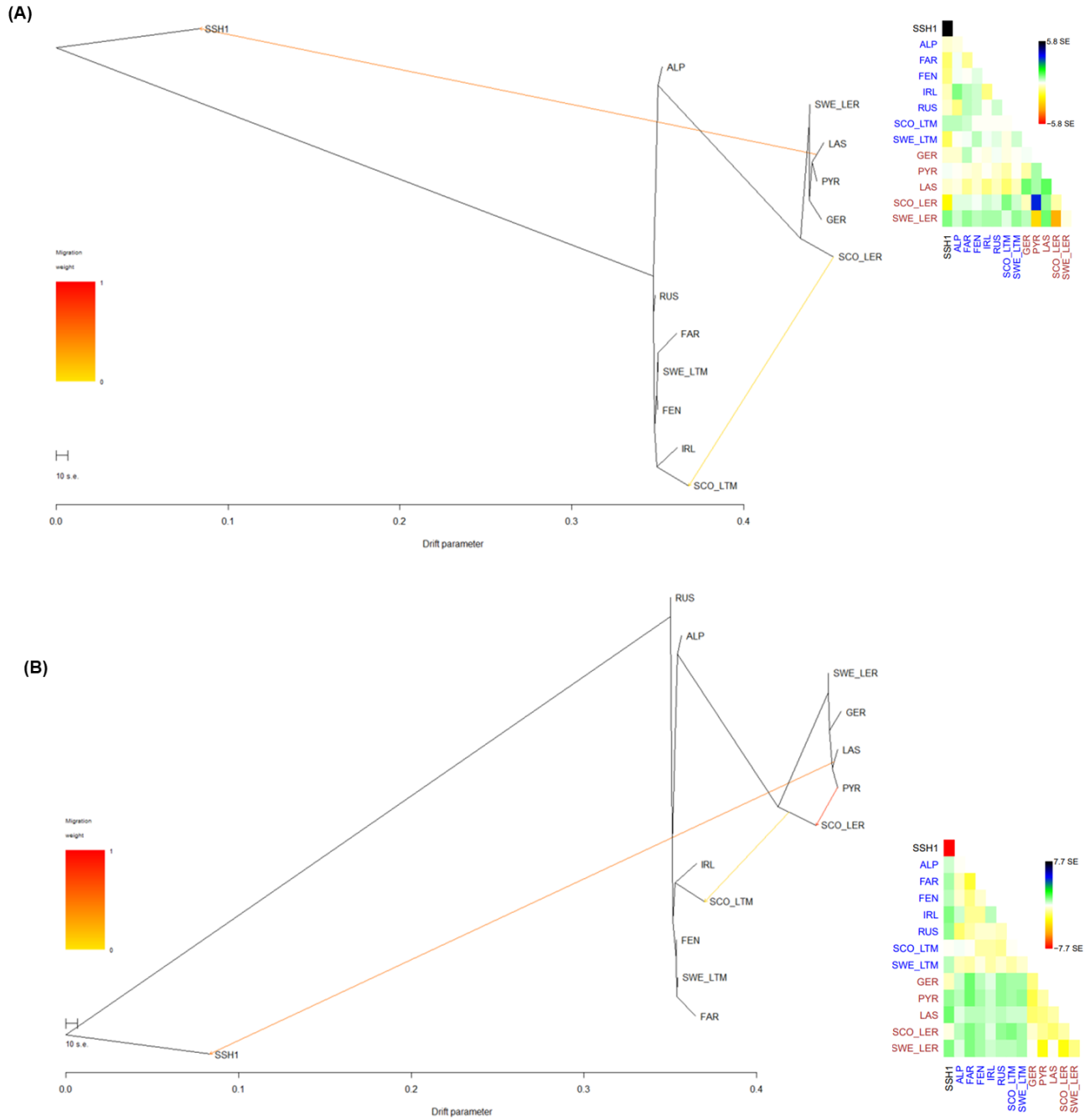

Figure S12: TreeMix phylogram with sample size correction with (A) two migration events and (B) three migration events, and its matrix of residuals refers to the fit of the model to the data.

Appendix IV- Broad genetic characterization of *L. europaeus* and *L. timidus* across the Scottish and Swedish contact regions

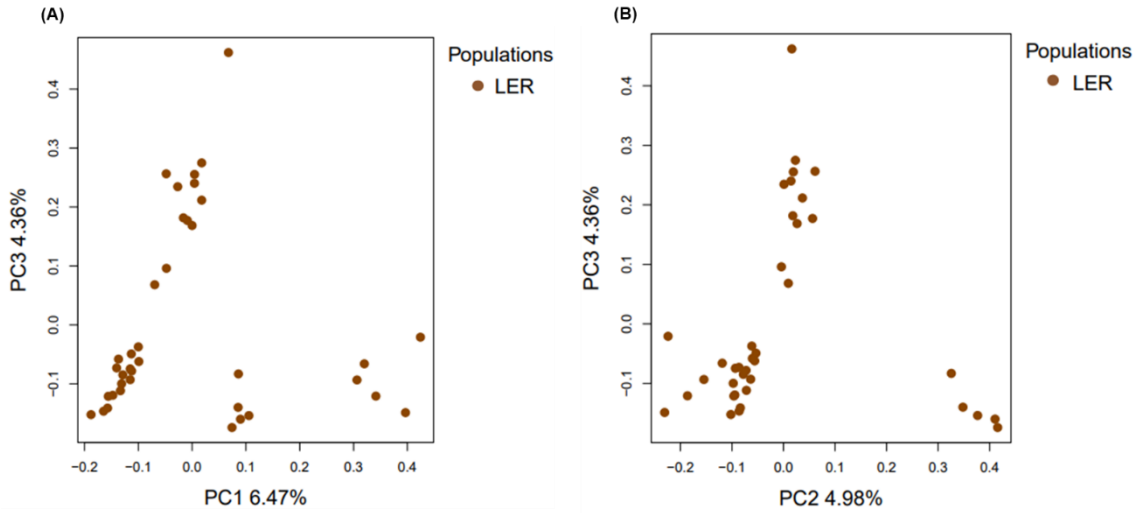

Figure S13: PCA plot Scottish population of *L. europaeus*. (A) PC1 vs PC3. (B) PC2 vs PC3.

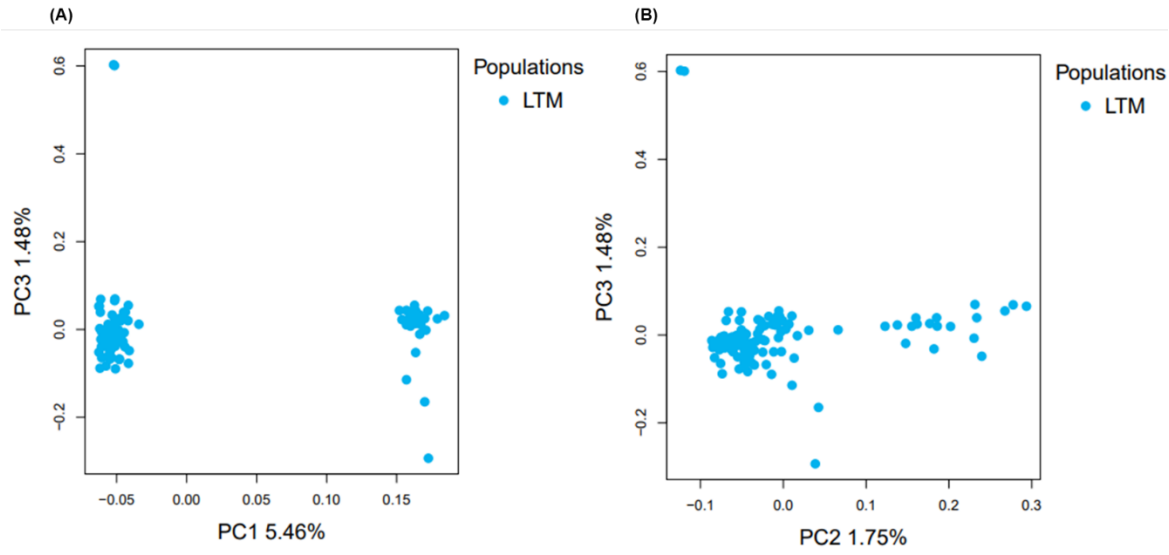

Figure S14: PCA plot Scottish population of *L. europaeus*. (A) PC1 vs PC3. (B) PC2 vs PC3.

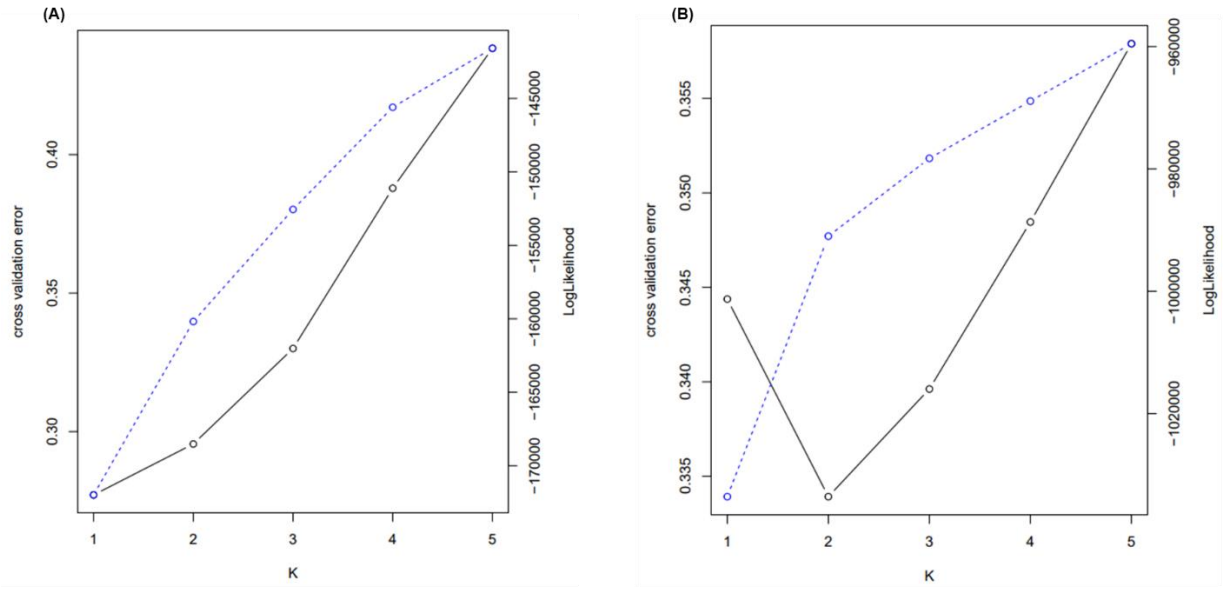

Figure S15: Cross validation error for Scottish populations of (A) *L. europaeus* and (B) *L. timidus*, for each K value.

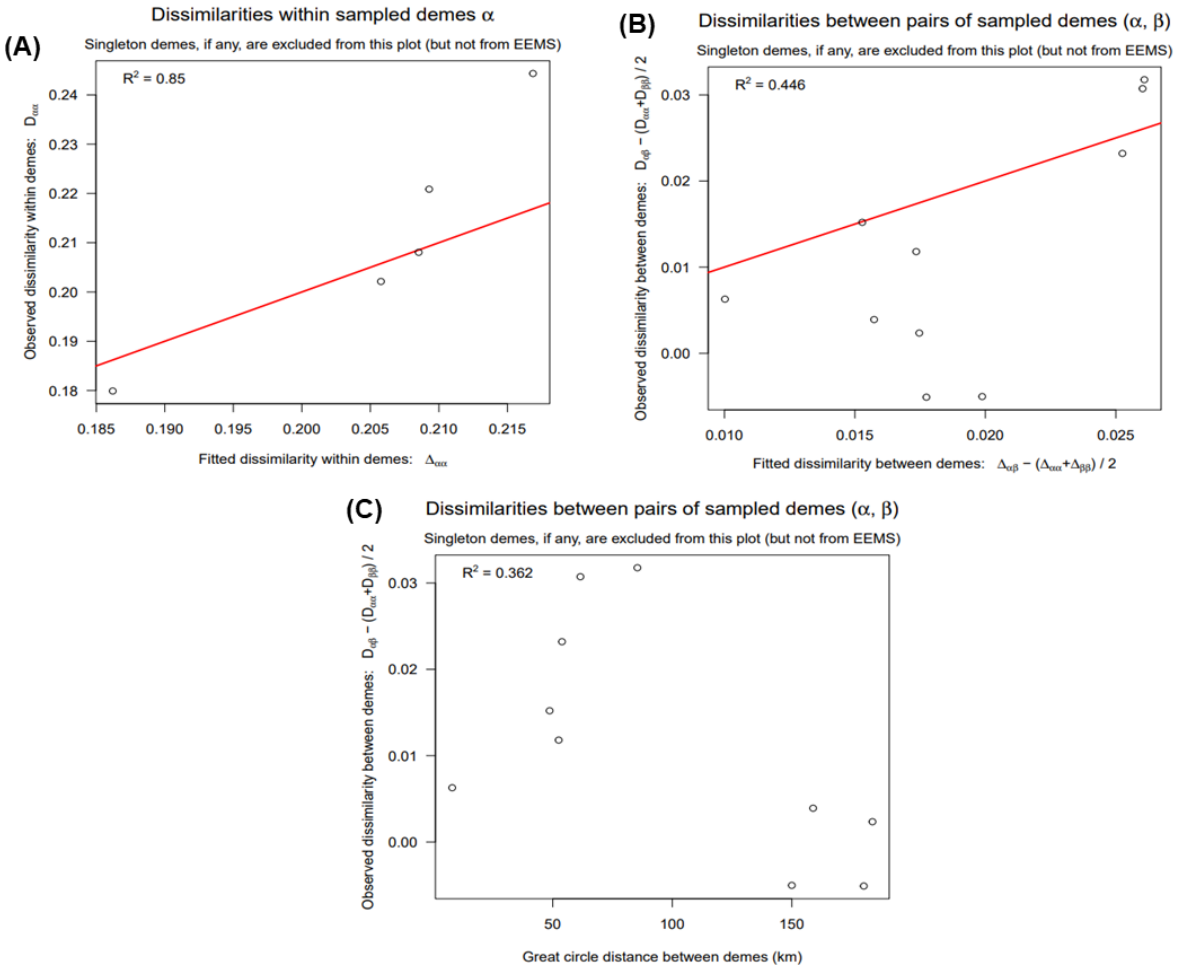

Figure S16: Diagnostic plots for EEMS model fitting for Scottish populations of *L. europaeus*. (A) Pairwise comparison of estimated and observed genetic dissimilarities between demes. (B) Pairwise comparison of estimated and observed genetic dissimilarities within demes. (C) Scatter plot of observed genetic distances with geographic distances between populations. The r^2 coefficient (shown at the top left of each plot) was estimated for each scatterplot.

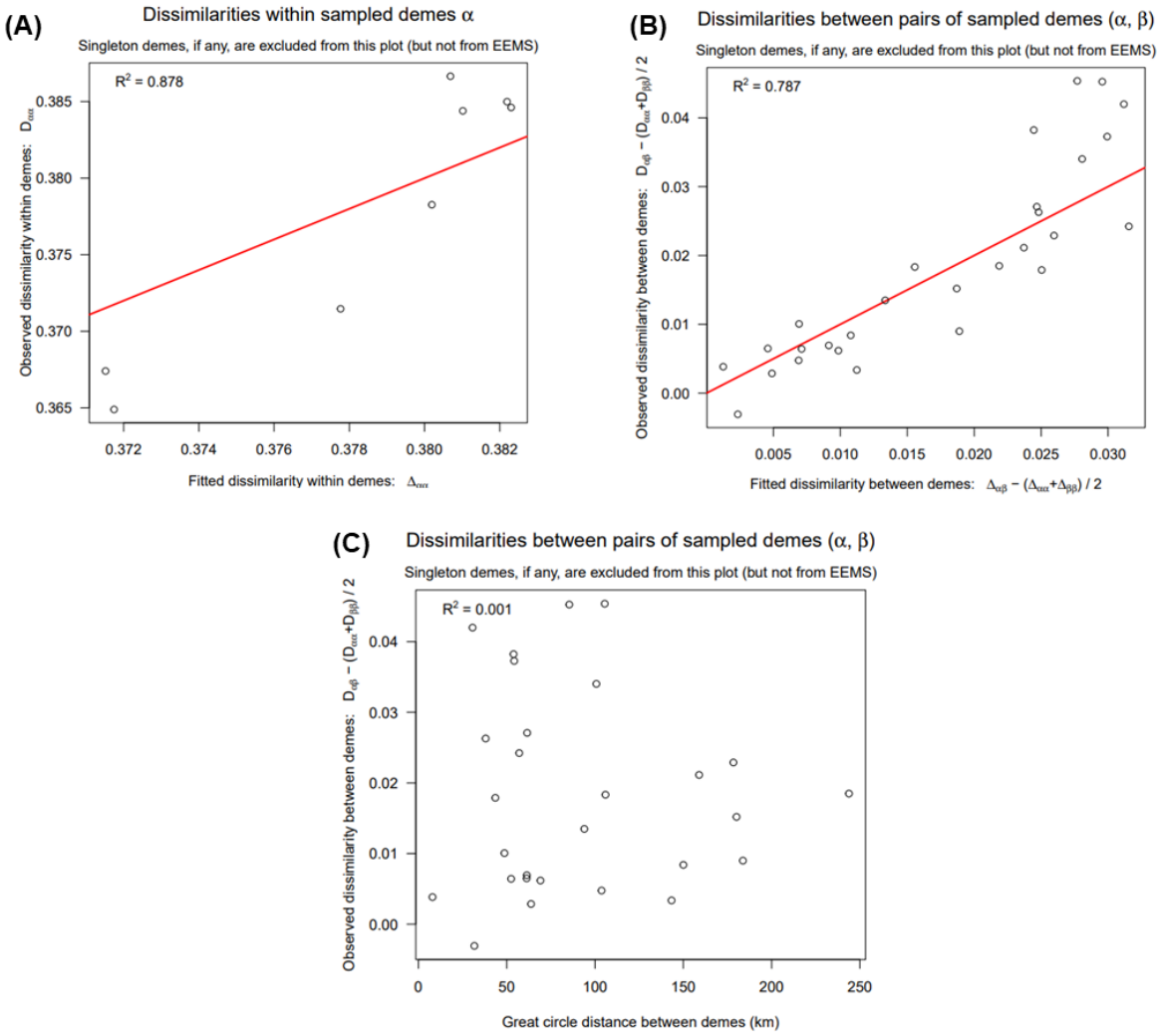

Figure S17: Diagnostic plots for EEMS model fitting for Scottish populations of *L. timidus*. (A) Pairwise comparison of estimated and observed genetic dissimilarities between demes. (B) Pairwise comparison of estimated and observed genetic dissimilarities within demes.(C) Scatter plot of observed genetic distances with geographic distances between populations. The r^2 coefficient (shown at the top left of each plot) was estimated for each scatterplot.

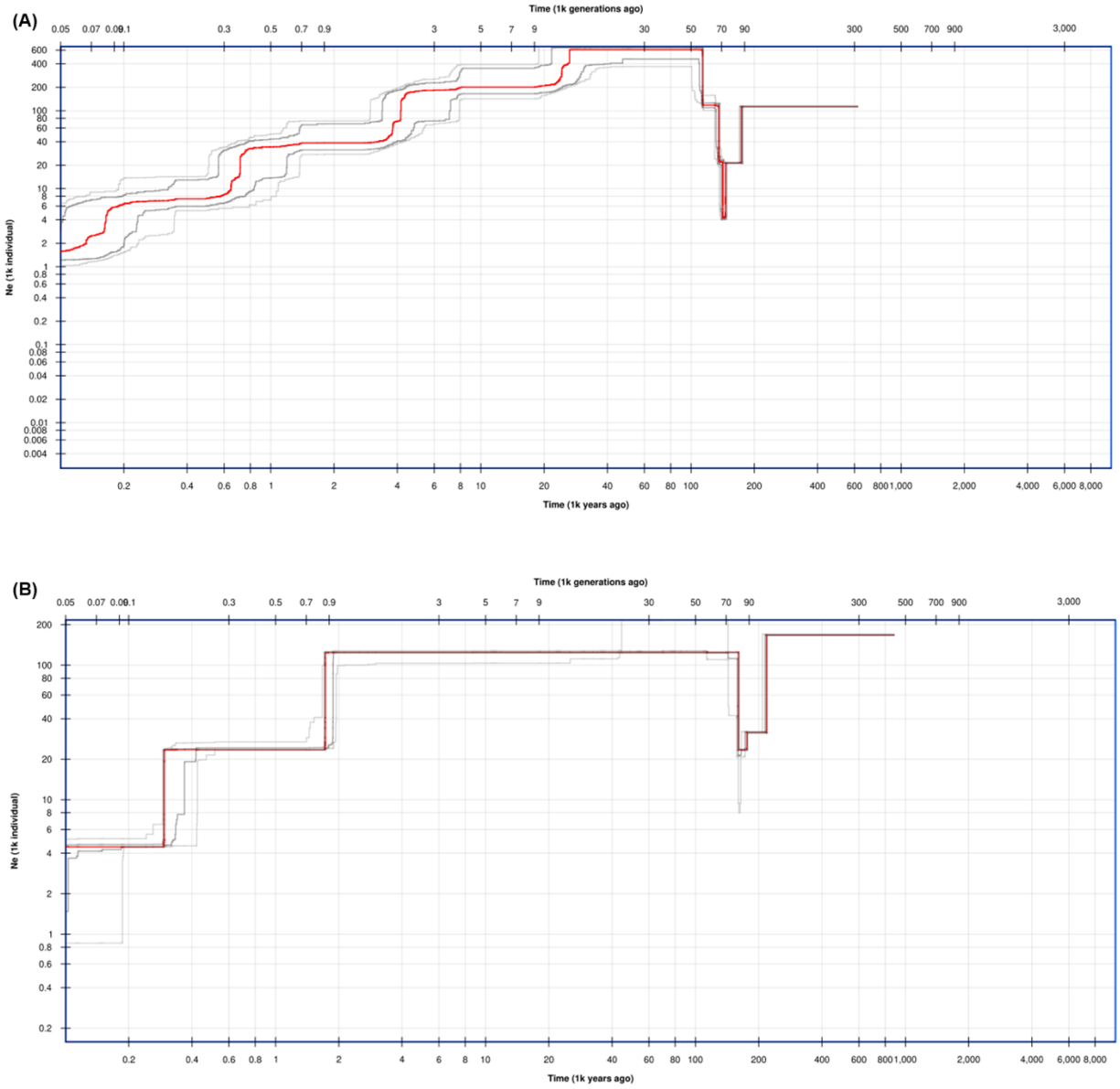

Figure S18: Stairway plot reconstructions of demographic history of (A) the Austrian population (LAS) of *L. europaeus* and (B) Russian population (URA) of *L. timidus*. The x-axis is the time in thousand years (lower scale bar) and in thousand generations (upper scale bar) before present. The y-axis is the effective population size in thousand individuals. The red line shows median of effective population size. The upper and lower thick and light gray lines show 75% and 95% confidence intervals, respectively.

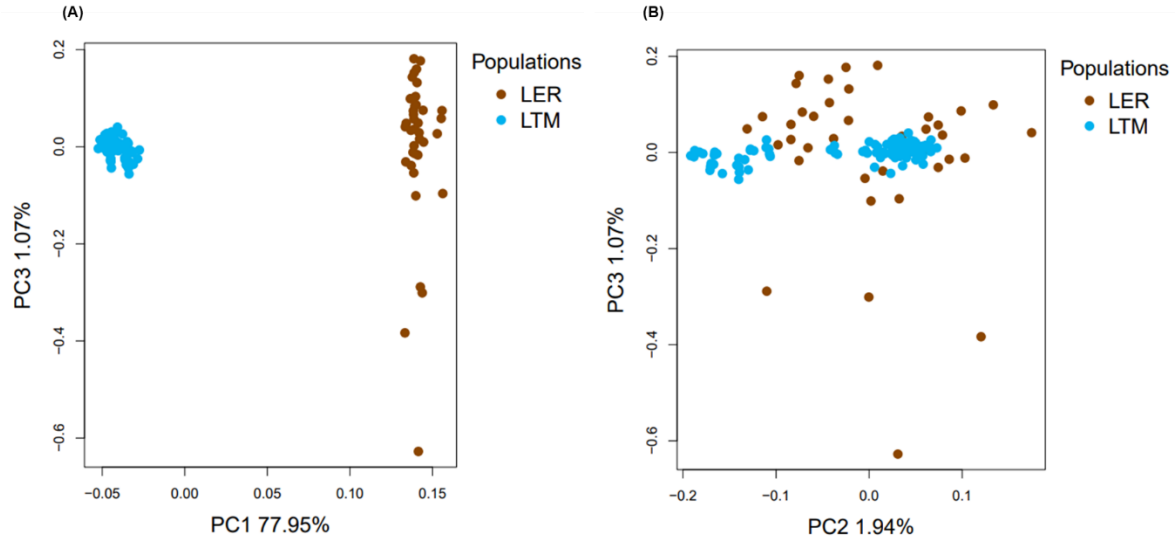

Figure S19: PCA plot for both Scottish populations: (A) PC1 vs PC3. (B) PC2 vs PC3. LER refers to *L. europaeus* and LTM to *L. timidus*.

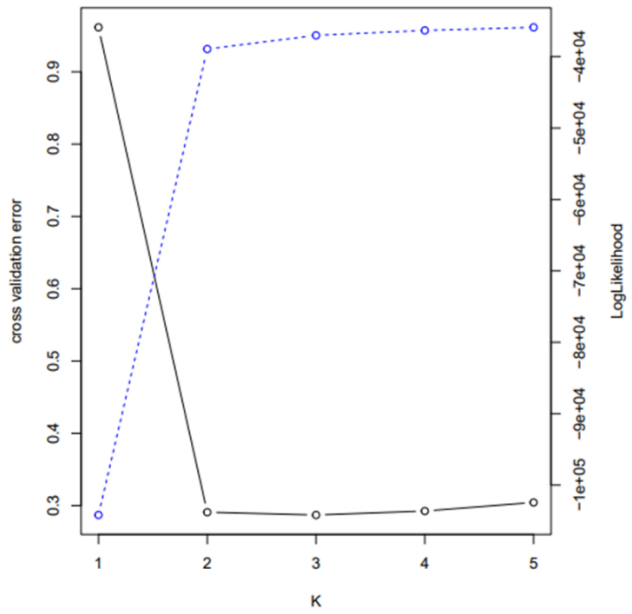

Figure S20: Cross validation error for Scottish populations of *L. europaeus* and *L. timidus*, for each K value between 1 and 5. K=3 was inferred as the best number of ancestral populations but K=2 is more informative for the present analysis.

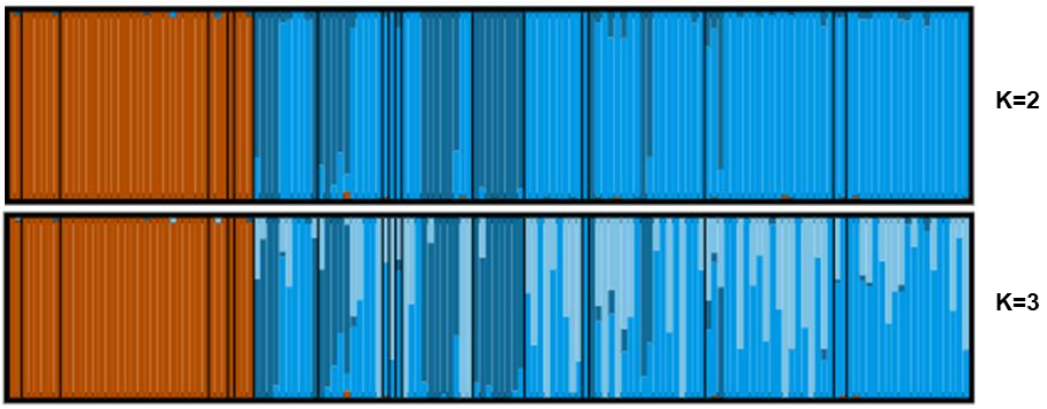

Figure S21: ADMIXTURE plots for K=2 and K=3. The analysis includes the Scottish population of *L. europaeus* (brown) and *L. timidus* (blue).

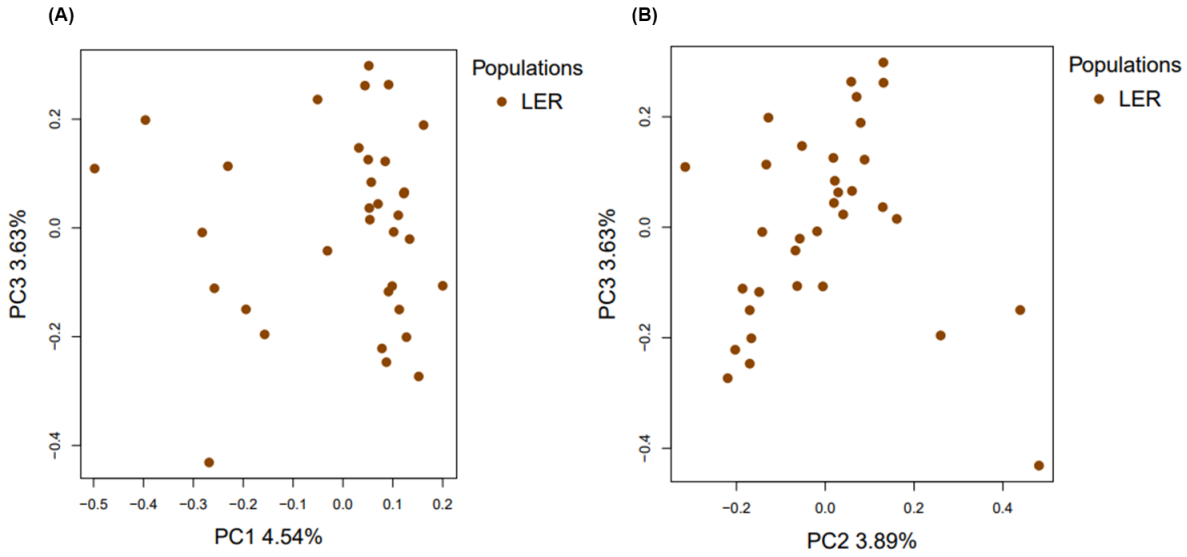

Figure S22: PCA plot Swedish population of *L. europaeus*. (A) PC1 vs PC3. (B) PC2 vs PC3

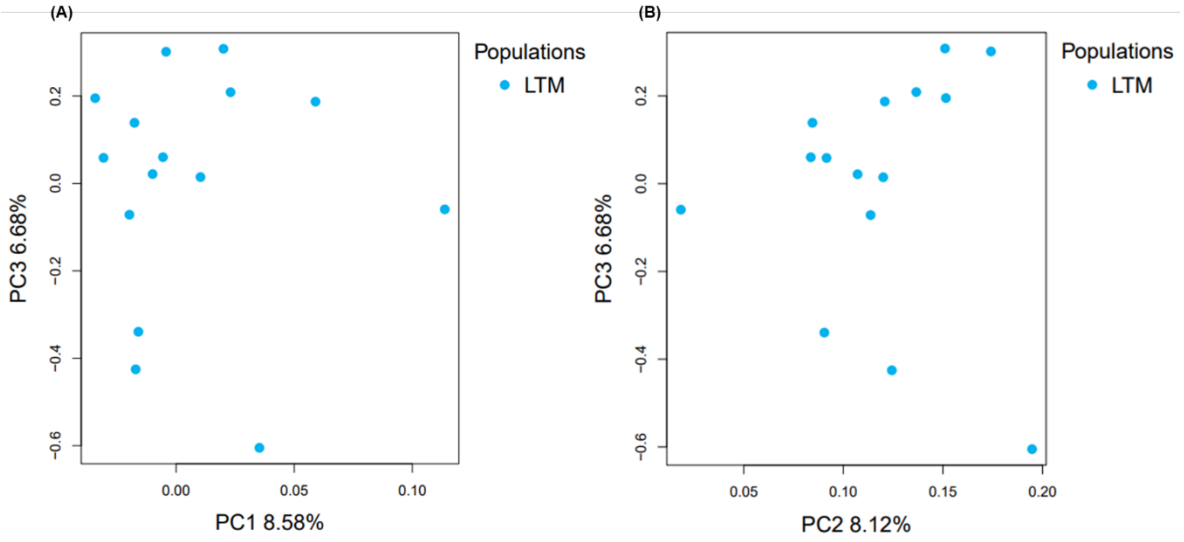

Figure S23: PCA plot Swedish population of *L. timidus*. (A) PC1 vs PC3. (B) PC2 vs PC3.

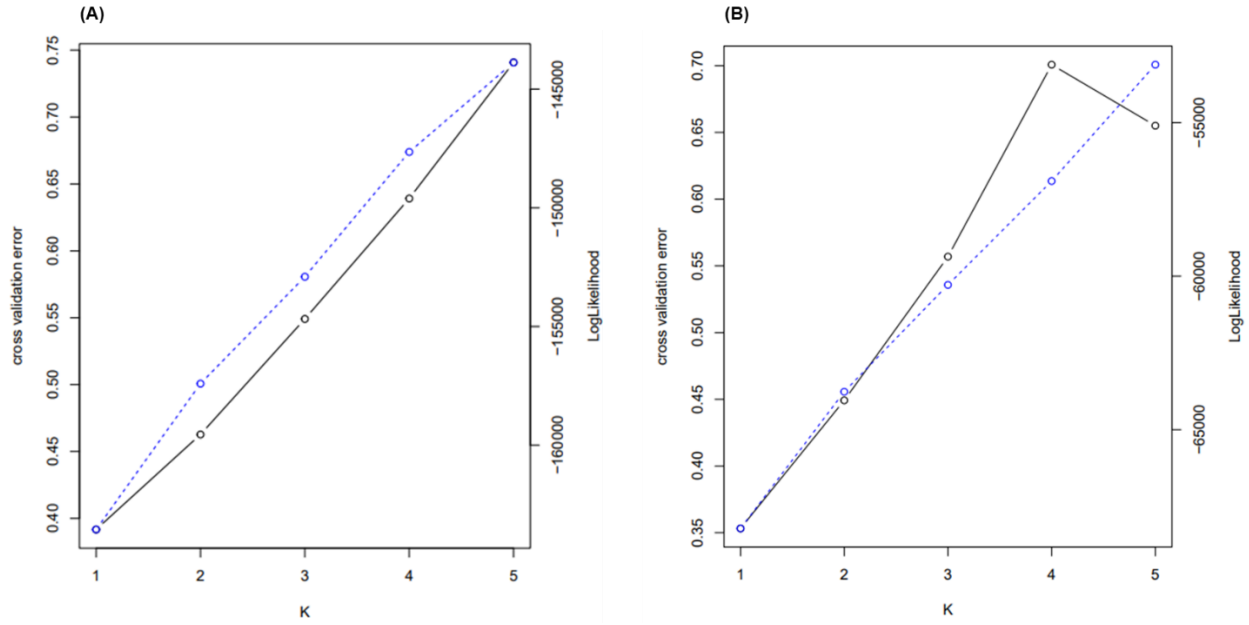

Figure S24: Cross validation error for Swedish populations of *L. europaeus* and *L. timidus*, for each K value between 1 and 5. K=1 was inferred as the best number of ancestral populations for both species.

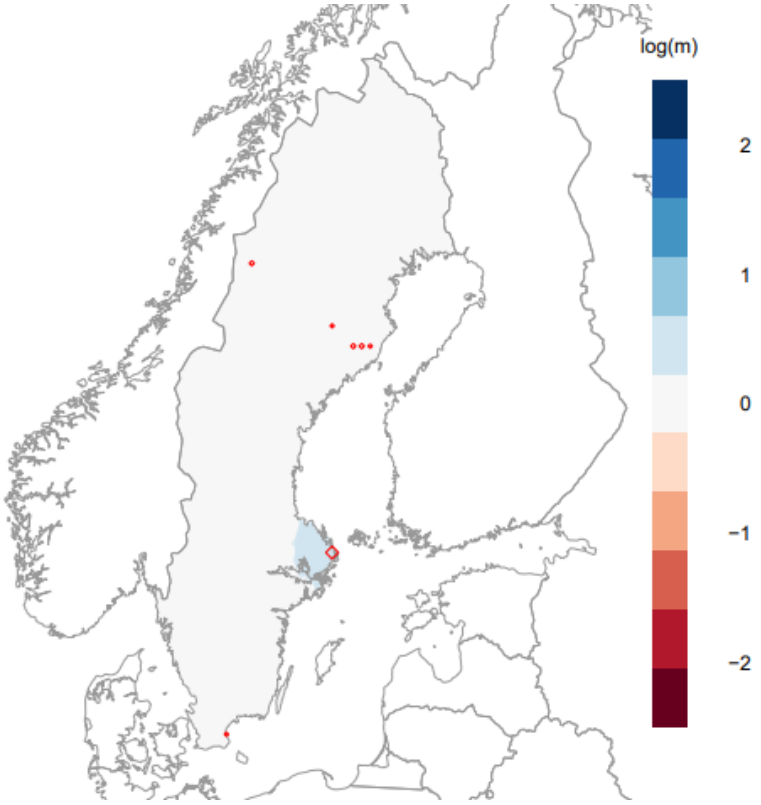

Figure S25: Effective Migration Maps estimated for the Swedish population of *L. timidus*. The plots were estimated with EEMS under a log10 scale and after mean centring. The blue indicates areas with an effective migration between populations above average (more genetic similarity) and the red regions with an effective migration below average (less genetic similarity).

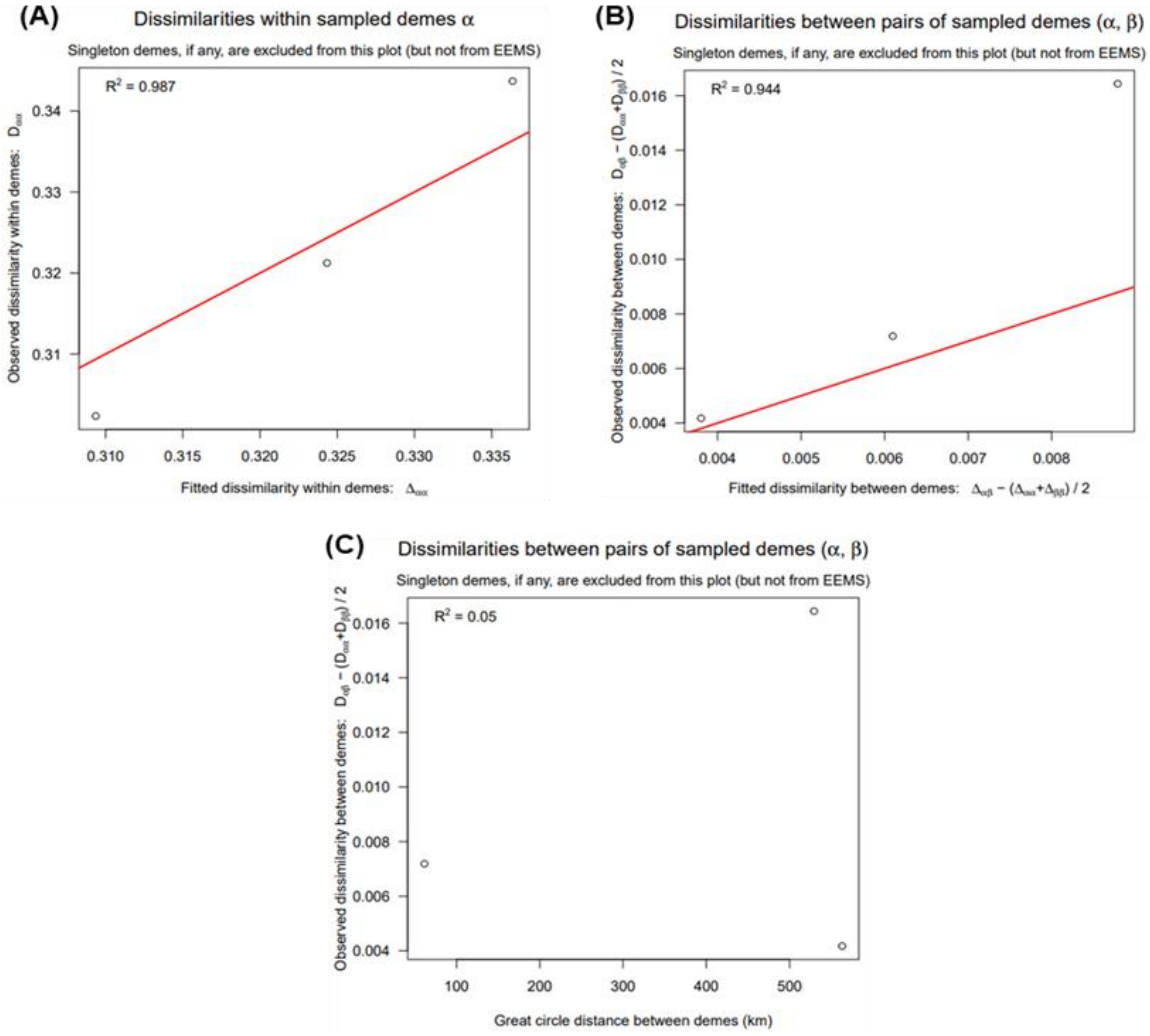

Figure S26: Diagnostic plots for EEMS model fitting for Swedish populations of *L. europaeus*. (A) Pairwise comparison of estimated and observed genetic dissimilarities between demes. (B) Pairwise comparison of estimated and observed genetic dissimilarities within demes.(C) Scatter plot of observed genetic distances with geographic distances between populations. The r^2 coefficient (shown at the top left of each plot) was estimated for each scatterplot.

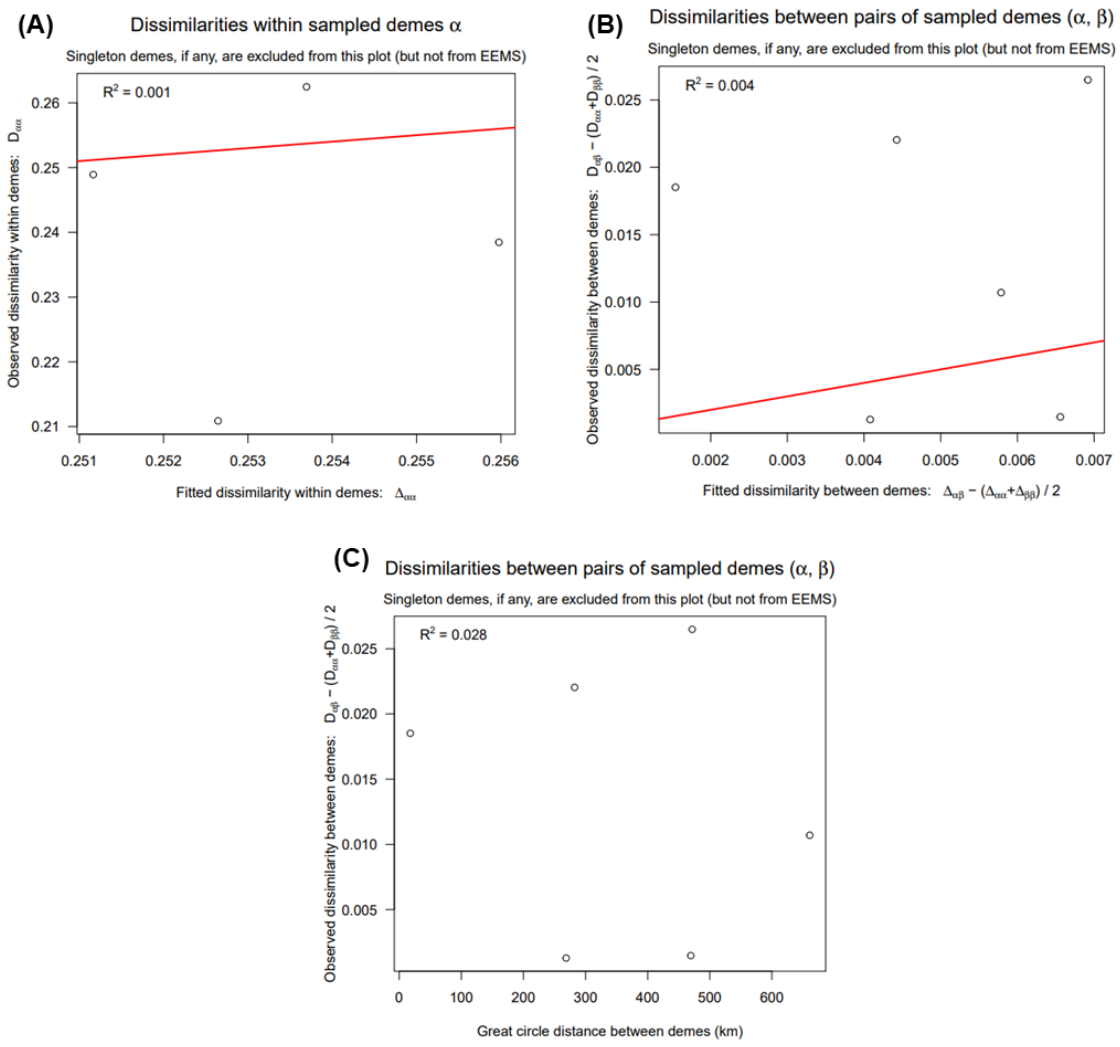

Figure S27: Diagnostic plots for EEMS model fitting for Swedish populations of *L. timidus*. (A) Pairwise comparison of estimated and observed genetic dissimilarities between demes. (B) Pairwise comparison of estimated and observed genetic dissimilarities within demes.(C) Scatter plot of observed genetic distances with geographic distances between populations. The r^2 coefficient (shown at the top left of each plot) was estimated for each scatterplot.

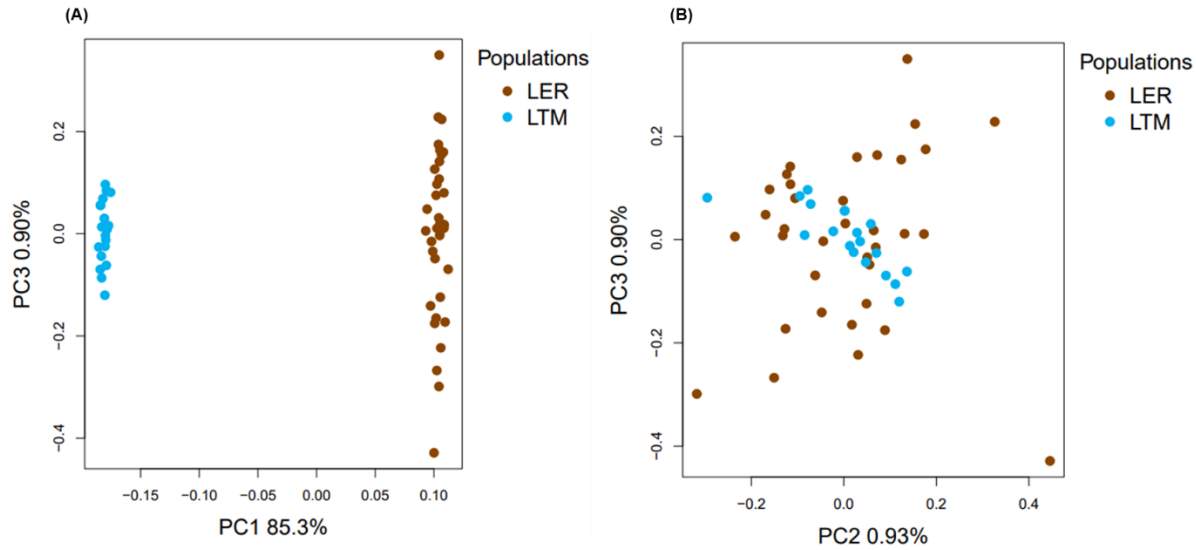

Figure S28: PCA plot for both Swedish populations: (A) PC1 vs PC3. (B) PC2 vs PC3. LER refers to *L. europaeus* and LTM to *L. timidus*.

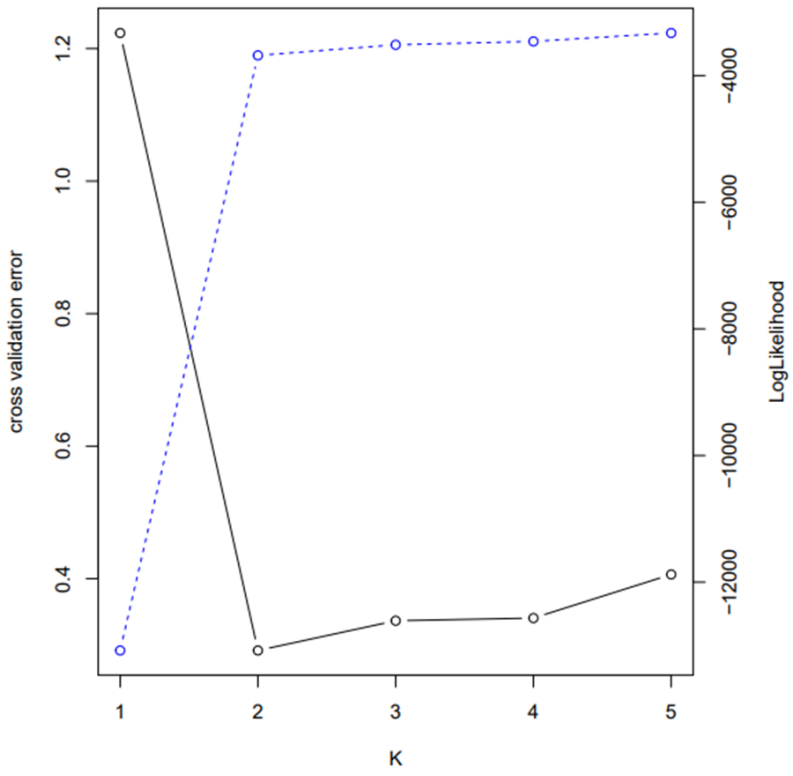

Figure S29: Cross validation error for Scottish populations of *L. europaeus* and L. *timidus*, for each K value between 1 and 5. K=2 was inferred as the best number of ancestral populations.