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Ferreira

Padrões ecológicos e genéticos da expansão do esquilo vermelho (*Sciurus vulgaris*) em Portugal

Uncovering ecological and genetic patterns of the eurasian red squirrel (*Sciurus vulgaris*) expansion in Portugal

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica do Doutor Carlos Manuel Martins Santos Fonseca, professor auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Rita Gomes Rocha, colaboradora do Departamento de Biologia da Universidade de Aveiro e do Doutor Eduardo Manuel Silva Loureiro Alves Ferreira, Professor Auxiliar Convidado e Investigador do Departamento de Biologia da Universidade de Aveiro

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Palavras-chave Sciuridae, distribuição, filogeografia, conservação, modelação, adequação de habitat, ciência cidadã.

Resumo O esquilo vermelho encontra-se amplamente distribuído nas florestas da Europa. Em Portugal, a espécie esteve extinta durante quase 400 anos devido à perda de habitat resultante da fragmentação florestal. A sua recente expansão no norte e centro do país deu-se após a recolonização natural a partir das populações espanholas e possivelmente a partir dos eventos de reintrodução nos anos 90. No entanto, padrões ecológicos e genéticos por trás da rápida expansão desta espécie no território nacional carecem de investigação. De forma a colmatar esta lacuna, foram realizadas análises moleculares com base em sequências de DNA mitocondrial obtidas em Portugal e uma série de sequências já conhecidas para a Europa, não tendo sido revelados sinais de expansão demográfica, contrariamente aos resultados obtidos para a população europeia. A população de Portugal partilha haplótipos ancestrais com Espanha e França, mostrando que ocorreu expansão natural desta espécie por indivíduos vindos de territórios vizinhos. No entanto, um indivíduo partilha o haplótipo de D-loop com um individuo do norte da Itália, sendo possivelmente uma linhagem proveniente de reintroduções passadas. Foi ainda criado um modelo preditivo de adequação de habitat com variáveis climáticas e de uso do solo, prevendo áreas de adequação do habitat para a espécie em ambientes urbanos e semiurbanos e evidenciando um padrão de proximidade às florestas. A precipitação e as áreas verdes urbanas, foram as variáveis que mais contribuíram para explicar a atual distribuição da espécie, tendo sido também prevista a futura expansão para sul do Tejo com base nestes indicadores e a proximidade à floresta. No entanto, é de ressaltar que este modelo pode estar enviesado devido aos dados geográficos de presença do esquilo terem sido obtidos através de um projeto de ciência cidadã, que apresenta algumas limitações como a ausência de dados em locais menos urbanizados. Questões relacionadas com a presença da espécie em ambientes urbanizados constituem um novo desafio para futura investigação sobre os padrões ecológicos que afetam a distribuição de esquilo vermelho neste tipo de habitat.

Keywords

Abstract

Red squirrels are widely distributed across most European forests, while in Portugal they have been absent for almost 400 years due to habitat loss and forest fragmentation. Recent expansion in the north and centre of the country derived from natural recolonization from Spanish population and possibly from reintroduction events in the 90s. However, ecologic and genetic patterns behind this species rapid expansion in the territory lacked scientific research. For that, a phylogeographic analysis was conducted using mitochondrial DNA sequences obtained in Portugal, showing no signs of demographic expansion, contrary to European population dataset. The population of the red squirrel in Portugal shares haplotypes with Spain and France, evidencing a pattern of natural colonization of the territory from neighbouring countries. However, the D-loop haplotype identified in one individual was shared with an individual from northern Italy, suggesting the possibility of representing a descendent of previous reintroductions. Additionally, environmental factors affecting red squirrel distribution were modelled to predict habitat suitability areas for the species based on climate and land cover variables. These models have predicted the occurrence of the red squirrel in urban and semi-urban environments and highlighted a pattern of proximity to forests. Precipitation and green urban areas were the variables contributing the most to explain species current distribution and were useful to predict future expansion southwards Tejo river, based on these predictors and proximity to forests. However, it is important to know that the models here generated may be affected by sampling bias due to data collection through a citizen science project. This approach has limitations, such as the smaller amount of observations recorded in less urbanized environments. These results provide insights that will help us to solve questions related with the presence of this species in human-shaped environments and pose a challenge for future research on the ecological patterns affecting the red squirrel distribution in these habitats.

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CHAPTER 1

GENERAL INTRODUTION

THE EURASIAN RED SQUIRREL (*Sciurus vulgaris,* Linnaeus 1758)

The Eurasian red squirrel (*Sciurus vulgaris*) is a small mammal of the order Rodentia, family Sciuridae, commonly widespread across most woodlands of Eurasia (Shar et al. 2016). This species occurs mainly in coniferous and mixed deciduous forests dominated by pine but also in urbanized environments such as city parks, gardens and small stands of conifers (Lurz et al. 2005; Shar et al. 2016; Gurnell & Pepper 1991).

Red squirrels do not present sexual dimorphism, as males and females are about the same size and colour. Although its common name may be confusing, red squirrels fur colour varies from deep brown to red brown, bright red or even greyish or blackish colours in dorsal coat, contrasting with whitish or creamy on the underside. Red squirrels grow prominent ear tufts throughout most of the year, which are a specific character of this species (see Figure 1.1) and enable its identification in the presence of other squirrel species (Gurnell et al. 2001, Lurz et al. 2005).

As a diurnal foraging species, the activity of *S. vulgaris* has been broadly studied. Although the red squirrel does not hibernate, it shows patterns of minimal activity during the colder months. During the rest of the year red squirrels show higher activity patterns, with peaks of activity during the morning (Tonkin 1983; Wauters & Dhondt 1987; Lurz et al. 2005). Such patterns favour squirrel sightings by citizens during

Figure 1.1. Eurasian red squirrel, *Sciurus vulgaris* (© Nelson Nascimento).

the day, which makes them a suitable species for the success of citizen science projects based on the volunteer observations of the species.

Red squirrels have a solitary behaviour and are territorial within sexes. They often use scent marks made of urine as signs of occupation of home range, social status and reproductive condition (Wauters & Dhondt 1992; Lurz et al. 2005). Female red squirrels can breed twice in a year, usually in winter and spring, and can produce two litters of one to six offspring when food resources are not scarce (Gurnell 1983; Wauters & Lens 1995).

Dietary sources in *S. vulgaris* are mainly composed of tree seeds and acorns, berries, fruits and fungi, although they may include others such as bark and sapwood, flowers, shoots and occasionally bird eggs, nestlings, invertebrates and lichens (Lurz et al. 2005; Krauze-gryz & Gryz 2015; Shar et al. 2016). Due to their behaviour of hoarding seeds on the ground, they are likely to promote seed germination and forest regeneration, while keeping food reserves for when seed availability is lower (Wauters & Lens 1995; Forget et al. 2005). Hence, S. vulgaris plays an important role in reforestation processes.

Major predators in most European woodland habitats include: pine marten (*Martes martes*); goshawk (*Accipiter gentilis*); buzzard (*Buteo buteo*); and owls (Petty et al. 2003; Wauters et al. 2004; Lurz et al. 2005). While foraging on the ground, red squirrels may also be predated by red fox (*Vulpes vulpes*) (Kenward & Hodder 1998) and by domestic cats (*Felis silvestris catus*) in urbanized environments (Magris & Gurnell 2002).

CONSERVATION STATUS IN EUROPE

The Eurasian red squirrel (*Sciurus vulgaris*) is currently protected over regulation of Appendix III of the Bern Convention and classified as *Least Concern* in Europe by the IUCN - International Union for the Conservation of Nature (Shar et al. 2016). The Eurasian red squirrel is widespread across most European regions (Figure 1.2), although some exceptions occur in the south-east of Iberian Peninsula and in most Mediterranean islands, where it is mainly absent. Although red squirrel distribution is ample, recent population declines have been widely documented, particularly in United Kingdom and Northern Italy (Gurnell & Pepper 1993; Ogden et al. 2005; Bertolino et al. 2014) where the invasive Eastern gray squirrel (*Sciurus carolinensis*) has ben introduced.

Competitive exclusion and subsequent replacement of native red squirrel populations due to the introduction of the invasive gray squirrel are well-known in Europe

(Gurnell & Pepper 1993; Lawton et al. 2010; Bertolino et al. 2014). More than thirty different occasions of intentional introduction of gray squirrels took place in Great Britain and Ireland between 1876 and 1929 (Middleton 1930; Gurnell & Pepper 1993; Teangana et al. 2000) and several more in Italy between 1948 and 1994 (Bertolino 2008; Martinoli et al. 2010).
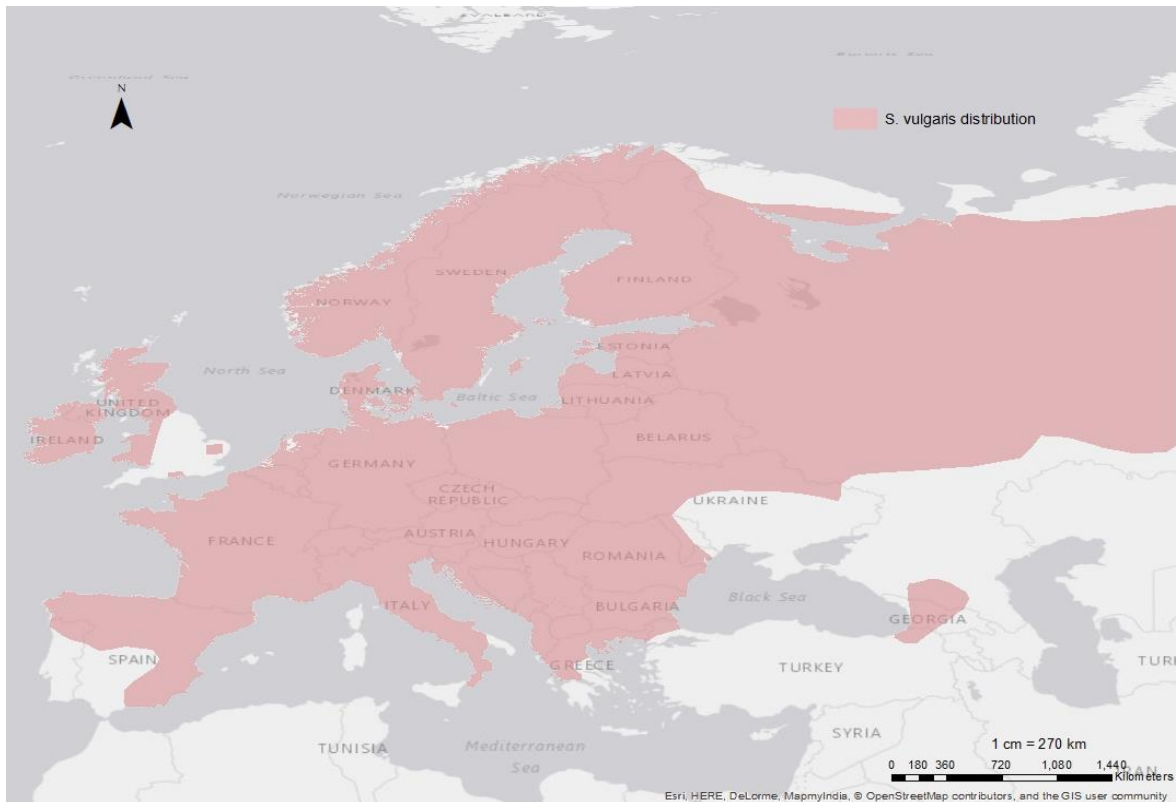
Figure 1.2. Current distribution of the Eurasian red squirrel in Europe (adapted from IUCN 2016).

Gray squirrel invasive settlement and expansion is a cause for concern for many ecologists and nature conservationists, as they have been replacing native species in overlapping regions, thus threatening the viability of red squirrel populations (Kenward & Hodder 1998; Mathias & Gurnell 1998; Rocha et al. 2014; Bertolino et al. 2014). Currently, the range of the gray squirrel in Europe covers most of England and Wales, a portion of Scotland and Eastern Ireland, as well as expanding areas in North Western and Central Italy (Bertolino 2008; Martinoli et al. 2010). Conservation measures and preventive actions are being implemented to protect red squirrel throughout invaded areas (Pepper & Patterson 1998; Bertolino & Genovesi 2003; Robertson et al. 2017).

CONSERVATION STATUS IN PORTUGAL

The Eurasian red squirrel *(Sciurus vulgaris)* is classified as *Least Concern* in Portugal (Cabral et al. 2005), being currently under expansion in this country (Rocha et al. 2017). However, the history of this species was not always as promising as nowadays.

The red squirrel became extinct in Portugal by the 16th century. Massive deforestation for the naval industry was pointed as the cause of accentuated declines in the red squirrel population due to forest fragmentation and subsequent habitat loss (Mathias & Gurnell 1998; Rocha et al. 2014).

During the 19th century, three attempts of red squirrel reintroduction have been carried in Portugal. The first two attempts were carried in the central region, in Sintra and Belas, with animals being brought from continental Europe (unspecified) and England, respectively (Mathias & Gurnell 1998). The population from Belas have survived for more than three decades before it was considered extinct. The last attempt was carried in the northern region, Fornos de Algodres, with specimens being brought from Belgium (Mathias & Gurnell 1998). All of them were considered unsuccessful as none of the animals was captured or seen in the next 50 years (Mathias & Gurnell 1998).

Since 1980, the species started to expand through natural recolonization from Spanish populations across Portuguese northern borders. In 1991 the red squirrel was classified as *Rare* and acquired legal protection (Mathias & Gurnell 1998). Shortly after that, three important and successful reintroductions took place in Portugal, which may have reinforced species dispersal into adjacent areas (Mathias & Gurnell 1998; Vieira et al. 2015): in 1993 (Parque Florestal de Monsanto, Lisboa); in 1994 (Jardim Botânico de Coimbra); and in 1997 (Parque Biológico de Gaia). The reintroduced animals were mainly from Madrid (Spain), though in Parque Biológico de Gaia donor specimens were from Azé, France (Vieira et al. 2015). The expansion of the red squirrel quickly succeeded in the next years and by 2001 it was already widely distributed up to the northern areas above Douro river (Ferreira et al. 2001). Currently, it is known that the species already crossed Tejo river in central Portugal and is rapidly expanding southwards (Rocha et al. 2017) but ecological factors behind its sudden expansion are yet to be explained.

The red squirrel, among many other species, is highly dependent on woodland habitats (Andrén & Lemnell 1992), although in Portugal almost 50% of forested areas

belong to monocultures of *Pinus pinaster* and *Eucalyptus spp.* (ICNF 2013). While coniferous forests constitute the habitat of selection for squirrels, providing abundance of food resources and shelter (Andrén & Lemnell 1992), other habitats may constitute low quality and low suitability fragments. Nevertheless, connectivity between fragments seems to be an important aspect favouring the expansion of the red squirrel in Portugal (Rocha et al. 2017). In addition, the increase of habitat suitability and food resources for the red squirrel within pine forests may also be related with likely expansion routes from the eastern border with Spain predicted by Rocha et al. (2014) and supported by Rocha et al. (2017) results.

DATA COLLECTION THROUGH CITIZEN SCIENCE

O Esquilo Vermelho em Portugal */ The Red Squirrel in Portugal*

1. Project description

A citizen science project was created in 2013 to monitor red squirrel distribution in Portugal, mainly by gathering local observations by citizens. This emerged from the need to understand red squirrel distribution in Portugal, due to the lack of studies in Portuguese territory since its recent expansion from 1980. The project was therefore used to collect, share and promote scientific knowledge on the species, its ecology and behaviour. Besides that, this project aims to raise public awareness about the red squirrel conservation and its

ecological functions in the ecosystem and to promote actions and change of attitude towards protection of the species and its natural habitat, conifer and deciduous forests.

Initially, a page was created on Facebook (https://www.facebook.com/pages/O-esquilo-vermelho-em-Portugal/547722785306913) to collect data and share information about the species and the project. Then, an online form was created to collect past and present squirrel observations nationally, in which about 500 citizens replied (please see Rocha et al. 2017). Local media and social networks were used to advertise and spread the project to the citizens. Currently, the Facebook page has about 4000 followers. In general, interactions with page followers, publication shares, likes, comments and private messages, are daily positive. The public can continuously contribute to the project by sending records of squirrel sightings and/or traces as well as roadkill animals, and their respective geographical locations. Besides that, citizens are still involved in a campaign to collect DNA samples such as hair tufts from roadkill animals, contributing to the study of population genetics of the red squirrel in Portugal (please see detailed methodology of data collection below). Furthermore, educational activities addressed to the community are being developed to raise awareness about the red squirrel conservation and protection of its natural habitat, encouraging citizens to respect nature, biodiversity and ecosystems.

2. Methodology for data collection

Geographical records: Citizens were asked to collect geographical records of red squirrels in Portugal and share information in our Facebook official page of the project. Citizens were also asked to include, if possible, exact location or coordinates, date, period of day (morning/afternoon), number of individuals, behaviour and photographic records or video.

Hair samples and roadkill animals: To study genetic patterns of the red squirrel population in Portugal two types of DNA material sources were used, hair samples and tissue (muscle) samples removed from dead animals, mainly victims of roadkill. Citizens were asked to collect hair samples or to bring the dead carcasses to our research laboratory in the University of Aveiro for proper storage and posterior muscle removal. A specific set of sample collection guidelines was made to guarantee citizens safety and prevent human DNA contamination from the collector. This included the use of disposable gloves, clean

paper tissues or other protective material while dealing with dead animals. Technical information was also given and collectors were asked to collect samples by pulling a few hairs with its respective roots, where cells containing DNA are located, and send in an envelope to our laboratory address properly identified and sealed.

3. Public engagement through content publication

The project "O Esquilo Vermelho em Portugal", as a citizen science project, was based on a compromise between the academy and the community. This meant that citizens were asked to send valuable and reliable information about red squirrels and, in exchange, researchers were responsible for sharing scientific knowledge and communicate science with them, either by social media or through educational activities. Nevertheless, this project was a major contribution to gather red squirrel geographical records in Portugal and to engage academy and community in nature conservancy.

To keep the public engaged and curious about the project, scientific and recreation content was created and shared on the Facebook page on a regular basis of 1-2 times a week. This content was developed concerning the following themes:

a. General information about the species;

b. Project dissemination through informative publications about squirrel observations, sample collection and traces and photo/video sharing sent by Facebook followers. These publications often included scientific information about the species and/or its natural behaviour related to the content of the photo/video. Author rights of photo and/or video was included in each dissemination action;

c. Dissemination of projects and events involving species conservation and/or its habitat;

d. Dissemination of activities promoted by this project or with its direct contribution;

e. Recent news on the species;

f. Important dates and curiosities.

OBJECTIVES OF THE THESIS

Main goal

The main objective of this study is to understand the expansion patterns of the Eurasian red squirrel *(Sciurus vulgaris*) in Portugal, by investigating expansion routes of the species and the effects of environmental factors on populations using species distribution models and molecular tools.

Specific goals

(I) To access environmental factors affecting the species expansion in Portugal. The knowledge about the ecology and the dispersal ability of the red squirrel is essential to access the viability of the red squirrel populations. Specifically, we aim to investigate trends of the species distribution in nature and identify environmental factors that may influence its distribution, and thus determine if the ongoing expansion of the species in the last years will continue or if it will stop or reverse in a near future.

(II) To understand the phylogeographic relationships of the red squirrel populations in Portugal and in the European background and to identify possible routes of expansion. The genetic characterization of the red squirrel population is fundamental to understand the current expansion of this species. We use mitochondrial molecular markers to understand population structure and the dynamics of the Portuguese North-South expansion, as well as to identify routes and colonization source areas of the species.

CHAPTER 2

UNCOVERING ECOLOGICAL AND GENETIC PATTERNS OF THE EURASIAN RED SQUIRREL (*SCIURUS VULGARIS*) EXPANSION IN PORTUGAL

INTRODUCTION

The Eurasian red squirrel (*Sciurus vulgaris*) is a rodent of the family Sciuridae found in most forests of Eurasia (Shar et al. 2016), occupying a wide range of coniferous and mixed-deciduous forests (Lurz et al. 2005). Although the red squirrel distribution is ample, being listed as *Least Concern* by the IUCN Red List (Shar et al. 2016), population declines have been well documented in several countries (Lloyd 1983; Bertolino 2008), particularly in United Kingdom and Northern Italy. These declines occurred following the introduction of the invasive Eastern gray squirrel *Sciurus carolinensis* (Gurnell & Pepper 1993; Ogden et al. 2005; Bertolino et al. 2014). This invasive squirrel is a direct competitor of the native red squirrel, showing ecological dominance over this species. The dominant character of the gray squirrel is a consequence of its higher body mass and more diverse foraging patterns, that results in a more efficient exploitation of food resources (Gurnell & Pepper 1993; Kenward & Hodder 1998; Wauters et al. 2001, 2002a,b), and because it acts as a reservoir for the squirrel poxvirus (SQPV). The gray squirrel is a healthy carrier of the virus that is highly pathogenic in the red squirrel, likely to cause the death of the native species within days (Rushton et al. 2000; Thomas et al. 2003; Tompkins et al. 2003).

Besides the interspecific competition with the invasive grey squirrel, other significant pressures, such as forest fragmentation, have been described to be causing red squirrels range fluctuations, leading to loss of habitat suitability and quality of food resources (Andrén & Delin 1994; Mathias & Gurnell 1998; Koprowski 2005; Verbeylen et al. 2009; Rima et al. 2010). Indeed, in Portugal, a large increase of logging, in order to supply naval construction, caused massive habitat destruction and fragmentation leading to red squirrel extinction by the 16th century (Mathias & Gurnell 1998; Rocha et al. 2014). Since 1980, after four centuries of absence, this scenario started to reverse as natural recolonization into national territory occurred due to the dispersal of red squirrel Spanish

populations across the northern borders (Mathias & Gurnell 1998; Ferreira et al. 2001). Later, in the 90s, red squirrels were successfully reintroduced in three Portuguese urban parks, boosting its expansion, at some extent, in the north and centre of the country (Mathias & Gurnell 1998; Vieira et al. 2015; Rocha et al. 2017). Currently, the red squirrel population is not threatened, but rather thriving and expanding southwards, at the limits of its distribution (Rocha et al. 2017). Both natural recolonization of newly reforested areas (Mathias & Gurnell 1998) and reintroduction events (Vieira et al. 2015) seemed to have contributed to the current expansion of the red squirrel in Portugal (Rocha et al. 2017). However, ecological and genetic factors underlying this expansion are still unknown.

In the last decades, technology advances provided important research tools such as species distribution models (SDMs) to uncover species distribution patterns, relating species geographical presences with the environmental and/or spatial variables in those sites (Guisan & Thuiller 2005; Franklin 2010; Elith et al. 2011). Species modelling offered great potential in predicting anthropogenic and climate effects on patterns of biodiversity, addressing important issues that support population ecology, phylogeography and conservation research (Guisan & Thuiller 2005). Most importantly, SDMs allow researchers to use geographical data of target species to produce habitat suitability maps with significant relevance to study biogeographical and evolutionary patterns, predict effects of climate change in ancient and contemporary time scales and hopefully, to acknowledge important areas for biodiversity conservation (Guisan & Thuiller 2005; Jiménez-Valverde et al. 2008; Goldstein et al. 2016). Considering this, SDMs will be used to uncover the environmental factors that shape red squirrel distribution, thus accessing future landscape suitability for the species and defining better strategies for forest management while reassuring species conservation.

On the other hand, molecular tools are particularly helpful in investigating the evolutionary history and geographical origin of individuals (Barratt et al. 1999), and have been previously used to study phylogeographic structure of red squirrel populations (Hale et al. 2004; Grill et al. 2009; Dozières et al. 2012; Barbosa et al. 2013; Simpson et al. 2013; Lucas et al. 2015). Combined, distribution models and molecular tools can help to investigate historical factors behind red squirrel expansion and to predict future dispersal movements of the species, offering powerful insights on the future viability of the red squirrel population in Portugal.

To achieve our objectives and gather red squirrel data to its maximum range, a citizen science project was implemented with the help of social media (see Rocha et al. 2017). Portuguese citizens have been continuously asked to collect geographical and genetic material from direct observation and roadkill animals, respectively. Based on the information gathered by the community, molecular tools and ecological modelling have been used in this study to uncover the biogeographical patterns underneath the red squirrel expansion in Portugal. This data was further used in ecological modelling and genetic analysis to investigate the main drivers of the red squirrel expansion in Portugal.

MATERIALS AND METHODS

Red squirrel geographical records

Red squirrel geographical records were collected from the citizen science project "O esquilo vermelho em Portugal" using the official Facebook page of the project (https://www.facebook.com/O-esquilo-vermelho-em-Portugal-547722785306913/) as described in (Rocha et al. 2017). Records were obtained from online surveys and messages sent by the observers to the page, containing information on red squirrel sightings, signs and roadkill. Species records were validated considering the animal description provided by the observer, indirect signs of presence (such as chewed pine cones) or photographs. Since the Eurasian red squirrel is the only native squirrel specie in Portugal accurate identification was guaranteed (Rocha et al. 2017).

Sample Collection and Storage

Genetic sample collection campaign was also run through our Facebook page. Citizens were asked to collect red squirrel hair tufts from road-killed animals, according to the following steps: collect a tuft of hairs from the animal by pulling them close to the roots; use gloves, a clean tissue or other material for personal safety and to prevent cross contamination; save them in an envelope; collect information about the surrounding habitat and GPS location; send to the laboratory address.

Roadkill animals were also collected by citizens and directly brought to the laboratory, which were identified with individual codes and stored in a refrigerator at -

20ºC. A small piece of muscle from the tongue or, in case the tongue was missing, from the right hind leg was removed from each animal and stored in a 2ml tube with 2/3 alcohol 95% at -20ºC.

All samples from muscle and hairs were given a code name SV followed by the sample number (SV1 to SV26).

DNA extraction, amplification and sequencing

Adequate protocols for each type of sample (muscle and hairs) were carefully chosen. DNA from muscle samples was isolated using a standard salt extraction (Bufford et al. 1992). DNA from hair samples was isolated using Invisorb® Spin Forensic Kit modified protocol 3 "DNA isolation from hair roots" (DDT replaced by β-mercaptoetanol) from STRATEC®. DNA isolation was carried out in a clean laboratory surface previously sterilized with bleach and alcohol. Furthermore, were included in all extraction procedures negative controls, aerosol resistant pipette tips and sterilized material to monitor and prevent cross-contamination.

Two mitochondrial DNA fragments corresponding to Cytochrome b (Cytb) and Control Region (D-loop) were amplified for each sample by polymerase chain reaction (PCR). These mitochondrial genes were chosen to evaluate the existence of polymorphisms in mitochondrial DNA sequences and identify possible new European haplotypes. PCR optimization was needed for each sample type since we were dealing with substantial variation in DNA quantity and quality, varying from good and high in muscle to poor and low in hair samples.

PCR preparation was conducted in a different room apart from the extraction room, using aerosol resistant pipette tips and sterilized material in a DNA UV-cleaner box, to ensure complete sterilization each time, before and after usage. Amplification was performed in a Veriti® 96-well thermal cycler (Applied Biosystems), using the following primers: MVZ05 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' (forward) and MVZ04 5'GCAGCCCCTCAGAATGATATTTGTCCTC-3' (reverse) and MVZ05 and MVZ16 5'-GCTGTGTCTGATGTGTAGTGTAT-3' (reverse) (Smith & Patton 1993) for Cytb gene, which amplified a small fragment of length 423bp and a larger fragment of 801bp, respectively; L15774M 5´-ACATGAATTGGAGGACAACCNGT-3´ and H651B

5´-AAGGCYAGGACCAAACCT-3´ (Fumagalli et al. 1997) including the D-Loop region of mtDNA, which amplified a large fragment of length 1301bp.

For the amplification of Cytb gene fragments MVZ05-MVZ16 the standard reaction mix included: 3μl of DNA template; 2,5μl of PCR buffer; 2,5μl of BSA; 1μl MgCl$_2$; 0,5μl dNTPs; 0,3μl of each primer solution; 0,3μl of Invitrogen Taq DNA Polymerase; and H2O up to 25μl of final reaction volume. The PCR profile used for amplification was: activation step at 94∘C for 2min; 39 cycles of denaturation at 94∘C for 32s, annealing at 48∘C for 45s and extension at 72∘C for 45s; final extension at 72∘C for 5min. Smaller fragments such as MVZ05-MVZ04 were amplified only if the larger fragment failed, due to low quantity or less quality DNA material under the following optimized conditions: 5μl of DNA template; 2,5μl of PCR buffer; 1 μl of BSA; 1μl MgCl$_2$; 0,5μl dNTPs; 0,3μl of each primer solution; 0,25μl of Invitrogen Taq DNA Polymerase; and H2O up to 25μl of final reaction volume. The PCR profile used for amplification was: activation step at 94∘C for 5min; 42 cycles of denaturation at 94∘C for 30s, annealing at 48∘C for 45s and extension at 72∘C for 45s; final extension at 72∘C for 5min.

For the amplification of D-Loop gene fragments the reaction mix included: 3μl of DNA template; 2,5μl of PCR buffer; 2,5μl of BSA; 1μl MgCl$_2$; 0,5μl dNTPs; 0,3μl of each primer solution; 0,3μl of Invitrogen Taq DNA Polymerase; and H2O up to 25μl of final reaction volume. The PCR profile used for amplification was: activation step at 94∘C for 2min; 35 cycles of denaturation at 94∘C for 30s, annealing at 48∘C for 1min30s and extension at 68∘C for 1min; final extension at 72∘C for 10 min.

PCR products were separated by electrophoresis in a 2% agarose gel stained with GelRed™ and a molecular-weight size marker with an interval of 100bp between consecutive bands and DNA fragments were visualized under UV light. Then, PCR products were purified through an enzymatic reaction (ExoSap-IT® - USB Corporation).

Sequencing was performed in an automatic sequencer ABIPRISM® 3730-XL DNA Analyzer from Applied Biosystems™. Fragments of the Cytb gene were sequenced in the forward direction and fragments of the D-loop gene were sequenced in both directions to obtain good quality sequences of the whole fragment.

Genetic diversity, population structure and neutrality tests

All generated sequences were edited manually using the software MEGA version 6.0 (Tamura et. al 2013). Only high-quality sequences were considered in the analysis. For further analyses within the Europe framework, we included an additional dataset of 21 Cytb and 455 D-loop sequences of Eurasian red squirrel generated by Barratt et al. (1999), Hale et al. (2004), Ogden et al. (2005), Finnegan et al. (2008) Grill et al. (2009) Simpson et al. (2013) and Barbosa et al. (2013). These sequences were retrieved from GenBank® and were aligned with mitochondrion genome of *Sciurus vulgaris* (accession number AJ238588).

Genetic analyses were performed for both genes separately. Number of haplotypes (h), polymorphic sites (S) and haplotype (Hd) and nucleotide (Π) diversities were estimated for both mtDNA genes using DnaSP v.6. (Rozas et al. 2017) Additionally, deviation from neutrality was calculated through Tajima's (1989) *D*, Fu's (1997) *Fs*, and Ramos-Onsins and Rozas's (2002) R_2 statistics using DnaSP v.6. Coalescence simulations with 1,000 replicates were applied to determine the p-value of each statistics. Significant p-values (< 0.05) were taken as evidence of a scenario of demographic expansion. Demographic expansion was verified with pairwise mismatch distribution analyses (Rogers & Harpending 1992), under a population growth–decline model in DnaSP v.6. In these tests, multimodal distributions correspond to demographic stability and unimodal patterns to rapid expansion (Slatkin & Hudson 1991). The raggedness index (r) with a low and non-significant values indicates that the observed distribution is not different from the expected while non-significant values indicates an expanding population.

In order to access phylogeographic relationship between Portuguese and other European red squirrel populations, we created Median-joining networks (Bandelt et al. 1999) in PopART software (Leigh & Bryant 2015). Phylogeographic relationships were estimated using Bayesian inference (BI, Lemey et al. 2009) using MrBayes 3.2.5 (Ronquist et al. 2012). BI was performed separately for each mitochondrial gene. The best model of nucleotide substitution was selected in MrModelTest (Nylander 2004) based on the Akaike Information Criterion (AIC) for each molecular marker (GTR+G for Cytb, and HKY+I+G for D-loop). Trees were sampled every 500 generations until Markov chain Monte Carlo (MCMC) became stationary, i.e., when standard deviation of split frequencies was below

0.01, which is an indicator of good convergence (Lemey et al. 2009). Stationary samples were obtained after 1 million generations for Cytb and 10 million generations for D-loop. A 50 % majority rule consensus tree was obtained after "burn-in" of 25% of the sample points to generate Bayesian posterior probabilities (BPP). Consensus trees were visualized in FigTree 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Ecological modelling of S. vulgaris

Ecological modelling was performed using presence records provided by Rocha et. al (2017) and posterior records. We used Maxent software package (v3.3.3k, Phillips et al. 2006) to create species distribution models of habitat suitability, crossing data of presence-only occurrences with environmental variables. Environmental variables used to model the distribution of the red squirrel in Portugal were divided in:

a) Bioclimatic variables and elevation: continuous raster dataset downloaded from *WorldClim - Global Climate Data* - http://www.worldclim.org/bioclim, at 30 arc seconds (~1 km resolution), which provides climate data between 1960-1990 for ecological modelling. Climate variables included 19 rasters of temperature and precipitation parameters referring to annual trends, seasonality and extreme or limiting environmental factors, according to the information given on the website (Hijmans et al. 2005). Variables are described in Appendix I.

b) Land-use variables: categorical raster dataset downloaded from Corine Land Cover 2000 (provided by the European Environment Agency) at 100-meter resolution. Dataset included land uses and vegetation variables set into five major categories: artificial areas (urbanizations, industry, roads and city parks) agricultural areas (crops, plantations and agroforestry), forest and semi-natural areas (forests and vegetation), wetlands (saltmarshes and saline) and water bodies (water courses, lagoons and estuaries). Variables are described in Appendix II.

Climatic and land cover variables, with available data for continental Portugal, were cut and rescaled to the same resolution and extent. As a result, the resolution for all environmental variables for habitat suitability mapping was 30 arc seconds (~1 km pixel)

on the WGS 84 projection. Most of climate variables had high correlations between them and correlated variables limit model scenario predictions (Merow et al. 2013). Thus, a set of highly correlated variables (r > 0,9) was calculated and removed using the tool "Remove highly correlated variables" in SDM Tool Box (Brown 2014), a complementary tool created to help with Maxent modelling in ArcGIS 10.4.1 (ESRI, 2016). From the 19 climate variables, 11 variables were selected to be used in the model.

Because observation records of the red squirrel were extremely biased due to the background of the collection procedure under the citizen science project, and biased samples strongly affect model prediction, a bias correction file was included in the models. This file was generated by Gaussian kernel density of sampling localities tool with a sampling bias distance of 0.5 decimal degrees (1 decimal degree = 111.32 km at equator), producing a bias grid used for ecological niche modelling (Elith et al. 2010; Fourcade et al. 2014). Models were built based on 20 replicates, *i. e.*, replicate sample sets were chosen by sampling with replacements, by selecting 'random seed', bootstrap and including 25% random test percentage for model fit testing. A Jackknife test was run to evaluate variables contribution to the model in isolation, by: creating a model with exclusion of variables in turns; then creating a model with each variable isolated; and, additionally, creating a model using all variables.

After that, occurrences and environmental variable datasets were introduced in Maxent and a first model was computed with all variables to evaluate general performance and variable contribution to the model. Several models were run to test settings and variable inclusion until the final model, using AUC (area under the Receiver Operating Characteristic curve) value as a reference to test and evaluate performance between models. An AUC value of 0.5 indicates that the performance of the model is no better than random, while values closer to 1.0 indicate better model performance (Young et al. 2011). Final model included response curves with the following selected environmental layers: Land_uses (categorical); and climate (continuous): altitude, bio10, bio12, bio13, bio16, bio17, bio18, bio19, bio5, bio7 and slope.

RESULTS

Red squirrel records

A total of 1813 records of both red squirrel sightings and roadkill were collected combining the data obtained by the citizen science project "*O esquilo vermelho em Portugal*" in Rocha et al. (2017) with new and more recent data. An additional dataset of 66 geographical records was extracted from GBIF (Global Biodiversity Information Facility). A total of 1879 presence records were used to study red squirrel distribution patterns in Portugal.

Mitochondrial DNA amplification and sequencing

In total, DNA was extracted from 24 samples: 15 tissue samples, obtained from red squirrel carcasses; and 9 hair samples from red squirrel hair tufts removed from roadkill animals during the project campaign between the year 2015 and 2017. A total of 24 sequences were successfully obtained for Cytb contemplating large and short fragments of 423bp (N=7) and 801bp (N=17), respectively. Only 16 sequences were successfully obtained for D-loop gene. Differences were verified between sample type and sample quality in amplification and sequence procedures. For Cytb gene, most hair samples were only successfully amplified and sequenced for shorter fragments (<500bp) while we had no difficulties in amplifying the larger fragment for tissue samples. On the other hand, for D-loop gene only 2 (out of 9) hair samples were successfully sequenced, while tissue samples were easily amplified for the expected fragment size (1301bp).

Contamination with human DNA was recorded in one hair sample, revealing possible lack of caution during collection by an untrained volunteer.

Genetic diversity, population structure and neutrality tests

A total of 5 and 4 haplotypes were recorded for Cytb and D-loop, respectively, for the Portuguese population (Table 2.1). From these, 4 Cytb and 1 D-loop haplotypes were new, when compared to the known European haplotypes. Haplotype diversity was higher

for the European dataset for both genes (Table 2.1). Overall diversity was higher for D-loop than Cytb (Table 2.1.)

| Gene	n	bp	h	S	Hd	Π	D	Fs	R_2	r
Cytb[PT]	28	801/339	5	4	0.672±0.078	0.00453±0.00062	1.289	0.438	0.192	0.193
Cytb[EU]	45	801/255	19	21	0.869±0.041	0.00848±0.00108	-1.792*	-13.048*	0.050*	0.172
D-loop[PT]	34	1301/252	4	9	0.316±0.097	0.00569±0.00183	-1.052	1.522	0.089	0.604
D-loop[EU]	470	1302/148	167	65	0.974±0.002	0.03275±0.00101	-1.780*	-269.856*	0.037	0.0104*

Neutrality tests are non-significant for both genes for the Portuguese populations, not supporting population expansion (Table 2.1). Pairwise mismatch distribution is multimodal for Cytb and bimodal for D-loop, also not supporting population expansion (Figure 2.1). On the other hand, significant values of neutrality tests for both genes (with exception of R_2 for D-loop) were found for the European population, supporting population expansion (Table 2.1). This expansion of the European populations is also confirmed by the unimodal pairwise mismatch distribution (Figure 2.1) and low and non-significant value of raggedness index (Table 2.1). Both Cytb and D-loop MJ networks show a star-like configuration (Figure 2.2 and 2.3, respectively), indicating a fast range expansion from a relatively small founding population. For Cytb, the ancestral haplotype (H1) is shared between Portugal (PT), Albania (AL), Germany (DE) and Poland (PL). All other Cytb Portuguese haplotypes (H16–H19) derive from the ancestral haplotype H1, and none of these haplotypes are shared with other countries. For D-loop there are several central haplotypes (H3, H58, H59), all shared by several countries, including Germany (DE), Italy (IT), Austria (AT), Hungary (HU), Czech Republic (CZ), Finland (FI), France (FR), United Kingdom (UK) and The Netherlands (NL) (Figure 2.3). Haplotype H57 is the most common haplotype in Portugal, being shared with Spain and France, followed by the haplotype H167, which is exclusive of Portugal and derived from a Spanish haplotype (H162). Portuguese haplotypes also include haplotype H84 which is shared with Italy and derived from the ancestor haplotype H3 (shared between Germany, Italy, Austria, Hungary, Czech Republic, Finland and France). This haplotype belongs to the same

individual (SV16 located in Leiria, Portugal) found in the ancestral haplotype (H1) of the cytochrome b network.
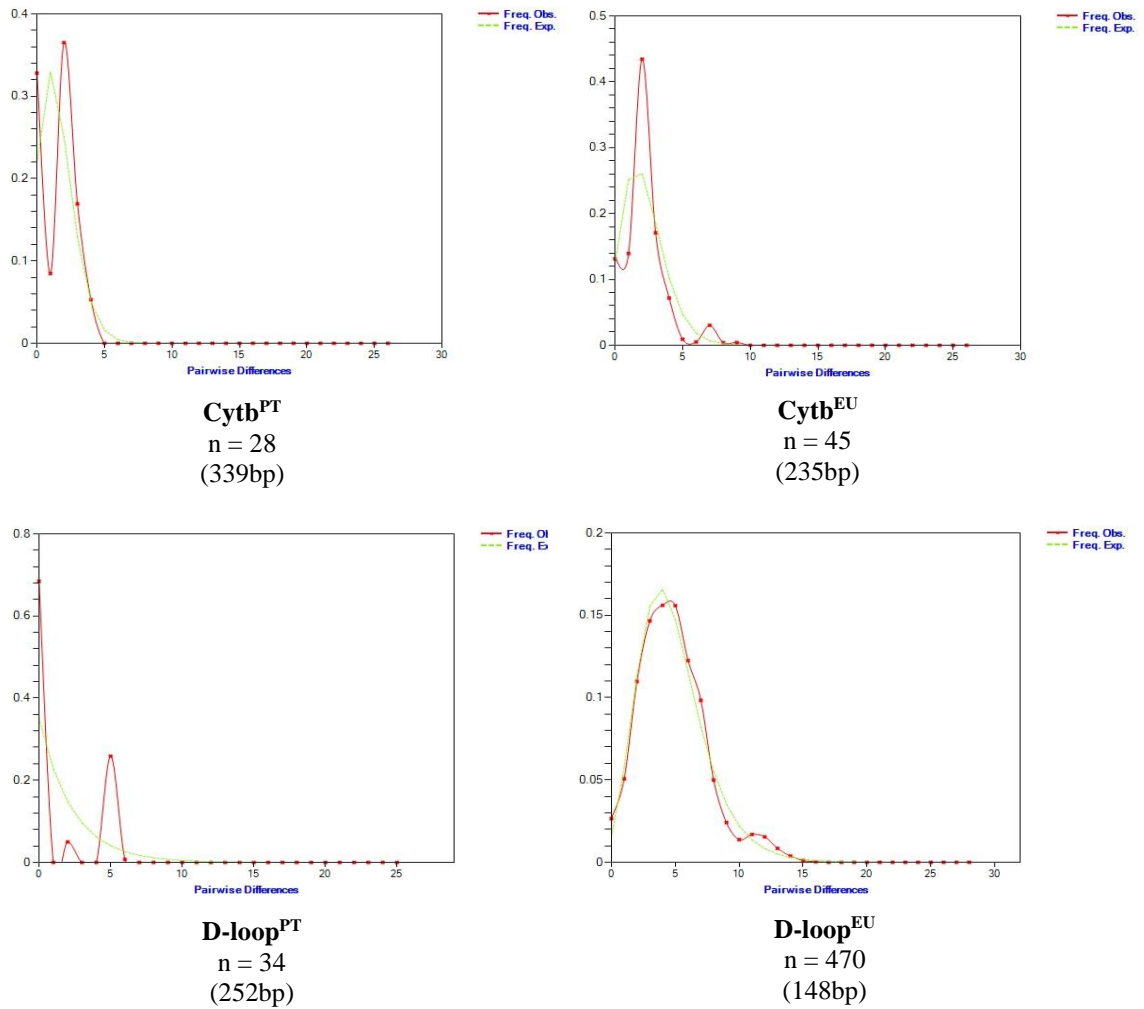
Figure 2.1. Mismatch distributions of *S. vulgaris* sequences of Cytb and D-loop for Portugal[PT] and Europe[EU].
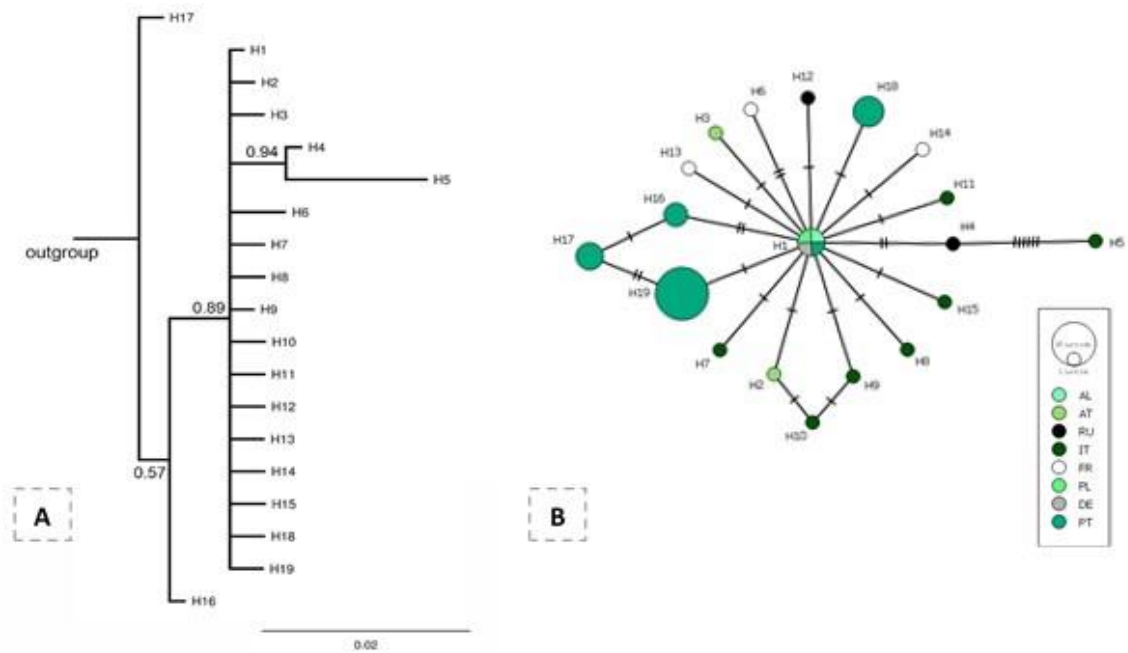
Figure 2.2. A- Bayesian Inference 50% majority consensus tree for *S. vulgaris* (Cytb) in Europe. **B -** Median-joining network of Cytb haplotypes for *S. vulgaris* sequences (N=45) in Europe. **C** – Map showing the distribution of samples of the *S. vulgaris* Cytb sequences in Europe. Each circle represents a different haplotype (from H1 to H19) and colours refer to the respective countries represented in the map (**C**). Horizontal dashes between circles represents mutations and circle sizes are proportional to haplotype frequency.
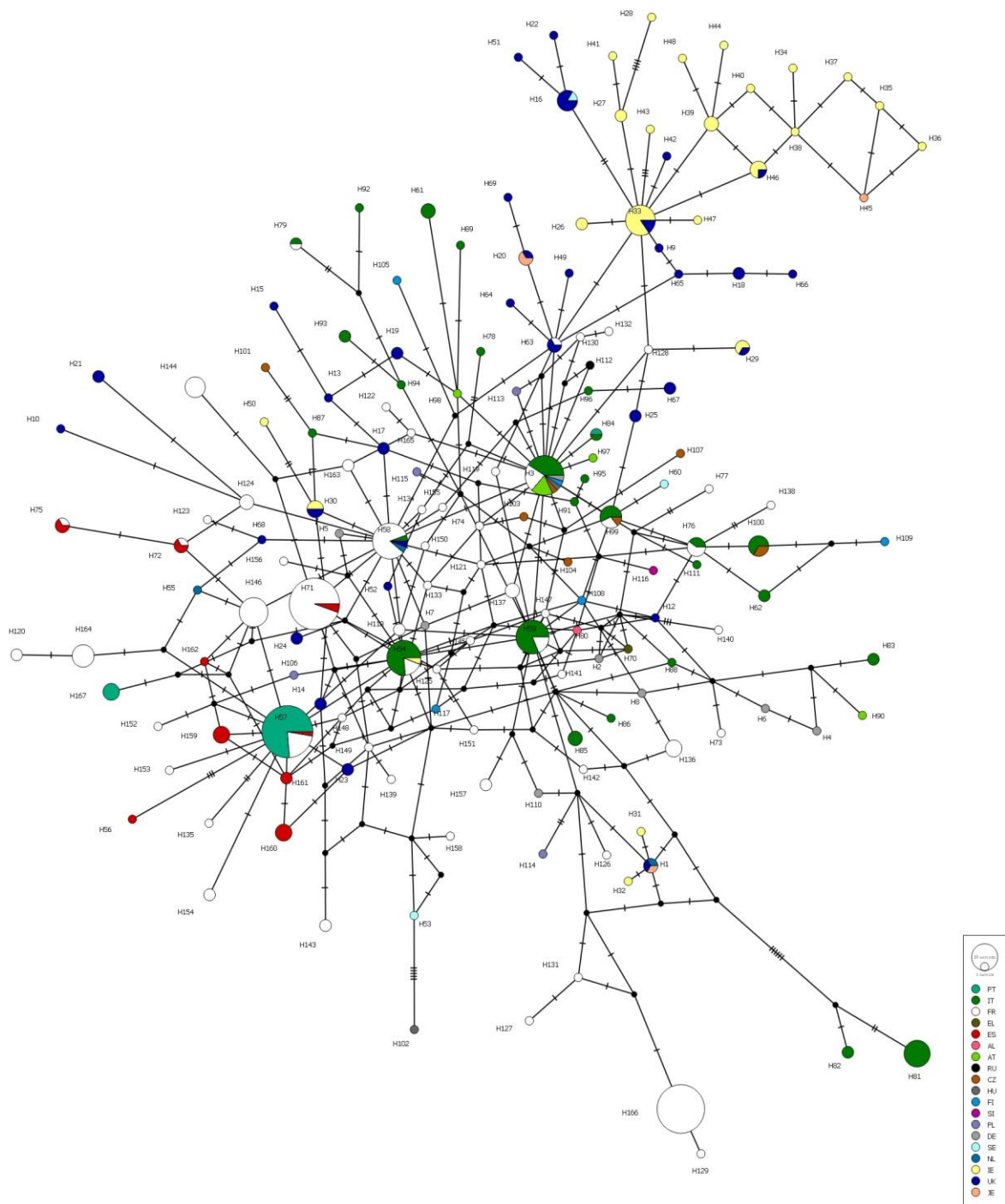
Figure 2.3. Median-joining network of D-loop haplotypes for *S. vulgaris* sequences (N=470) in Europe. Each circle represents a different haplotype (from H1 to H167) and colours refer to the respective countries represented in the map (Figure 2.4). Horizontal dashes between circles represents mutations and circle sizes are proportional to haplotype frequency.
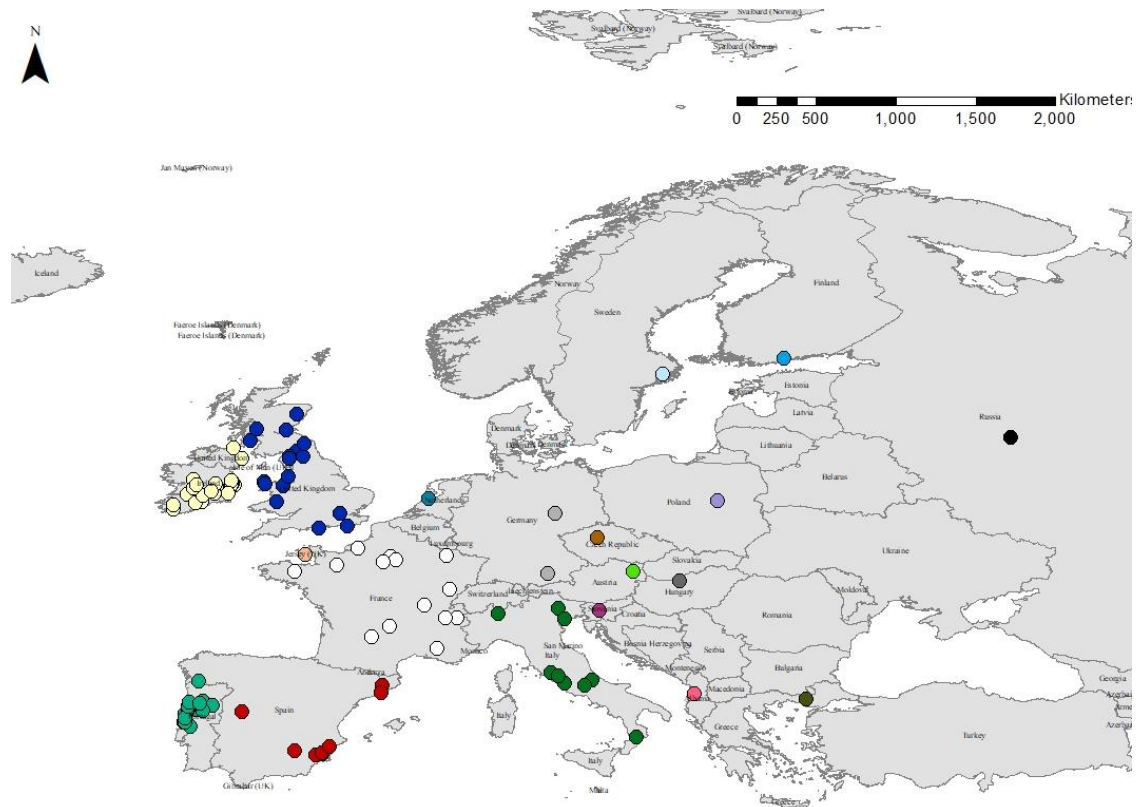
Figure 2.4. Map showing the distribution of samples of *S. vulgaris* D-loop sequences in Europe.

Both Cytb and D-loop Bayesian Inference trees recovered a large polytomy concerning red squirrel haplotypes (Figure 2.2 A and Appendix III), not allowing to determine phylogenetic relationships among European haplotypes. Species monophyly was only recovered in D-loop Bayesian Inference tree (BPP = 1.00, Appendix III).

Ecological Modelling of the red squirrel distribution in Portugal

The best model for predicted habitat suitability for the red squirrel distribution was expressed on a map using the mean value (point-wise) of the 20 replications (Figure 2.5). This model has a mean AUC value of 0.820 and a standard deviation of 0.005. High suitability areas are concentrated mostly in urban areas (Figure 2.5) depicting an extremely biased framework affecting the model, despite bias correction has been included. The red squirrel distribution is predicted to occur southwards Tejo river but not in the southern extreme of the country, where conditions seem to be unfavourable for the species presence, represented as blue colour.
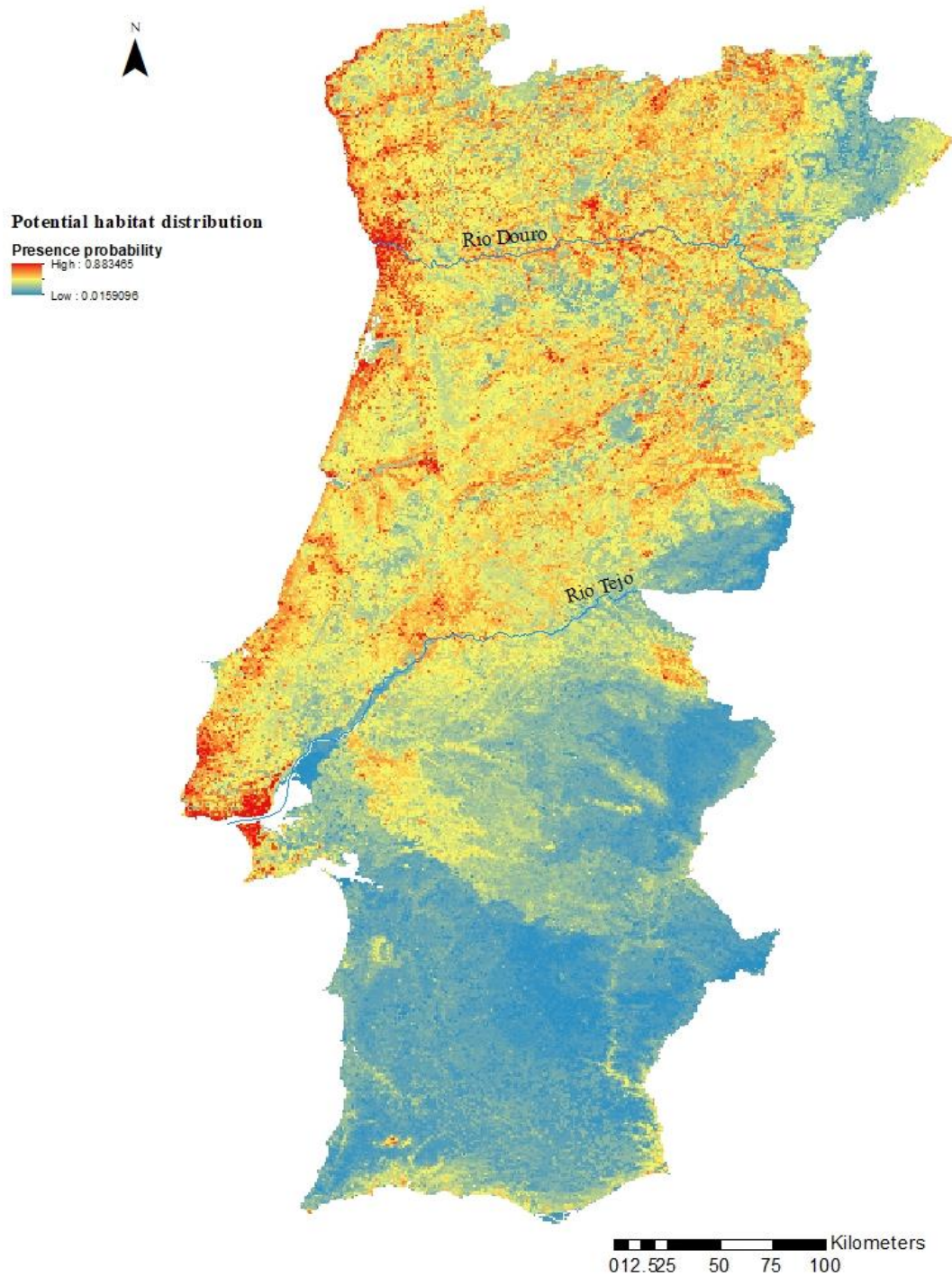
Figure 2.5. Maxent habitat suitability map for *S. vulgaris* in Portugal. Habitat suitability colour ranges from low values coloured blue (low suitability), medium values coloured yellow (medium suitability) and high values coloured red (high suitability).

Variables contribution to the model

The response curves of the 12 environmental predictor variables showed that probability of presence for the red squirrel: increased with slope and all precipitation variables (bio12, bio13, bio16, bio17, bio18, bio19); decreased with altitude and temperature annual range (bio7); and varied with maximum temperatures in the warmest periods (bio5, bio10) (Figure 2.6). Land uses categorical response showed higher probability of presence in a single category, green urban areas (10) (Figure 2.6). Continuous and discontinuous urban fabric (1 and 2) plus sport and leisure facilities (11) predicted species presence shortly over 50%. All other categories within land uses were presumed unsuitable for the species with probability of presence lower than 50% (Figure 2.6).
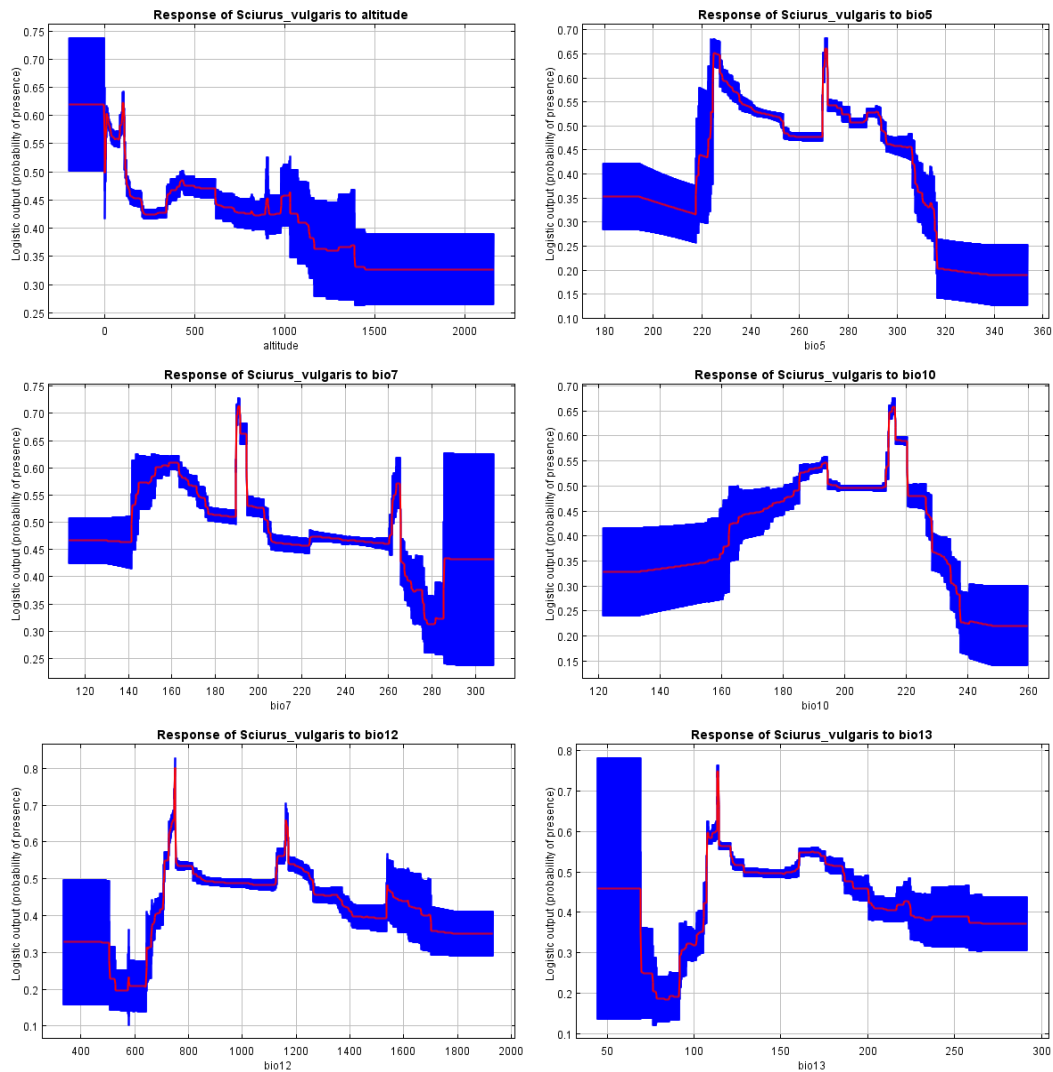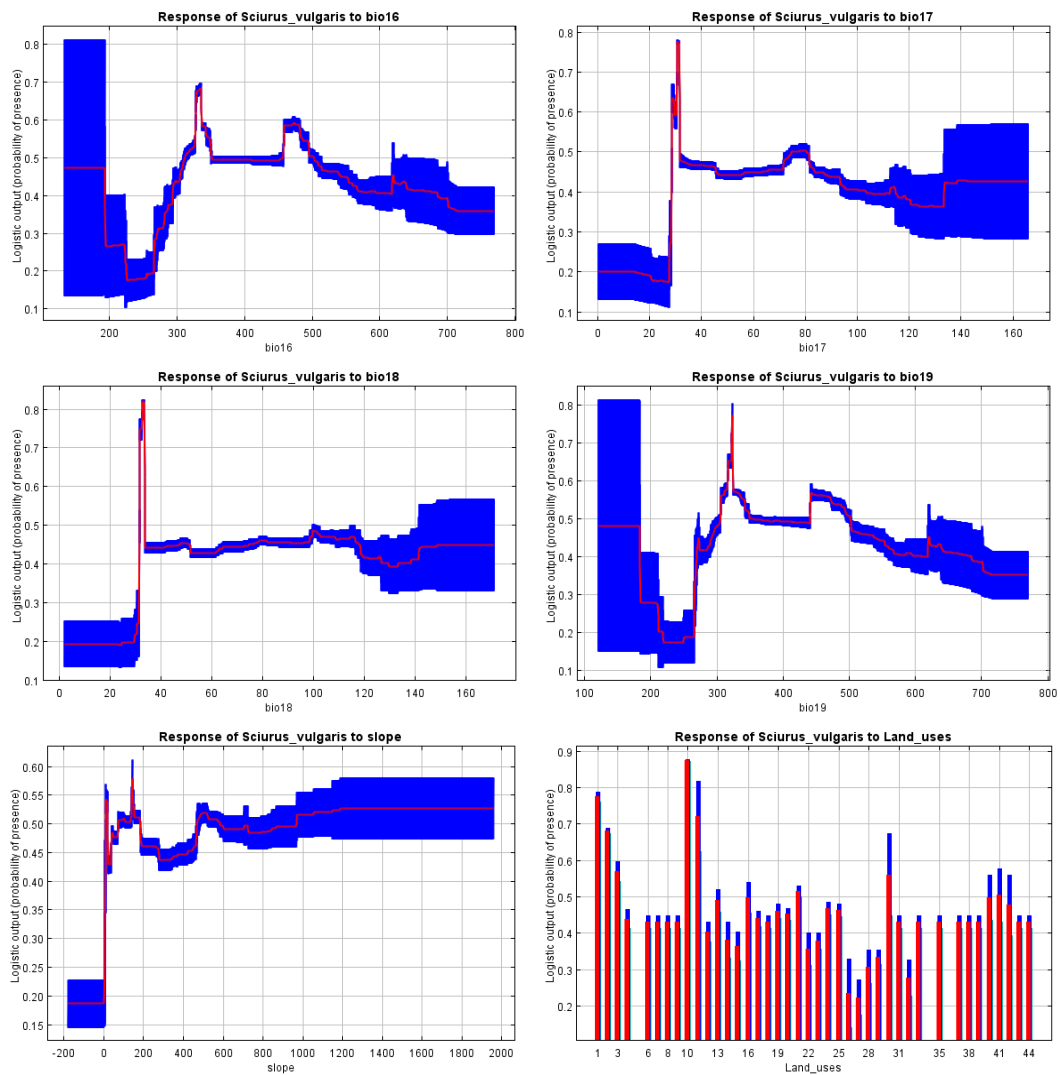
Figure 2.6. Response curves of climate and land uses variables related with red squirrel presence (0-1). These curves show the mean response of the 20 replicate Maxent runs (red) and the mean +/- one standard deviation (blue). Plots reflect the dependence of predicted suitability on the selected variable. On the Y-axis, logistic output (probability of presence) and on the X- axis (variable value). Abbreviations: bio 5 - max temperature of warmest month; bio 7 - temperature annual range; bio 10 - mean temperature of warmest quarter; bio 12 - annual precipitation; bio 13 - precipitation of wettest month; bio 16 - precipitation of wettest quarter; bio 17 - precipitation of driest quarter; bio 18 - precipitation of warmest quarter; bio 19 - precipitation of coldest quarter.

Relative contributions of environmental variables to the global model were higher for land uses, altitude, slope and precipitation (bio 18, bio13) (Table 2.2). Permutation importance, measuring the dependence of the model to each variable, was higher for land uses (Table 2.2), showing a high dependence of the final model to this variable.

Table 2.2. Contribution (%) and permutation importance (%) of the environmental variables to the model.

Variable	Contribution	Permutation importance
Land_uses	60.8	22.4
altitude	8.8	5.8
Bio18	5.1	4.1
Slope	4.7	4.7
Bio13	4.4	5.2
Bio5	2.6	4.4
Bio16	2.5	7.5
Bio7	2.4	1.6
Bio17	2.4	21.5
Bio12	2.3	9.1
Bio10	2.3	3.8
Bio19	1.7	9.8

Jackknife evaluation showed higher contributions to the final prediction of species presence for precipitation related variables (bio16, bio13, bio19, bio12) and land uses with AUC > 0.7 (Figure 2.7).
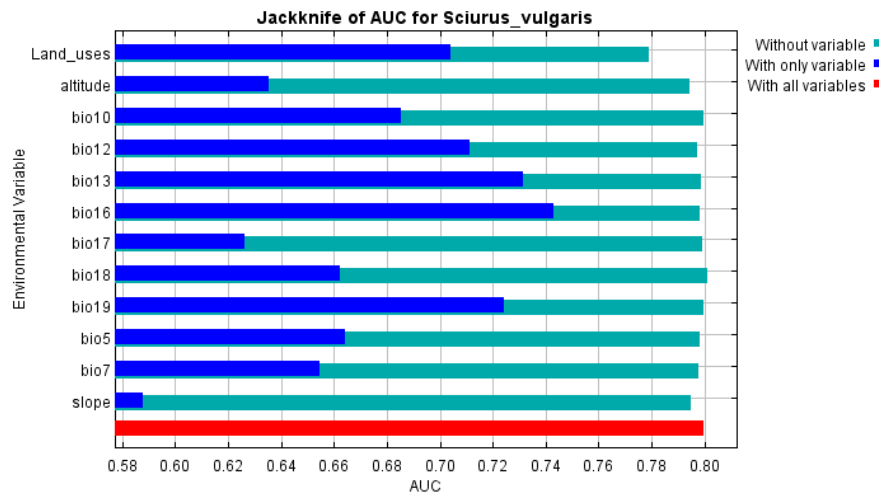
Figure 2.7. Jackknife AUC test to evaluate variable contribution.

When compared in isolation it was possible to identify a strong pattern between species occurrence and urban areas, which was that the red squirrel was only observed when forests were present nearby. In isolated urban areas with no forest patches the species was not observed, even if there were observations in proximate areas. The opposite was also observed, where continuous forests were present and urbanizations were distant, records were absent, showing a strong biased relation. Based on the predictive sites mapped on Maxent, forecasting habitat suitability areas for the red squirrel in urban

environments (Figure 2.5), we suggest future directions of the red squirrel distribution southwards after including its main habitat type, the forests, within the limitations of a biased sample (Figure 2.8).
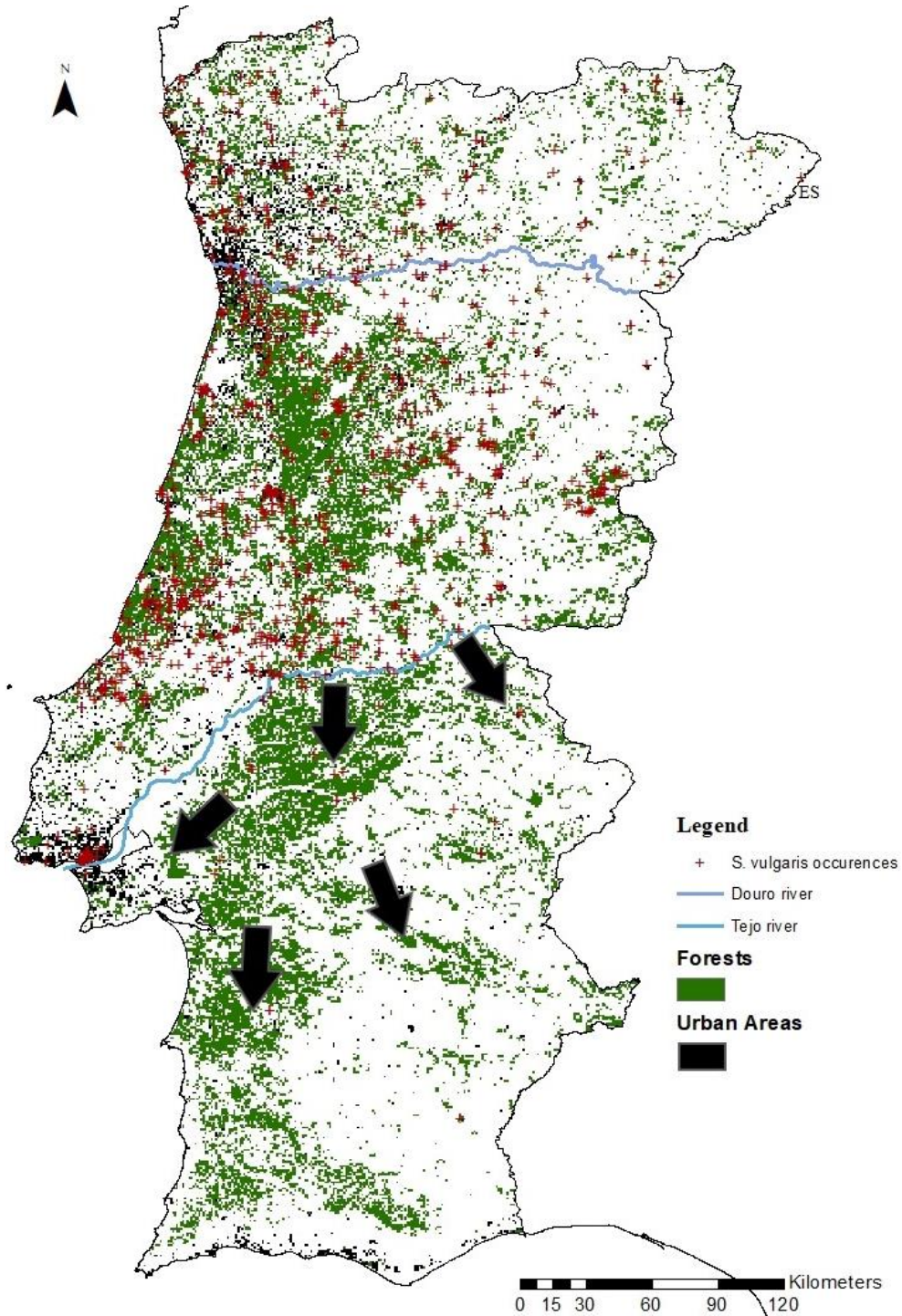
Figure 2.8. Predictive map of the red squirrel distribution and future directions considering proximity to forest habitats. Black arrows mark probable dispersal directions of the squirrel in Portugal.

DISCUSSION

Which factors, environmental and genetic, may be driving red squirrel expansion in Portugal?

Genetic patterns of the red squirrel

Low haplotype diversity is found in the Portuguese population. As suggested by Grill et al. (2009) and Trizio et al. (2005) low haplotype diversity may be related with recent population bottlenecks after significant forest fragmentation events, which might have affected genetic diversity levels of Iberian populations. Indeed, Portuguese population was extinct during almost 4 centuries, and recent expansion is reported only for the past 4 decades (Mathias & Gurnell 1998; Ferreira et al. 2001), which may explain low genetic diversity as previously reported by Grill et al. (2009).

Genetic analysis supported both natural and reintroduced origins of the red squirrel population in Portugal. Allele similarity have been used to discriminate between natural colonizers and reintroduced animals (Stewart et al. 2017). Thus, haplotype sharing between Portugal and close countries such as Spain and France support the natural expansion from colonizers of close countries. This supports the suggestion of previous authors that recent recolonization of the Portuguese territory occurred from individuals that crossed the Spanish borders when favourable habitat became available (Ferreira et al. 2001; Rocha et al. 2017). However, three successful reintroductions of red squirrels were conducted in Portugal in the 90s, with individuals coming from the Iberian Peninsula and France (Mathias & Gurnell 1998; Vieira et al. 2015). Thus, our results do not exclude the contribution of these reintroductions in the expansion of the red squirrel in Portugal.

On the other hand, haplotype sharing between Portugal and distant countries such as Italy, does not fit neither of the above-mentioned possibilities (descendants from reintroduced animals in the 90's and/or natural dispersal movements from Spanish borders into the Portuguese territory). Considering this, a third hypothesis must be highlighted: this similarity is probably the result of past reintroduction events. Mathias et al. (1998) pointed out several recreational reintroductions in Portugal from central Europe in the 19th century, which turned out be considered unsuccessful as individuals were no longer seen after. The finding of this haplotype suggests that some individuals may have survived those events, or

recent unknown reintroductions may have occurred. Considering its aesthetic value and high appreciation by the public, squirrels represent a major trend in the pet market worldwide, and easily became the cause of new and undesirable introductions (Bertolino 2009).

Additionally, star-like configuration of median-joining networks and results of neutrality tests support the rapid expansion of this species in Europe. Recent phylogeographic studies of the Eurasian red squirrel state that in the past there was an ancestor widely distributed that suffered several bottlenecks and local extinctions until intermediate populations disappeared, remaining only within the limits of their distribution (Grill et al. 2009; Dozières et al. 2012; Lucas et al. 2015). The finding of an ancestral haplotype shared by individuals from relatively distant countries (central Europe, Albania and Portugal) supports the existence of a refuge during the glacial period from which the species after rapidly expanded. The same ancestral haplotype found in Cytb analysis has been reported by Grill et al (2009) and Lucas et al. (2015), containing sequences from China, the Balkans and Iberia favouring the hypothesis of an Iberian refuge during the great glaciation.

Although the Iberian, Italian and Balkan Peninsulas are referred as potential refuges for the species after the great glaciation (Taberlet et al. 1998), there is still no phylogeographic evidence to support this hypothesis for the Iberian Peninsula (Dozières et al. 2012; Lucas et al. 2015) neither for the Italian peninsula. Though Grill et al. (2009) suggests a differentiated population in Calabria resulting from isolation within the glacial refuge itself, in which population did not expand after this period and therefore remained isolated. In contrast, Grill et al (2009) found genetic differentiation within Portuguese population using microsatellites but not for the Calabrian population, although the meaning of these results is not clearly understood. Considering this, molecular markers such as microsatellites may help to define similarities between introduced and colonizer populations and, on the other hand offer better insights on the phylogeography of Portuguese populations (Grill et al. 2009; Stewart et al. 2017).

Ecological patterns affecting red squirrel distribution

Our results on Maxent modelling showed habitat suitability for the red squirrel within urban and semi-urban environments, with high probability of species presence in

green urban areas and, in a lesser extent, urban fabric and outdoors leisure facilities. Nevertheless, there was an undeniable pattern visible along the distribution of the red squirrel, which was the presence of forested areas close to urbanizations. Considering previous knowledge on the red squirrel ecology and habitat preferences (Krauze-gryz & Gryz 2015) we considered forests to be underrepresented in the final model, probably due to significative sample bias. The context of sample bias resulting from citizen science records, *i. e*., oversampling of residential and accessible areas and undersampling in inaccessible areas, was already discussed by Dickinson et al. (2010). Regarding our results, the nature of species records collection through citizen science does not reflect natural habitat suitability but instead, habitat suitability inside human shaped habitats. However, to some extent, it is possible to conclude that the red squirrel is well adapted in urban environments. One way to overcome bias limitation would be to carry standard visual surveys comprising less accessible and more extensive woodlands (Gurnell et al. 2009). This study is nonetheless an opportunity to understand species distribution in urbanized ecosystems. Additionally, higher densities in red squirrel populations have already been documented in urban parks, either because of the existence of biodiverse and abundant conifer species and fewer predators or because supplementary food sources were plentiful (Babińska-Werka & Żółw 2008; Jokimäki et al. 2017). In fact, red squirrels can persist in isolated city parks or even fragmented habitats if trees can provide enough seeds through the year and connectivity between fragmented and good quality patches exists (Mathias & Gurnell 1998; Babińska-Werka & Żółw 2008; Verbeylen et al. 2009). Verbeylen et al. (2009) while studying red squirrel home range differences in fragmented habitats, concluded that red squirrels were more affected by patch quality regarding abundance of food resources than patch size of forest fragments, especially, because female dispersal movements are more affected by food availability and male dispersion by distribution of females. Nevertheless, benefits for red squirrel conservation include proper forest management concerning age structure and tree composition in a biodiverse landscape and maintenance of ecological corridors in fragmented habitats (Mathias & Gurnell 1998; Gurnell, J. & Pepper 1991). Thus, city parks with natural vegetated areas should be carefully managed to favour biodiversity based on species requirements in the natural environment, providing more suitable habitats for highly adaptable species such as the red squirrel.

Besides land cover categories, precipitation-related variables were also an important predictor in the red squirrel distribution model. Low suitability areas were predicted for the red squirrel in the southern extreme of Portugal. Regarding the specie's patterns of activity some studies refer that extreme temperatures and heavy precipitation decrease red squirrel diurnal activity (Lurz et al. 2005) contrasting with cloud-cover effect, that seems to do the opposite (Tonkin 1983). Given the Mediterranean climate variation from north to south regions in Portugal, characterized by higher humidity and precipitation in the north and higher temperatures in the south (Miranda et al. 2002), low suitability of the species for this region may be explained by climate.

This study represents a stepping-stone for further analysis of the red squirrel distribution in Portugal. Important questions remain to be answered regarding red squirrel presence in relation to urbanized environments, including: which characteristics are driving species occurrences closer to urban and suburban areas? Is it proximity to natural areas? Favourable climate conditions? Supplementary feeding sites? Absence of predators? Escape and shelter after wildfires? Are green urban areas truly suitable for red squirrels? Thus, more studies are needed to identify red squirrel distribution patterns in response to a diverse human shaped landscape in Portuguese urbanized networks.

CHAPTER 3

FINAL CONSIDERATIONS

We aimed to understand which factors, environmental and genetic, may be driving red squirrel expansion in Portugal. For such a complex question with so many factors to consider and evaluate, the answer could not be easy. Although interesting insights were obtained in both ecological and genetic approaches, some limitations did not allow us support findings, leaving more questions open and more possibilities for future research.

Genetic analysis supported both natural and reintroduced origins of the red squirrel population in Portugal. Haplotype sharing with close countries such as Spain and France support the natural expansion, whereas haplotype sharing with Italy support a reintroduction origin, possibly from past reintroductions occurred in the 19th century. Although, population expansion was not supported for the Portuguese population, this expansion was supported in the European context. Our results of demographic equilibrium were similar to other peninsulas representing scenarios for possible refuges during the last glaciation. Further analysis using fast-evolving molecular markers, namely microsatellites, may shed light to the recent expansion occurring in the last decades in Portugal.

Spatial predictions of species expansion through modelling projected habitat suitability mostly in urban and semi-urban environments, probably due to a biased sample. Nevertheless, forest areas in proximity were considered important to explain species presence and support future predictions, considering ecology of the red squirrel and its dependence to forested habitats. In this scenario, precipitation and green urban areas were the main variables predicting the probability of presence of the red squirrel in the territory. In sum, our results showed that red squirrel expansion southwards is likely to occur in the next years, though the southern extreme has less favourable climate conditions to support species occurrence.

Still, many questions concerning red squirrel presence and ecology in semi urban environments remain to be answered, posing important questions and doubts useful for future research on red squirrel habitat use and prosperity in urban areas.

Limitations found

Some limitations have been observed in this study. Regarding the genetic analysis, hair samples from red squirrels showed less DNA quality and quantity, in general, resulting in lower amplification and sequencing success rates. Although poor quality of hair samples may be related with the sample itself, storing hair samples in ethanol at -20ºC prior to extraction as suggested by (Ogden et al. 2005) could be tested as an alternative strategy to preserve DNA. Additionally, one sample was contaminated with human DNA. Voluntary citizens of citizen science projects usually have no experience in collecting genetic material for research purposes and this can be a problem when dealing with samples with low quantity of DNA, such as hairs. To compensate the low amounts of genetic material obtained in this study, sample collection should be complemented with hair-tube surveys within the red squirrel distribution to better understand recolonization and/or reintroduction patterns in the species (Gurnell et al. 2009).

As a recent method for predicting species distributions, ecological modelling has still many limitations regardless of fast-evolving solutions to improve biological reality (Merow et al. 2013). Limitations found in this study included sampling bias through a citizen science project, which should be complemented with standardized surveying to minimize the impact of spatial bias on the final model, probable correlation between variables and misleading projections if models are not interpreted cautiously regarding specific aspects of the species ecology (Guisan & Thuiller 2005).

Citizen science input and future perspectives

Public response to the red squirrel project in Portugal was an overall successful and positive. Being a diurnal species and easy to observe both in the wild and the city parks, the red squirrel has many admirers who wish to know more about the species and its natural behaviour. In this case, digital social network publications were very important to share scientific information and facts about the species, which has helped public to relate and commit with natural ecosystems and wildlife conservation.

"The red squirrel in Portugal" team took part in several activities related with environmental education and wildlife conservation in the last years. The project took part

in activities and interventions such as native tree sowing, seed bombing and plantation events and workshops as well as removal of invasive species with BioLiving, Quercus and University of Aveiro, in Aveiro and Lousada. Additionally, we endorsed wildlife awareness activities with groups and participated in project dissemination at conservation events such as the "Biodiversity Fest" in Fornos de Algodres and "ZURRA", in Trofa.

Moreover, during the project some positive actions and attitudes derived from the environmental education activities and social media awareness. Individually or in groups, these activities inspired chain reactions for the red squirrel awareness and conservation. For example, a class of 5-year-old children from Vila Nova de Cerveira developed a collective book about the red squirrel and planted a forest of oaks on a burnt area near the school. The preschool teacher accompanied the whole project which was requested by the children and mostly based on information shared on our Facebook page. The result was a colourful book of the red squirrel designed by the students. Another example happened after our presence in a conservation fest which motivated a 7-year-old to develop a presentation about the species to her school class.

Other good examples followed, from the placement of squirrel feeders near houses, to information requests for school papers, scout activities and public presentations in photography festivals.

The project will continue to gather information on the red squirrel from the citizens and there will be a continuous management of the project page on Facebook and other social networks. We also intend to promote local activities of public awareness for biodiversity conservation related with the red squirrel and its habitat such as sowing workshops and autochthonous tree planting on damaged forests by fires or other projects whether native forest can be implemented to expand squirrel habitat and improve its regeneration while scientific knowledge of ecological relations in the forest biodiversity is being taught to the volunteers.

An official website of the project is halfway under construction to provide a creative and conscientious database of the project concepts, gathering as much as informative material as possible on the Eurasian red squirrel in Portugal. The site will have an online form to collect observations that will be shown on an interactive map, which will enable people to see all squirrel observations in geographical records by year and name of the observer.

CHAPTER 4

REFERENCES

Andrén, H., & Lemnell, P. (1992). Population fluctuations and habitat selection in the Eurasian red squirrel *Sciurus vulgaris*. *Ecography*, *15*(3), 303–307.

Andrén, H., & Delin, A. (1994). Habitat selection in the Eurasian red squirrel, *Sciurus vulgaris*, in relation to forest fragmentation. *Oikos*, *70*, 43–48.

Babińska-Werka, J., & Żółw, M. (2008). Urban populations of the red squirrel (*Sciurus vulgaris*) in Warsaw. *Fennici — Ann. Zool. Fennici*, *45*, 270–276.

Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, *16*(1), 37–48.

Barbosa, S., Pauperio, J., Searle, J. B., & Alves, P. C. (2013). Genetic identification of Iberian rodent species using both mitochondrial and nuclear loci: Application to noninvasive sampling. *Molecular Ecology Resources*, *13*(1), 43–56.

Barratt, E. M., Gurnell, J., Malarky, G., Deaville, R., & Brufords, M. W. (1999). Genetic structure of fragmented populations of red squirrel (*Sciurus vulgaris*) in the UK. *Molecular Ecology*, *8*, 55–63.

Bertolino, S. (2009). Animal trade and non-indigenous species introduction: The worldwide spread of squirrels. *Diversity and Distributions*, *15*(4), 701–708.

Bertolino, S., di Montezemolo, N. C., Preatoni, D. G., Wauters, L. A., & Martinoli, A. (2014). A grey future for Europe: *Sciurus carolinensis* is replacing native red squirrels in Italy. *Biological Invasions*, *16*(1), 53–62.

Bertolino, S., & Genovesi, P. (2003). Spread and attempted eradication of the grey squirrel (*Sciurus carolinensis*) in Italy, and consequences for the red squirrel (*Sciurus vulgaris*) in Eurasia. *Biological Conservation*, *109*, 351–358.

Bertolino Sandro (2008). Introduction of the American grey squirrel (*Sciurus carolinensis*) in Europe: a case study in biological invasion.

Brown, J. L. (2014). SDMtoolbox: A python-based GIS toolkit for landscape genetic, biogeographic and species distribution model analyses. *Methods in Ecology and Evolution*, *5*(7), 694–700.

Bufford, M., Hanotte, O., Brookeld, J., Burke, T., 1992. Single-locus and DNA fingerprinting. In: Hoelzel, A. (ed) Molecular genetic analyses of populations. A pratical approach: 225-269. Oxford: IRL Press.

Cabral, M. J., Almeida, J., & Almeida, P. R. (2005). Livro Vermelho dos Vertebrados de Portugal. *Habitat*, 283–284.

Dickinson, J., Zuckerberg, B., & Bonter, D. (2010). Citizen Science as an Ecological Research Tool: Challenges and Benefits. *Annual Review of Ecology, Evolution and Systematics*, *41*(1), 149–172.

Dozières, A., Chapuis, J. L., Thibault, S., & Baudry, E. (2012). Genetic Structure of the French Red Squirrel Populations: Implication for Conservation. *PLoS ONE*, *7*(10).

Elith, J., Kearney, M., & Phillips, S. (2010). The art of modelling range-shifting species. *Methods in Ecology and Evolution*, *1*(4), 330–342.

Elith, J., Phillips, S. J., Hastie, T., Dudík, M., En Chee, Y., & Yates, C. J. (2011). A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions*, *17*, 43–57.

ESRI. ArcGis 10.4. Environmental Systems Research Institute, Inc. Redlands, CA. 2016.

Ferreira, A. F., Guerreiro, M., Álvares, F., & Petrucci-Fonseca, F. (2001). Distribución y aspectos ecológicos de *Sciurus vulgaris* en Portugal. *Galemys*, *13*, 155–170.

Finnegan, L. A., Edwards, C. J., & Rochford, J. M. (2008). Origin of, and conservation units in, the Irish red squirrel (*Sciurus vulgaris*) population. *Conservation Genetics*, *9*(5), 1099–1109.

Forget, P. M., Lambert, J. E., Hulme, P. E., & Vander Wall, S. B. (2005). *Seed Fate: Predation, dispersal and seedling establishment. Cabi Publishing*.

Fourcade, Y., Engler, J. O., Rödder, D., & Secondi, J. (2014). Mapping species distributions with MAXENT using a geographically biased sample of presence data: A performance assessment of methods for correcting sampling bias. *PLoS ONE*, *9*(5), 1–13.

Franklin, J. (2010). Mapping species distributions. Spatial inference and prediction. *Ecology, Biodiversity and Conservation*, *53*(9), 340.

Fumagalli, L., Pope, L. C., Taberlet, P., & Moritz, C. (1997). Versatile primers for the amplification of the mitochondrial DNA control region in marsupials. *Molecular Ecology*, *6*(12), 1199–1201.

Goldstein, E. A., Butler, F., & Lawton, C. (2016). Modelling future range expansion and management strategies for an invasive squirrel species. *Biological Invasions*, *18*(5), 1431–1450.

Grill, A., Amori, G., Aloise, G., Lisi, I., Tosi, G., Wauters, L. A., & Randi, E. (2009). Molecular phylogeography of European *Sciurus vulgaris*: Refuge within refugia? *Molecular Ecology*, *18*(12), 2687–2699.

Guisan, A., & Thuiller, W. (2005). Predicting species distribution: Offering more than simple habitat models. *Ecology Letters*, *8*(9), 993–1009.

Gurnell, J. (1983). Squirrel numbers and the abundance of tree seeds. *Mammal Review*, *13*(2–4), 133–148.

Gurnell, J. & Pepper, H. W. (1991). Conserving the red squirrel. *Great Britain, Forestry Commission, Research Division*.

Gurnell, J., & Pepper, H. (1993). A critical look at conserving the British Red Squirrel *Sciurus vulgaris*. *Mammal Review*, *23*(3–4), 127–137.

Gurnell, J., Lurz, P., McDonald, R., & Pepper, H. (2001). Practical techniques for surveying and monitoring squirrels. *Forestry Commission. Practice Note*, 12.

Gurnell, J., Lurz, P., McDonald, R., & Pepper, H. (2009). Practical techniques for surveying and monitoring squirrels. *Forestry Commission. Practice Note*, 12.

Hale, M. L., Lurz, P. W. W., & Wolff, K. (2004). Patterns of genetic diversity in the red squirrel (*Sciurus vulgaris* L.): Footprints of biogeographic history and artificial introductions. *Conservation Genetics*, *5*, 167–179.

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, G., & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, *25*(15), 1965–1978.

ICNF. (2013). IFN6 – Áreas dos usos do solo e das espécies florestais de Portugal continental em 1995, 2005 e 2010. *6º Inventário Florestal Nacional*, 34.

Jiménez-Valverde, A., Hortal, J., Lobo, J. M., Borges, P. A. V, Abreu, C., Aguiar, A. F., Azevedo, E. B., Boieiro, M., Fontinha, S., Jardim, R., Oliveira, P., Sérgio, C., Serrano, A. R. M., Sim-Sim, M., Nunes, D. (2008). Using predictive models of species distribution to validate biodiversity data: case studies for Madeira island. *A list of the terrestrial fungi, flora and fauna of Madeira and Selvagens archipelagos*, 27–56.

Jokimäki, J., Selonen, V., Lehikoinen, A., & Kaisanlahti-Jokimäki, M. L. (2017). The role of urban habitats in the abundance of red squirrels (*Sciurus vulgaris*, L.) in Finland. *Urban Forestry & Urban Greening*, *27*, 100–108.

Kenward, R. E., & Hodder, K. H. (1998). Red squirrels (*Sciurus vulgaris*) released in conifer woodland: the effects of source habitat, predation and interactions with grey squirrels (*Sciurus carolinensis*). *Journal of Zoology*, *244*(1), 23–32.

Koprowski, J. L. (2005). The response of tree squirrels to fragmentation: a review and synthesis. *Animal Conservation*, *8*, 369–376.

Krauze-gryz, D., & Gryz, J. (2015). A review of the diet of the red squirrel (*Sciurus vulgaris*) in different types of habitats. *Red Squirrels: Ecology, Conservation & Management in Europe*.

Lawton, C., Cowan, P., Bertolino, S., Lurz, P. W. W., & Peters, A. R. (2010). The consequences of introducing non-indigenous species: two case studies, the grey squirrel in Europe and the brushtail possum in New Zealand. *Revue Scientifique et Technique*, *29*(2), 287–298.

Leigh, J. W., & Bryant, D. (2015). POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, *6*(9), 1110-1116.

Lloyd, H. G. (1983). Past and present distribution of Red and Grey squirrels. *Mammal Review*, *13*(2–4), 69–80.

Lemey, P., Rambaut, A., Drummond, A. J., & Suchard, M. A. (2009). Bayesian phylogeography finds its roots. *PLoS computational biology*, *5*(9), e1000520.

Lucas, J. M., Prieto, P., & Galián, J. (2015). Red squirrels from south–east Iberia: low genetic diversity at the southernmost species distribution limit. *Animal Biodiversity and Conservation*, *38*(1), 129-138.

Lurz, P. W. W., Gurnell, J., & Magris, L. (2005). *Sciurus vulgaris*. *Mamalian Species*, 1–10.

Magris, L., & Gurnell, J. (2002). Population ecology of the red squirrel (*Sciurus vulgaris*) in a fragmented woodland ecosystem on the Island of Jersey, Channel Islands. *Journal of Zoology*, *256*, 99–112.

Martinoli, A., Bertolino, S., Amiano, D. G., Balduzzi, A., Marsan, A., Genovesi, P., Tosi, G., Wauters, L. A. (2010). Headcount 2010: the multiplication of the grey squirrel introduced in Italy. *Hystrix, the Italian Journal of Mammalogy*, *21*(2), 127–136.

Mathias, M. da L., & Gurnell, J. (1998). Status and Conservation of the Red squirrel (*Sciurus vulgaris*) in Portugal. *Hystrix*, *the Italian Journal of Mammalogy, 10*(2), 13–19.

Merow, C., Smith, M. J., & Silander, J. A. (2013). A practical guide to Maxent for modelling species' distributions: What it does, and why inputs and settings matter. *Ecography*, *36*(10), 1058–1069.

Middleton, A. D. (1930). The ecology of the American grey squirrel in the British Isles. *Journal of Zoology*, *100*(3), 809–843.

Miranda, P., Coelho, F. E. S., Tomé, A. R., Valente, M. A., Carvalho, A., Pires, C., Pires, H. O., Pires, V. C. & Ramalho, C. (2002). 20th century Portuguese climate and climate scenarios. *Climate Change in Portugal. Scenarios, Impacts and Adaptation Measures—SIAM Project (Santos F. D., Forbes K., Moita R., eds). Lisbon: Gradiva Publishers*, 23-83.

Nylander, J. A., Ronquist, F., Huelsenbeck, J. P., & Nieves-Aldrey, J. (2004). Bayesian phylogenetic analysis of combined data. Systematic biology, 53(1), 47-67.

Ogden, R., Shuttleworth, C., McEwing, R., & Cesarini, S. (2005). Genetic management of the red squirrel, *Sciurus vulgaris*: A practical approach to regional conservation. *Conservation Genetics*, *6*(4), 511–525.

Pepper, H., & Patterson, G. (1998). Red Squirrel Conservation. *Forestry Commission*, 1–8.

Petty, S. J., Lurz, P. W. W., & Rushton, S. P. (2003). Predation of red squirrels by northern goshawks in a conifer forest in northern England: Can this limit squirrel numbers and create a conservation dilemma? *Biological Conservation*, *111*(1), 105–114.

Phillips, S. B., Aneja, V. P., Kang, D., & Arya, S. P. (2006). Modelling and analysis of the atmospheric nitrogen deposition in North Carolina. *International Journal of Global Environmental Issues*, *6*(2–3), 231–252.

Rima, P. C., Cagnin, M., Aloise, G., Preatoni, D., & Wauters, L. A. (2010). Scale-dependent environmental variables affecting red squirrel (*Sciurus vulgaris meridionalis*) distribution. *Italian Journal of Zoology*, *77*(1), 92–101.

Robertson, P. A., Adriaens, T., Lambin, X., Mill, A., Roy, S., Shuttleworth, C. M., & Sutton-Croft, M. (2017). The large-scale removal of mammalian invasive alien species in Northern Europe. *Pest Management Science*, *73*(2), 273–279.

Rocha, R. G., Vieira, B. P., Rodrigues, V., & Fonseca, C. (2017). Public engagement offers insights on the Eurasian red squirrel distribution. *European Journal of Wildlife Research*, *63*(6), 87.

Rocha, R. G., Wauters, L. A., Mathias, M. da L., & Fonseca, C. (2014). Will an ancient refuge become a modern one? A critical review on the conservation and research priorities for the red squirrel (*Sciurus vulgaris*) in the Iberian Peninsula. *Hystrix, the Italian Journal of Mammalogy, 25*(1), 9–13.

Rogers, A. R., & Harpending, H. (1992). Population growth makes waves in the distribution of pairwise genetic differences. *Molecular biology and evolution*, *9*(3), 552-569.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology*, *61*(3), 539-542.

Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA Sequence Polymorphism Analysis of Large Datasets. *Molecular Biology and Evolution*, 1–4.

Rushton, S. P., Lurz, P. W. W., Gurnell, J., & Fuller, R. (2000). Modelling the spatial dynamics of parapoxvirus disease in red and grey squirrels: a possible cause of the decline in the red squirrel in the UK?. *Journal of Applied Ecology*, *37*(6), 997-1012.

Shar, S., Lkhagvasuren, D., Bertolino, S., Henttonen, H., Kryštufek, B., & Meinig, H. (2016). *Sciurus vulgaris*. *The IUCN Red List of Threatened Species 2016: e.T20025A115155900.*

Simpson, S., Blampied, N., Peniche, G., Dozières, A., Blackett, T., Coleman, S., Cornish, N., Groombridge, J. J. (2013). Genetic structure of introduced populations: 120-year-old DNA footprint of historic introduction in an insular small mammal population. *Ecology and Evolution*, *3*(3), 614–628.

Slatkin, M., & Hudson, R. R. (1991). Pairwise comparisons of mitochondrial DNA sequences in stable and exponentially growing populations. *Genetics*, *129*(2), 555-562.

Smith, M. F., & Patton, J. L. (1993). The diversification of South American murid rodents: evidence from mitochondrial DNA sequence data for the akodontine tribe. *Biological Journal of the Linnean Society*, *50*(3), 149–177.

Stewart, F. E. C., Volpe, J. P., Taylor, J. S., Bowman, J., Thomas, P. J., Pybus, M. J., & Fisher, J. T. (2017). Distinguishing reintroduction from recolonization with genetic testing. *Biological Conservation*, *214*, 242–249.

Taberlet, P., Fumagalli, L., Wust-Saucy, A. G., & Cosson, J. F. (1998). Comparative phylogeography and postglacial colonization routes in Europe. *Molecular ecology*, *7*(4), 453-464.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution, 30(12), 2725-2729.

Teangana, D. O., Reilly, S., Montgomery, W. I., & Rochford, J. (2000). Distribution and status of the Red Squirrel (*Sciurus vulgaris*) and Grey Squirrel (*Sciurus carolinensis*) in Ireland. *Mammal Review*, *30*(1), 45–55.

Thomas, K., Tompkins, D. M., Sainsbury, A. W., Wood, A. R., Dalziel, R., Nettleton, P. F., & McInnes, C. J. (2003). A novel poxvirus lethal to red squirrels (*Sciurus vulgaris*). *Journal of General Virology*, *84*(12), 3337–3341.

Tompkins, D. M., White, A. R., & Boots, M. (2003). Ecological replacement of native red squirrels by invasive greys driven by disease. *Ecology Letters*, *6*(3), 189–196.

Tonkin, J. M. (1983). Activity patterns of the Red squirrel (*Sciurus vulgaris*). *Mammal Review*, *13*(2–4), 99–111.

Trizio, I., Crestanello, B., Galbusera, P., Wauters, L. A., Tosi, G., Matthysen, E., & Hauffe, H. C. (2005). Geographical distance and physical barriers shape the genetic structure of Eurasian red squirrels (*Sciurus vulgaris*) in the Italian Alps. *Molecular Ecology*, *14*(2), 469–481.

Verbeylen, G., Wauters, L. A., De Bruyn, L., & Matthysen, E. (2009). Woodland fragmentation affects space use of Eurasian red squirrels. *Acta Oecologica*, *35*(1), 94–103.

Vieira, B. P., Fonseca, C., & Rocha, R. G. (2015). Critical steps to ensure the successful reintroduction of the Eurasian red squirrel. *Animal Biodiversity and Conservation*, *38*(1), 49–58.

Wauters, L. A., & Dhondt, A. A. (1987). Activity budget and foraging behaviour of the Red squirrel (*Sciurus vulgaris* Linnaeus, 1758) in a coniferous habitat. *Zeitschrift Für Säugetierkunde*, *52*(6), 341–353.

Wauters, L. A., & Lens, L. (1995). Effects of food availability and density on red squirrel (*Sciurus vulgaris*) reproduction. *Ecology*, *76*(8), 2460–2469.

Wauters, L. A., Gurnell, J., Martinoli, A., & Tosi, G. (2001). Does interspecific competition with introduced grey squirrels affect foraging and food choice of Eurasian red squirrels? *Animal Behaviour*, *61*(6), 1079–1091.

Wauters, L. A., Gurnell, J., Martinoli, A., & Tosi, G. (2002a). Interspecific competition between native Eurasian red squirrels and alien grey squirrels: Does resource partitioning occur? *Behavioral Ecology and Sociobiology*, *52*(4), 332–341.

Wauters, L. A., Tosi, G., & Gurnell, J. (2002b). Interspecific competition in tree squirrels: Do introduced grey squirrels (*Sciurus carolinensis*) deplete tree seeds hoarded by red squirrels (*S. vulgaris*)? *Behavioral Ecology and Sociobiology*, *51*(4), 360–367.

Wauters, L. a., Zaninetti, M., Tosi, G., & Bertolino, S. (2004). Is coat-colour polymorphism in Eurasian red squirrels (*Sciurus vulgaris* L.) adaptive? *Mammalia*, *68*(1), 37.

Wauters, L., & Dhondt, A. A. (1992). Spacing behaviour of red squirrels, *Sciurus vulgaris*: variation between habitats and the sexes. *Animal Behaviour*, *43*(2), 297–311.

Young, N., Carter, L., & Evangelista, P. (2011). A MaxEnt Model v3.3.3e Tutorial (ArcGIS v10).

APPENDIX I

Climate Variables Description

Table A.1. Climate rasters downloaded from the WorldClim online database for the period 1960-1990 (Hijmans et al., 2005).

VAR CODE	RESOLUTION	DESCRIPTION
BIO1		Annual Mean Temperature
BIO2		Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3		Isothermality (BIO2/BIO7) (* 100)
BIO4		Temperature Seasonality (standard deviation *100)
BIO5		Max Temperature of Warmest Month
BIO6		Min Temperature of Coldest Month
BIO7		Temperature Annual Range (BIO5-BIO6)
BIO8		Mean Temperature of Wettest Quarter
BIO9	30 arc-seconds	Mean Temperature of Driest Quarter
BIO10	(~1 km)	Mean Temperature of Warmest Quarter
BIO11		Mean Temperature of Coldest Quarter
BIO12		Annual Precipitation
BIO13		Precipitation of Wettest Month
BIO14		Precipitation of Driest Month
BIO15		Precipitation Seasonality (Coefficient of Variation)
BIO16		Precipitation of Wettest Quarter
BIO17		Precipitation of Driest Quarter
BIO18		Precipitation of Warmest Quarter
BIO19		Precipitation of Coldest Quarter
ALTITUDE		Elevation above sea level

APPENDIX II

# Land Cover Variables Description

Table A.2. Land cover variables description from CORINE LAND COVER (CLC 2012) raster data at 100m resolution. Each code number represents the respective variable of categorical layer "Land uses" in Maxent modelling results.

CODE	LABEL 1	LABEL 2	LABEL 3
1	Artificial surfaces	Urban fabric	Continuous urban fabric
2	Artificial surfaces	Urban fabric	Discontinuous urban fabric
3	Artificial surfaces	Industrial, commercial and transport units	Industrial or commercial units
4	Artificial surfaces	Industrial, commercial and transport units	Road and rail networks and associated land
5	Artificial surfaces	Industrial, commercial and transport units	Port areas
6	Artificial surfaces	Industrial, commercial and transport units	Airports
7	Artificial surfaces	Mine, dump and construction sites	Mineral extraction sites
8	Artificial surfaces	Mine, dump and construction sites	Dump sites
9	Artificial surfaces	Mine, dump and construction sites	Construction sites
10	Artificial surfaces	Artificial, non-agricultural vegetated areas	Green urban areas
11	Artificial surfaces	Artificial, non-agricultural vegetated areas	Sport and leisure facilities
12	Agricultural areas	Arable land	Non-irrigated arable land
13	Agricultural areas	Arable land	Permanently irrigated land
14	Agricultural areas	Arable land	Rice fields
15	Agricultural areas	Permanent crops	Vineyards
16	Agricultural areas	Permanent crops	Fruit trees and berry plantations
17	Agricultural areas	Permanent crops	Olive groves
18	Agricultural areas	Pastures	Pastures
19	Agricultural areas	Heterogeneous agricultural areas	Annual crops associated with permanent crops
20	Agricultural areas	Heterogeneous agricultural areas	Complex cultivation patterns
21	Agricultural areas	Heterogeneous agricultural areas	Land principally occupied by agriculture, with significant areas of natural vegetation
22	Agricultural areas	Heterogeneous agricultural areas	Agro-forestry areas

CODE	LABEL 1	LABEL 2	LABEL 3
23	Forest and semi natural areas	Forests	Broad-leaved forest
24	Forest and semi natural areas	Forests	Coniferous forest
25	Forest and semi natural areas	Forests	Mixed forest
26	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Natural grasslands
27	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Moors and heathland
28	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Sclerophyllous vegetation
29	Forest and semi natural areas	Scrub and/or herbaceous vegetation associations	Transitional woodland-shrub
30	Forest and semi natural areas	Open spaces with little or no vegetation	Beaches, dunes, sands
31	Forest and semi natural areas	Open spaces with little or no vegetation	Bare rocks
32	Forest and semi natural areas	Open spaces with little or no vegetation	Sparsely vegetated areas
33	Forest and semi natural areas	Open spaces with little or no vegetation	Burnt areas
34	Forest and semi natural areas	Open spaces with little or no vegetation	Glaciers and perpetual snow
35	Wetlands	Inland wetlands	Inland marshes
36	Wetlands	Inland wetlands	Peat bogs
37	Wetlands	Maritime wetlands	Salt marshes
38	Wetlands	Maritime wetlands	Salines
39	Wetlands	Maritime wetlands	Intertidal flats
40	Water bodies	Inland waters	Water courses
41	Water bodies	Inland waters	Water bodies
42	Water bodies	Marine waters	Coastal lagoons
43	Water bodies	Marine waters	Estuaries
44	Water bodies	Marine waters	Sea and ocean
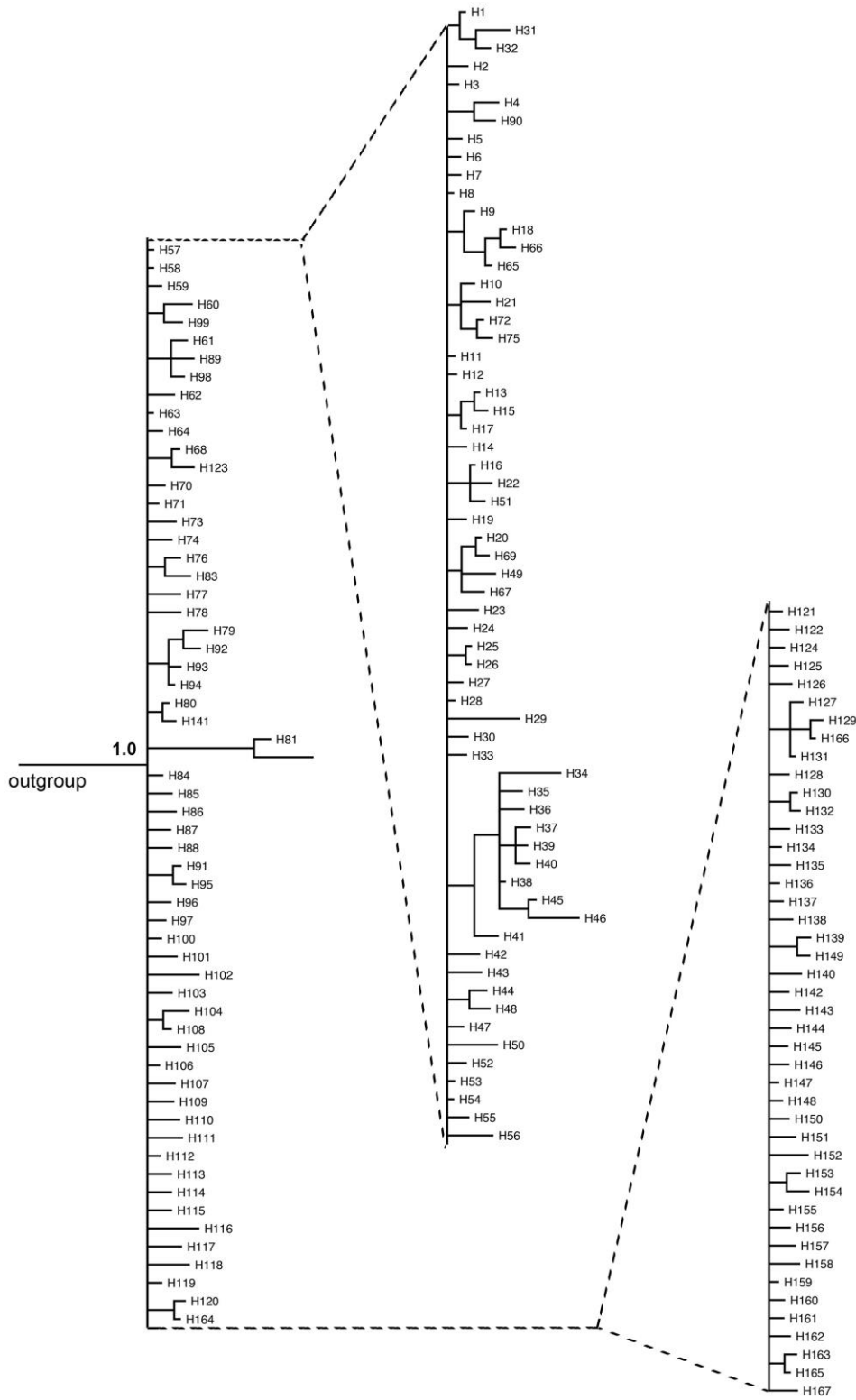
Figure A. 1. Bayesian Inference 50% majority consensus tree for D-loop in Europe