Assessment of biological invasions phenomena in reptiles of Mediterreanean islands: a biogeographic, genetic and ecological perspective.

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Nota Prévia

Na elaboração desta tese, e nos termos do número 2 do Artigo 4 do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31 do Decreto-Lei 74/2006, de 24 de Março, com a nova redação introduzida pelo Decreto-Lei 230/2009, de 14 de Setembro, foi efetuado o aproveitamento total de um conjunto coerente de trabalhos de investigação já publicados ou submetidos para publicação em revistas internacionais indexadas e com arbitragem científica, os quais integram alguns dos capítulos da presente tese. Tendo em conta que os referidos trabalhos foram realizados com a colaboração de outros autores, o candidato esclarece que, em todos eles, participou ativamente na sua conceção, na obtenção, análise e discussão de resultados, bem como na elaboração da sua forma publicada. A instituição de origem da candidata foi a Faculdade de Ciências da Universidade do Porto, tendo o trabalho sido realizado sob orientao do Doutor Miguel A. Carretero, Professor Auxiliar no Departamento de Biologia da Faculdade de Ciências da Universidade do Porto e Investigador Principal do Centro de Investigação em Biodiversidade e Recursos Genéticos (CIBIO-InBio) e sob co-orientação do Doutor Daniele Salvi, Professor Associado na Universidade de L'Aquila e Investigador do CIBIO/InBIO. A instituição de acolhimento foi o Laboratoire d'Ecologie Alpine (LECA), sob a coorientação do Professor Doutor Gentile Francesco Ficetola. O trabalho laboratorial foi realizado no CIBIO-InBio, no LECA e Serveis Cientifico-Tècnics (University of Barcelona, Spain)

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“How to get the best of it all? One must conquer, achieve, get to the top; one must know the end to be convinced that one can win the end - to know there’s no dream that mustn’t be dared. . . Is this the summit, crowning the day? How cool and quiet! We’re not exultant; but delighted, joyful; soberly astonished. . . Have we vanquished an enemy? None but ourselves. Have we gained success? That word means nothing here. Have we won a kingdom? No. . . and yes. We have achieved an ultimate satisfaction. . . fulfilled a destiny. . . To struggle and to understand - never this last without the other; such is the law. . ”

George Mallory

Climbing Everest: The Complete Writings of George Mallory

Abstract

The movement of species across suitable areas occur naturally according to species' dispersal abilities, but since humans started to explore the world, they also started to translocate species to a place where they could not arrive by their own means, thus reshaping the world's biota. In a fraction of cases, such organisms establish naturalized populations in the new range, expand and eventually become detrimental to native species. As a result, biological invasions already represent one of the major causes of biodiversity loss, but at the same time, they provide an opportunity to deepen our knowledge regarding key ecological and evolutionary questions.

The Mediterranean basin is the result of a complex history encompassing profound geological processes, climatic shifts, and biogeographical interchanges harbouring one of the world's biodiversity hotpots. It has been also a meeting point of several civilizations, with a long history of human-biota interaction which makes it a good model to study different layers of biological invasions phenomena. Mediterranean islands, in particular, can represent a live laboratory to test evolutionary and ecological hypothesis, due to their geographic isolation and limited number and taxa of organisms that can naturally colonize these ecosystems. These features, on the other hand, make island ecosystems particularly vulnerable to biological invasions.

Reptiles are a vertebrate group particularly vulnerable to biological invasions but at the same time one of the groups that are more introduced. The long history of interactions between humans and reptiles in Mediterranean islands has caused a mix of multiple ancient and recent introductions of non-native species. Such history makes it difficult to distinguish between alien and native species on this region. At the same time, the diversity of islands together with repeated introduction events provide an excellent opportunity to investigate factors related to introductions and success of alien species in new environments.

The main aim of this thesis is to determine the patterns and to infer the main drivers of biological invasions on reptiles' of Mediterranean Islands at different levels, using a combination of biogeographic, ecologic and genetic tools. First, we focused on the broad Mediterranean basin; we combined genetic, distribution and biological data on Mediterranean reptiles together with island information, to identify the factors promoting the presence of alien reptiles on islands, considering human-related, geographic and species traits. A literature review on native/alien status of reptiles on more than 900 Mediterranean islands was also incorporated in the database. Spatially-explicit models show a complex signal of geographic features, species traits and human-related factors. A reptile record is most likely to be alien if the species feeds on plants and is present on island's populations

far from the native range and with large human population size. Furthermore, traditional data sources underestimate the frequency of alien reptiles in Mediterranean islands and the use of genetic information to assess the status of populations provides a more accurate representation of the factors related to alien populations.

Second, we narrowed the study scale to one ‘paradigmatic’ archipelago, the Balearic Islands: here alien reptiles exceed the number of native reptile species and mutliple alien snake species have been recently introduced, endangering the few native species present. We used phylogenetic approaches combined with other information sources (literature and direct observations) to identify the origin and introduction pathways of the four introduced snake species (*Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus* and *Rhinechis scalaris*). We also used ecological niche models to deduce their patterns of invasion and expansion based on current and future suitability. Iberian Peninsula is the potential origin for most of the alien populations. However, the low phylogeographic structure of these species prevented the identification of more precise source-areas. Ecological niche models’ show a current low habitat suitability in the Balearic Islands, but with tendency to increase significantly in the next few decades under climate change scenarios. From direct observations and spatial analyses, we identified as the most common pathway, the nursery trade, especially of olive tree importation from the Iberian Peninsula.

Third, we focused on the Italian Wall lizard, *Podarcis sicula*, introduced in several places in the world, from the Mediterranean region to North America, where is frequently invasive and so a good model to understand the history and traits that make a reptile succeed in different systems. Available studies support an invasive behavior with interference with native species and a fast-changing capacity when in novel environments. Genomic data were used to assess the alien/native status of island populations combined with stable isotopes and morphological analyses to assess the genetic and phenotypic changes between alien (islands) and native (mainland) populations. The genomic data was also used to perform an outlier loci analysis, in order to test for selection. Three systems were used: Aegadian (Erice, Birgi, Marettimo, Favignana and Levanzo), Aeolian (Milazzo, Vulcano, Salina, Filicudi and Stromboli) and Tuscany (Talamone, Capalbio, Argentario, Giglio, Giannutri and Formica Grande). The results confirm the difficulty in inferring the status of a certain species on Mediterranean Islands. In Tuscany, the genetic evidence suggests an ancient colonization of islands, introduced by human-mediated introductions in ancient times or by natural means during the Last Glacial Maximum (LGM). In the Aegadians, the genetic closeness of Marettimo to mainland population suggests a recent introduction, while the genetic homogeneity between islands and mainland suggests a continuous gene flow. Finally, in the Aeolian system the genetic closeness between Vul-

cano and Stromboli to mainland population rather than to other islands suggests intermittent ongoing introductions. Genomic analyses indicate that populations from islands of Tuscany display the most evident signal of selection, followed by Aeolians and Aegadians. Also, lizards from islands of Tuscany reveal the broadest trophic niche and the largest shifts in body size, supporting the time-for-evolution hypothesis. All these results support the species capacity to disperse and successful establish in new environments but also illustrate the complexity of the interactions between evolutionary history, biogeographic background and environmental conditions promoting shifts in genotype and phenotype.

Overall, these results provide multiple layers of knowledge about biological invasions on the Mediterranean Islands. All the layers have in common the complexity of the phenomena on this region, by a combination of signals related to a deep human role and the natural adaptation or plasticity of species to the new environments. The integration of several tools to understand different stages of the process allowed a clear but complex picture. The Mediterranean Islands harbour systems with hidden complexity of alien reptiles, which are able to take advantage of their inherited traits and also of the human help, to succeed and expand. Such complexity brought additional questions providing directions for further studies.

Resumo

O movimento de espécies sempre ocorreu naturalmente de acordo com as capacidades de dispersão de cada espécie. No entanto, quando os seres humanos iniciaram a exploração do mundo, começaram também a transportar espécies do seu local de origem para locais onde as espécies nunca chegariam pelos seus próprios meios, redefinindo assim a biota de todo o mundo. Em alguns casos, os organismos transportados foram capazes de estabelecer as suas populações no novo local, expandindo-se e eventualmente tornando-se prejudiciais para as espécies nativas. Como resultado, as invasões biológicas representam hoje uma das maiores causas de perda de biodiversidade, e, ao mesmo tempo, proporcionam uma oportunidade de aprofundar o nosso conhecimento sobre questões chave em ecologia e evolução.

A bacia do Mediterrâneo é o resultado de uma história complexa que engloba processos geológicos profundos, mudanças climáticas e intercâmbios biogeográficos, sendo considerada um dos hotspots de biodiversidade do mundo. Esta região tem sido o ponto de encontro de várias civilizações, com uma longa história de interação entre humanos e biota, fazendo com que seja um bom modelo para estudar as diferentes camadas do fenómeno de invasões biológicas. As ilhas do Mediterrâneo, em particular, representam um laboratório vivo para testar hipóteses ecológicas e evolutivas, devido ao seu isolamento geográfico e limitado número e taxa de organismos que podem colonizar estes ecossistemas naturalmente. Estas características, por outro lado, fazem com que os ecossistemas insulares sejam particularmente vulneráveis a invasões biológicas.

Os répteis são um grupo de vertebrados particularmente vulnerável a invasões biológicas, mas ao mesmo tempo é um dos grupos mais introduzidos em novos locais. A longa relação entre humanos e répteis nas ilhas do Mediterrâneo tem causado uma mistura de introduções antigas e recentes, o que torna difícil a distinção entre as espécies exóticas e as nativas nesta região. Simultaneamente, a diversidade de ilhas, juntamente com os eventos de introdução repetidos, oferece uma excelente oportunidade de investigar os fatores relacionados com a introdução e sucesso de espécies exóticas em novos ambientes.

O principal objetivo da tese é determinar os padrões e inferir os principais fatores responsáveis pelo fenómeno de invasões biológicas, a diferentes níveis, nos répteis das ilhas do Mediterrâneo, usando uma combinação de ferramentas biogeográficas, ecológicas e genéticas.

Primeiramente, focamo-nos na ampla bacia do Mediterrâneo; combinamos genética, distribuição e dados biológicos em répteis do Mediterrâneo com informação de ilhas, de forma a identificar os fa-

tores que promovem a presença de répteis exóticos em ilhas, tendo em conta variáveis geográficas, humanas e características das espécies. Adicionalmente, foi realizada uma revisão bibliográfica para determinar a condição dos répteis (nativa/exótica) nas mais de 900 ilhas do Mediterrâneo, informação que foi incorporada na base de dados criada. Os modelos espaciais mostram um sinal complexo que mistura as características geográficas, os traços das próprias espécies e os fatores relacionados com o ser humano. Um registo de um réptil é mais provável ser exótico se a espécie for capaz de se alimentar de plantas e estiver presente em ilhas que se encontram longe da sua área nativa e com uma maior população humana. As fontes tradicionais de dados subestimam a frequência de répteis exóticos nas ilhas do Mediterrâneo, enquanto que o uso de informação genética para avaliar a condição das populações concede maior precisão na inferência dos fatores relacionados com as espécies exóticas.

Segundo, nós restringimos a escala de estudo para um arquipélago paradigmático, as ilhas Baleares: neste arquipélago os répteis exóticos ultrapassam o número de répteis nativos e, recentemente, múltiplas espécies de cobras têm vindo a ser introduzidas, colocando em maior perigo as poucas espécies nativas presentes. Foram usados métodos filogenéticos combinados com outras fontes de informação (literatura e observações diretas) para identificar a origem e vias de introdução de quatro espécies de cobra introduzidas (*Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus* and *Rhinechis scalaris*). Usamos também modelos de nicho ecológico para deduzir padrões de invasão e expansão, baseados na adequação das ilhas no presente e no futuro. A Península Ibérica é a origem mais provável para a maioria das populações exóticas. Contudo, a fraca estrutura filogeográfica das espécies não permite a identificação das áreas de origem com precisão. Os modelos de nicho ecológico mostram uma baixa adequabilidade do habitat no presente, no entanto com tendência para aumentar significativamente sob cenários de alteração climática nas próximas décadas. As observações directas juntamente com as análises efectuadas permitiram a identificação da via de introdução mais comum, o comércio de plantas ornamentais, especificamente a importação de oliveiras da Península Ibérica.

Terceiro, focamo-nos na lagartixa-italiana, *Podarcis sicula*, que foi introduzida em vários locais do mundo, desde a região do Mediterrâneo à América do Norte, onde é frequentemente considerada invasora e, portanto, um bom modelo para perceber a história e características que fazem um réptil prosperar em diferentes sistemas. Estudos realizados por outros autores suportam um comportamento invasivo com interferência nas espécies nativas e uma grande capacidade de mudança quando em novos ambientes. Os dados de genómica foram utilizados para inferir a condição exótica/nativa das populações insulares e foram combinados com análises de isótopos estáveis

e análises morfológicas para deduzir as mudanças genéticas e fenotípicas entre as populações exóticas (ilhas) e nativas (continente). Os dados genómicos foram também utilizados para realizar uma análise de outlier loci, de forma a testar a seleção nas populações exóticas. Foram três os sistemas usados neste estudo: Aegadian (Erice, Birgi, Marettimo, Favignana e Levanzo), Aeolian (Milazzo, Vulcano, Salina, Filicudi e Stromboli) e Toscânia (Talamone, Capalbio, Argentario, Giglio, Giannutri and Formica Grande). Os resultados confirmam a dificuldade em inferir a condição de algumas espécies nas ilhas do Mediterrâneo. Na Toscânia, a evidência genética sugere uma colonização antiga das ilhas, que pode ter sido mediada pelo ser humano ou resultado de uma dispersão natural durante o último máximo glacial. No sistema Aegadian, a maior proximidade dos indivíduos de Marettimo a populações do continente sugere uma introdução recente, ao passo que a homogeneidade genética entre ilhas e continente sugere um fluxo genético contínuo. Finalmente, no sistema Aeolian, as populações de Vulcano e Stromboli encontram-se mais próximas à população do continente em vez das restantes ilhas, sugerindo introduções intermitentes. As análises de genómica indicam, também, que as populações das ilhas da Toscânia demonstram um sinal mais evidente de seleção, seguida das ilhas do Aeolian e do Aegadian. Adicionalmente, as lagartixas das ilhas da Toscânia revelam um nicho trófico mais amplo e as maiores mudanças no cumprimento do corpo, suportando uma hipótese de tempo-para-evoluir. Todos estes resultados suportam a capacidade da espécie para dispersar e estabelecer-se com sucesso em novos locais, mas também demonstra a complexidade das interações entre a história evolutiva, a base biogeográfica e as condições ambientais que promovem as mudanças ao nível do genótipo e do fenótipo.

De uma forma geral, estes resultados proporcionam múltiplas camadas de conhecimento acerca das invasões biológicas em ilhas do Mediterrâneo. Todas estas camadas têm em comum a complexidade dos fenómenos nesta região, pela mistura de sinais relacionados com o papel do ser humano e a adaptação natural ou plasticidade das espécies aos novos ambientes. A integração de várias ferramentas para um melhor entendimento das diferentes etapas do processo permitiu uma imagem mais clara, mas complexa das invasões. As ilhas do Mediterrâneo albergam sistemas com uma complexidade escondida de répteis exóticos que são capazes de tirar proveito dos seus traços herdados e da ajuda humana para serem bem-sucedidos e se expandirem. Tal complexidade levantou questões adicionais, definindo a direção de estudos futuros.

Keywords

Mediterranean islands, biological invasions, alien species, reptiles, biogeography, adaptation, phenotypic plasticity, diet, human-features, ddRAD-seq, stable isotopes, morphology, climate change, GAMM, ecological niche modelling, mitochondrial DNA, Balearic Islands, *Podarcis sicula*, *Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus*, *Rhinechis scalaris*

Palavras - Chave

Ilhas do Mediterrâneo, invasões biológicas, espécies exóticas, répteis, biogeografia, adaptação, plasticidade fenotípica, dieta, fatores humanos, ddRAD - seq, isótopos estáveis, morfologia, mudanças climáticas, GAMM, modelação do nicho ecológico, DNA mitocondrial, Ilhas das Baleares, *Podarcis sicula*, *Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus*, *Rhinechis scalaris*

Abbreviations

ABC - Approximate Bayesian Computation

AIC - Akaikes Information Criterion

AUC - Area Under the Curve

BCE - Before Common Era

BP - Before Present

C - Carbon

CBD - Convention on Biological Diversity

CD - Mean Distance to the Centroid

Cyt*b* - Cytochrome*b*

ddRAD-seq - double-digested Restriction Associated DNA sequencing

DNA - Deoxyribonucleic Acid

GAMM - Generalized Additive Mixed Models

GBIF - Global Biodiversity Information Facility

IUCN - International Union for Conservation of Nature

LGM - Last Glaciation Maximum

MESS - Multivariate Environmental Similarity Surfaces

ML - Maximum Likelihood

Myr - Million years

MyrBP - Million years before present

N - Nitrogen

NE - North-East

NJ - Neighbour-Joining

PCA - Principal Component Analysis

PCR - Polymerase Chain Reaction

ROC - Receiver Operated Characteristics

SE - South-East

SEA - Standard Ellipse Area

SNPs - Single Nucleotide Polymorphisms

SVL - Snout-Length Length

TSS - True skill statistics

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Chapter 1

General Introduction

1.1 Part I: Biological invasions

1.1.1 Biological invasions and invasion science

The distribution of fauna in time and space under natural conditions is shaped by the availability of suitable habitat and the dispersal abilities of each species. Nevertheless, since humans started to colonize the Earth, they also started to reshape the worlds biota by translocating species to a place where they could not arrive by their own means (Lockwood, Hoopes, and Marchetti 2009). Human impact on the global distribution of species has grown over time, and especially after the II World War when massive globalization started to occur, biological invasions gained strength playing an increasingly important role on the definition of the Anthropocene Era (Hui and Richardson 2017). Elton (1958) was the first acknowledging this phenomenon by his pioneer book The Ecology of Invasions by Animals and Plants where he explored and proposed several hypothesis about invaders and invaded ecosystems. He is, then, considered the father of invasion science. This new science has been developed by multiple studies through the years becoming nowadays a mature discipline. Blackburn et al., (2011) has attempted to unify concepts in order to provide a simplified and clear definition of biological invasions processes and their underlying concepts (Fig. 1.1; Table 1.1), so all the scientists, policies and community are synchronized and understand each other.

In conservation biology, biological invasions are currently considered a major cause of biodiversity loss. This is because the effects of an alien species introduction are often severe and largely unpredictable. Overall, several major impacts are acknowledged by scientists: biotic homogenization (Mckinney, Mckinney, and Lockwood 2016; Toussaint et al. 2016; Olden and Rooney 2006; Lososová et al. 2016), disruption of food-webs (Fritts and Rodda 1998; David et al. 2017; Walsh, Carpenter, and Vander Zanden 2016; Galiana et al. 2014), alteration of community structure (Faraone et al. 2008; Craven et al. 2017; Traveset and Riera 2005) or genetic pollution by hybridization with native species (Lamer et al. 2015; Pliszko and Zalewska-Gaosz 2016). Furthermore, this phenomenon can also cause severe social and economic losses (Fritts, McCoid, and Haddock 1990; Barbier 2001; Perrings, Williamson, and Dalmazzone 2000; Mazza et al. 2014), as well as loss of scientific knowledge regarding the original species and its ecological relationships and dynamics.

The phenomenon of biological invasion is characterized in three main stages (Fig.1.1): (1) Transport/Introduction; (2) Establishment; and (3) Spread in the new range (Blackburn et al. 2011). The first stage involves the transportation of species by humans either intentionally due to its economic value or to provide services, or unintentionally where species are inadvertently transported in cargos being introduced in a new place. The establishment represents the step in which the population introduced is able to reproduce with success, due to the concordance between biotic and abiotic conditions and the needs of the species which arrive to the new range (Bomford, Kraus, and Barry 2009). The lower presence of predators, competitors, parasites and disease in the non-indigenous area can also give alien species an advantage over the native species, potentiating their expansion and success. Another determinant factor for the establishment of alien species is the propagule pressure (i.e. number of individuals released), because if species are released more often at more sites or in a great number, the probability of their success is higher (Williamson 1996; Kolar and Lodge 2001; Hayes and Barry 2008; Ficetola, Bonin, and Miaud 2008; Cassey et al. 2018). Furthermore, the life-history and behavioral characteristics of each species, as well as the invasional meltdown (i.e. earlier invaders benefit later arriving aliens) can also enhance the survival and reproduction in the new area (Simberloff and Holle 1999). Finally, in the last stage of the

Table 1.1: Main terminology used in biological invasions (source: Blackburn et al., 2011)

Concept	**Definition**
Native species (synonyms: indigenous)	Species that evolved in a given area or that arrived there by natural means, without the intervention of humans.
Alien species (synonyms: exotic, introduced, non-indigenous, non-native)	Species whose presence in a region is attributable to human actions that enabled them to overcome fundamental biogeographical barriers (i.e. human-mediated extra-range dispersal).
Naturalized species (synonyms: established species)	Alien species able to sustain self-replacing populations for several life cycles or a given period without direct intervention of people, or despite human intervention.
Invasive species	Alien species that sustain self-replacing populations over several life cycles produce reproductive offspring, often in very large numbers at considerable distances from the parent and/or site of introduction and have the potential to spread over long distances. Invasive species are a subset of naturalized species, but not all the naturalized species become invasive. The Convention on Biological Diversity (CBD) and the World Conservation Union (IUCN) definition add that an invasive species causes impacts on the economy, environment or health.
Introduction	Movement of a species, intentionally or accidentally, due to human activity, from an area where it is native to a region outside that range. The act of introduction may or not lead to invasion.
Invasion	The multistage process whereby an alien organism negotiates a series of potential barriers in the naturalization-invasion continuum.

invasion process, the spread can happen either slowly or in an explosive way, depending on the species characteristics. In several cases, introduced populations starts to grow in an undetectable way, normally called of lag phase, which makes difficult to detect an invasion in early stages, making any act on the management of the original biota difficult. In islands, the first and second steps (transport/introduction and establishment) are the most relevant to develop preventive measures towards new invasions, and hence, biogeographical and ecological studies should be performed to understand the distribution of species.

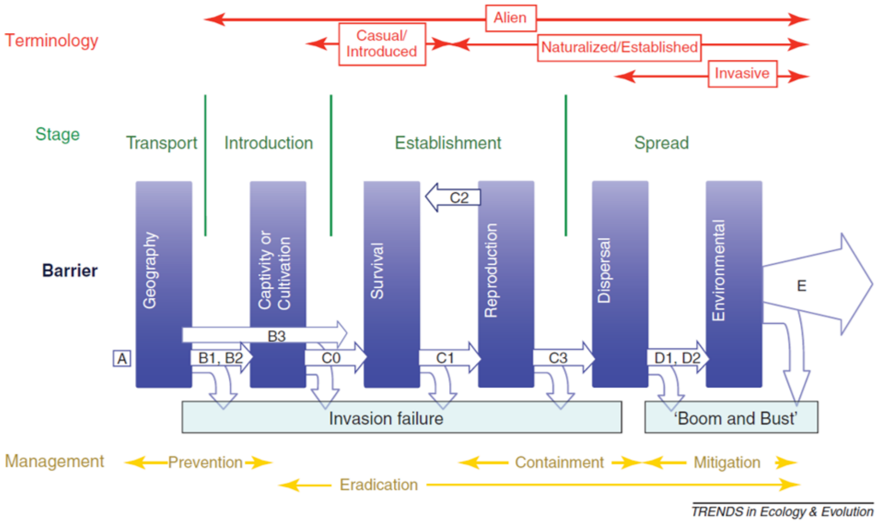

Figure 1.1: The unified framework proposed by Blackburn et al. (2011) for biological invasions. This framework represents the invasion process which can be divided in several stages, as described in the main text.

1.1.2 Island biogeography of biological invasions

> "In islands we have the facts of distribution often presented to us in their simplest forms, along with others which become gradually more and more complex; and we are therefore able to proceed step by step in the solution of the problems they present."
>
> Wallace (1880, p. 234)

Islands represent living laboratories where we can test several evolutionary and ecological hypotheses in a simplified manner. They are geographically isolated from the continent, limiting the number and taxa of organisms that can naturally reach and colonize these ecosystems. As a result, islands have special properties, such as fewer species, often fewer populations and/or smaller population sizes, lower predation and interspecific competition, and fewer parasites and diseases (MacArthur and Wilson 1967; Whittaker and Fernández-Palacios 2007). Therefore, islands are particularly vulnerable to biological invasions, which can cause great impacts on native and endemic biota not familiar with predators or new diseases (van Kleunen et al. 2015; Courchamp et al. 2015; Blackburn et al. 2004).

The theory of island biogeography states that species richness on islands depends on the equilibrium between species extinction, species colonization and speciation (Fig. 1.2, Whittaker and Fernández-Palacios, 2007). However, the globalization and exploration of islands by humans lead to a disruption of this equilibrium due to the destruction of habitat, the introduction of alien species and the increase of extinction rate of native species. The biogeography of island biodiversity is nowadays influenced not only by the species-area relationship, but also by different human factors. Numerous studies have been performed to identify those factors. Ficetola and Padoa-Schioppa (2009) have suggested that human population size and exchange (e.g. transportation) are key artificial factors explaining the reptiles distribution on islands. Islands with more human impact would host fewer native species and higher number of alien species. Blackburn and co-authors (2015) propose the same for plant and bird distributions, the islands with higher human population size being those with the highest number of alien species. Furthermore, Helmus and co-authors (2014) suggest that economic isolation would have an important role on the dispersion of alien species, with islands with higher importance in the trade being the ones more susceptible to invasion.

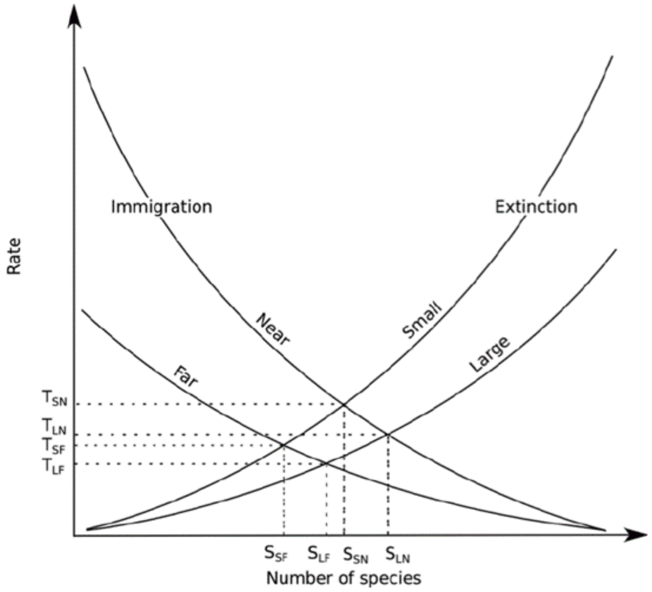

Figure 1.2: Equilibrium model of island biogeography as proposed by MacArthur and Wilson (1967). Graph represent the effects of island size small (S) and large (L) and island isolation near (N) and far (F) on the number of species (S) and the rate of species turnover (T). Source: MacArthur and Wilson, 1967.

In a context of human-dominated ecosystems, other authors are thus testing if biogeographic rules would stay the same. Burns (2015) proposes a modified version of the Theory of Island Biogeography keeping some principles in common with the former but adding a distinction on the dynamics of alien species from the dynamics of native species. This modified model predicts that the dynamics of native and alien species differ only on the early stages of the invasion and then converge long-term into the same pattern. Capinha and co-authors (2015) suggest that biogeographical barriers will be broken by human globalization, and the compositional dissimilarities will be reduced among distant regions. Furthermore, they suggest that novel species assemblages will be dominated by relatively few but widespread alien species, namely competitive generalists. The distribution of the species will be explained mostly by human-factors (such as trade) and climate, being the ecophysiological limitations of species the main responsible for a higher prevalence of biotic homogenization among areas sharing similar environments.

1.1.3 Climate change and biological invasions

It is now well-known that climate change is having significant impacts on native plants and animals of the world. The same is expected to happen with the invader species. Climate change can impact them either directly by promoting physiological shifts and changing its demography, either indirectly by modifying communities, environments and landscapes which they will occupy. On the other hand, in a combined force, humans will also have an additional role to play because climate change will change human behavior (e.g. economy and trade), which will have an impact on the transport and establishment of the alien species (Lockwood, Hoopes, and Marchetti 2009), and ultimately on its distribution in the world. This complex interplay has been studied by several authors in order to evaluate if the alien species will benefit or suffer the impact of the climate change, as the native species. Walther and co-authors (2009) performed a review on climate-mediated biological invasions of plants, invertebrates, fishes and birds, suggesting how climate change can influence these alien species. In summary, climate change can facilitate transport, increase success of survival and growth of alien species, enable the successful reproduction and establishment, and enhance the competitive and dispersal ability in the new environment. On the other hand, Sorte and co-authors (2013) and Rahel and Olden (2008) argue that ecosystems can respond differently to climate change: if climate becomes more suitable then the invasion risk is high, whereas if it shifts to severer conditions, it will become more resilient to biological invasions. Additionally, other authors, such as Hulme (2017), suggest that it is complex to establish a direct relationship between climate change and biological invasions, exactly due to the inherent complexity and interaction among the main drivers: climate, humans and species traits.

The importance of understanding the factors driving the distribution of species in the world stresses the role of studies analyzing alien species populations and their spread, which can greatly help on the development of conservation strategies but also on the acquisition of knowledge on species biology and ecology.

1.1.4 Ecological shifts and evolution of alien species

The arrival of an alien species into a new environment provides the opportunity to study ecological and evolutionary changes over contemporary timescales. The colonization and spread over a new area will subject the species to new environmental conditions, such as climate, novel edaphic (i.e. soil types) and biotic environments which can include different microbial, herbivore and predator communities, distinct competitive regimes, lack of mutualists or novel food resources such as new prey species. The proper phenotypic shift in the alien species will allow it to establish, reproduce and be successful on the new environment (García-Ramos and Rodríguez 2002). This shift can be attained by genetic changes, phenotypic plasticity or both.

The genetic diversity is generally divided in two main types: neutral and adaptive. Neutral diversity does not have a direct effect on individual fitness unless associated to adaptive components of the genome. However, measuring neutral genetic diversity can give important insights regarding several processes, namely gene flow between populations and admixture, population demographic history and species invasion routes (Kolbe et al. 2007; Dlugosch et al. 2015), which are important to understand the invasion dynamics and process. On the other hand, adaptive genetic diversity is affected by natural selection, which is expected to suffer important shifts in the novel environment and can give information regarding the genetic background in life history traits and the processes generating this variation, which is very important to determine potential invasion success (Colautti and Lau, 2015; Dlugosch et al., 2015).

Introduced populations are generally expected to have lower genetic diversity than populations from the native range. This loss of neutral genetic variation results from several processes like bottlenecks and founder effects (i.e. low number of invading individuals). However, many species are able to survive and be successful in the environment even when they should be suffering from deleterious losses of genetic diversity; this has been described as the genetic paradox applied to invasions. Dlugosch and Parker (2008) suggest that the neutral genetic loss is only moderate, with species losing about 19% of their heterozygosity during a single invasion event. But even if the loss is higher, this decrease in neutral genetic diversity do not necessarily lead to an immediate reduction in adaptive genetic variation in fitness-related traits. An example of this is the invasive shrub *Hypericum canariense* L. which has shown that rapid evolution can occur in founding populations that had suffered a bottleneck (Dlugosch and Parker 2008). Nevertheless, a loss on adaptive genetic variation would be more limitative of species potential for local adaptation, since natural selection requires genetic variation, e.g. in morphological traits (Lee 2002). Population genetic studies have demonstrated that a high genetic variation would allow a faster response to selection which increase the potential to adapt to the new environment and, consequently, increases the colonization and establishment success of the alien species (Lee 2002; García-Ramos and Rodríguez 2002; Parker, Rodriguez, and Loik 2003). Furthermore, the invasion process can underly a complex introduction history, and there is increasing evidence that successful non-native populations can be the result of multiple introductions instead of one single event. The admixture of these multiple sources of diversity can allow the increase of standing genetic variation in ecologically important traits leading to the production of novel phenotypes, both of which could facilitate adaptation in novel environments (Kolbe et al. 2004; Rius and Darling 2014). Furthermore, the hybridization with native species can also happen (i.e. interspecific hybridization) and this may also facilitate the establishment and growth success of the invader by providing a source of locally adapted genomic pool (Mesgaran et al. 2016; Roy et al. 2015). Finally, a constant gene flow can maintain or increase the genetic variability of the invader, if coming from divergent populations, or restrict the local adaptation of the invader, if coming from populations where individuals are not suitable to adapt to the conditions of the new environmental range (Dlugosch and Parker, 2008b; Sakai et al., 2001).

The alien species can also have already the proper genotype for adaptation in a novel environment, where there will not be an incorporation of new genes, but the expression of different phenotypes, according to different environmental requirements phenotypic plasticity. One hypothesis is that this process will allow populations to tolerate and adapt to new conditions, even if they have low genetic diversity (Ghalambor et al. 2007). Alternatively, natural selection will favor genotypes with increased phenotypic plasticity, thus leading to a rapid evolution of plasticity in invading species and consequently, facilitating its invasion and colonization (Richards et al. 2006). Lande (2015) suggests that there is no general tendency for increased plasticity or environmental tolerance in alien species, depending this process outcome of several parameters such as the optimal phenotype, the predictability of the environment or the cost of plasticity for the species.

Overall, the study of the origin, genetic diversity and changes on genetic and phenotypic traits allows understanding of the species invasion potential, as well as the biological invasions process itself, and could be used to develop guidelines for informed conservation policies.

1.2 Study area and species

1.2.1 The Mediterranean basin

The biodiversity of the Mediterranean Basin is the result of a complex history encompassing profound geological processes, climatic shifts, and biogeographical interchanges (Blondel et al. 2010) (Fig. 1.3). The Mediterranean Basin is localized between Africa, Asia and Europe, where geological processes occurred and lead to its current form (Cavazza and Wezel 2003). Along the geological time, this Sea was shaped by the destruction of old oceanic crust in subduction zones, continental plate collision, the volcanic arcs and the deformation of the active crust interacting through the course of Mediterranean formation and explaining the formation of various sea basins and numerous islands with different shapes, sizes and distribution (Arnold 2008). For the biota, the most influential geological events were: 1) the Messinian Salinity Crisis (from 5.9 to 5.3 Myr ago) during which the Mediterranean Sea went into a nearly complete desiccation allowing the last substantial faunal interchanges between land masses; and 2) the Pleistocene climatic oscillations between glacial and interglacial episodes (especially in the last Myr), which played a key role in molding and shaping present-day biota, including species range shifts and extinctions (Blondel et al. 2010). Nowadays, the Mediterranean Basin and adjacent regions are considered one of the global biodiversity hotspots (Myers et al. 2000), with islands sharing common species with the mainland of Europe, Asia and Africa but also with high levels of endemism due to a complex history with multiple vicariance and dispersal events.

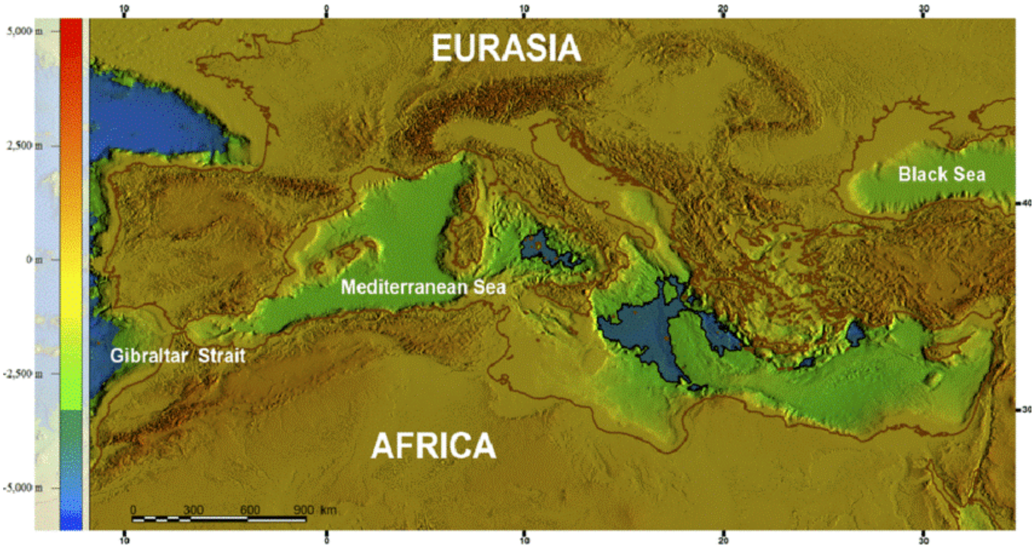

Figure 1.3: Paleogeographic reconstruction of the Mediterranean during the Messinian salinity crisis. In blue is the estimated Messinian extension of the Mediterranean basin (coastlines are in black). In brown are the current coastlines. Source: (Anzidei et al. 2014)

During the Holocene, and especially since the Neolithic, humans started playing a substantial role and contributed to great changes on the Mediterranean basin. Such effect already lasts millennia and directly impacts land-use patterns, resource management, and biota composition. For instance, recent evidence from archaeological sites in Cyprus reveals that human colonization of that island began very soon after the end of the last glacial episode (Abulafia 2011). Since ancient times, the Mediterranean was the meeting point of several civilizations which used the Mediterranean Sea and its islands as networks for their commercial trade (Fig. 1.4), namely the Phoenicians, the Greeks and the Romans in antiquity, Genoese, Venetians and Catalans in Middle Ages and Dutch, English and Russian navies in the centuries before 1800 (Abulafia 2011). After 1850, the Mediterranean started losing its central role on the world affairs and commerce; however, in recent times, a new threat to its biota arrived with the intensification of tourism.

Figure 1.4: Main routes during Medieval time. Source: http://www.icks.org/hugo33kim

Historical trade networks and more recent touristic activities have been defining the distribution of species within the Mediterranean region, with humans introducing species in islands where they were not present or causing the extinction of endemic species. Humans have been introducing animals and plants to new environments for several reasons, which can vary from a food resource to a religious rite. In general, when humans move to new places, they often try to match the local environment with familiar species of plants and animals threatening the native biota. Species can also be introduced by accident as stowaways among cargo.

This long interaction between humans and biota causes a mix of ancient and recent introductions making difficult to disentangle the alien species from the native ones. Alien reptile species do not make an exception, and while some species are long known to be invaders, the alien or native status of many others is yet not so clear.

1.2.2 Reptiles as a model group

Reptiles are ectotherms; hence their temperature requirements could potentially limit over-water dispersal. Additionally, some species have small size, and this also limits the long-distance over-water dispersal, essential on islands environments. However, within reptiles, we can find many examples of species that have been able to disperse naturally by rafting from hundreds up to thousands of kilometres, such as the day geckos *Phelsuma* in Andaman islands (5700 km; Rocha et al. 2009) and the skinks *Cryptoblepharus* in West

Africa (6000km; Rocha et al. 2006; Gamble et al. 2008). Indeed, reptiles are prone to be introduced by man and to establish in new environments. Depending on the species, they have several characteristics which make them good invaders: (1) they are very susceptible to be transported by human networks, either due to their small size (in some species) or secretive behavior, which make difficult to detect them, or to the use of human manufactures and ornamental plants as a refuge (Pitt, Vice, and Pitzler 2005; Valdeón et al. 2010); (2) they are resistant to starvation during transportation and able to withstand until two years without feeding (De Vosjoli 2012; McCue 2010, 2013); (3) some species, namely lizards and snakes, have cryptic behaviors, allowing the development of populations in an undetectable way until is readily established; (4) some species have generalist diets enabling many reptiles to adapt to the resources available on the novel environment (Pitt, Vice, and Pitzler 2005); (5) they generally have high reproductive rates which facilitate the rapid growth of population and the recovering of stochastic events; (6) their low metabolic rates and higher resistance to desiccation compared to endotherms allow to maintain a viable population in harsh environments, namely very small islets.

As a result of molecular studies, many species assumed to be native to Mediterranean islands are now known to be the result of ancient introduction. As one example of this complexity, we have the viper *Vipera aspis*, that was thought to be autochthonous in Montecristo Islands and even described as an endemic species, but in the light of genetic evidence has been revealed as an introduction likely by ancient militia from Magna Graecia during centuries VIII to III BCE (Barbanera et al. 2009). Another example is the case of *Emys orbicularis* in Sardinia, where there are fossils suggesting a continuous presence since the Late-Middle Pleistocene pointing a native status, but the low genetic differentiation between those island populations and the mainland suggest a recent re-introduction. Indeed, Pedall and co-authors (2011) propose that the observed phylogeographic pattern of this species could be indicative that native populations got extinct in those islands and that extant turtles were introduced later. These are only two examples on how the biological invasions patterns can be misleading in the Mediterranean islands justifying the need for clarification, especially on ancient and historical introductions. Hence, besides a biogeographical perspective of patterns on alien reptiles, there is a need for studies focused on relevant species or areas which unfold the biological invasions processes happening the Mediterranean Islands.

The Balearic Islands represent a paradigmatic case of multiple biological invasions within the Mediterranean Basin, with a much higher number of alien reptiles (i.e. lizards, snakes and turtles/tortoises) than native (Pinya and Carretero 2011). This archipelago is isolated from the continent since the Messinian (5.33 MyrBP) and nowadays host only two native and endemic terrestrial reptiles: the Lilfords lizard *Podarcis lilfordi* and the Ibiza wall lizard *Podarcis pityusensis*. During the last century, tourism development, the pet trade, and cargo transport of ornamental plants have produced a new wave of biological invasions. Recently introduced snakes are of special concern (Fig. 1.5), since the effect of predation may seriously threaten the remaining native reptiles in the main islands and the endemic subspecies in surrounding islets. Most of the introduced species are partially saurophagous and potentially very harmful for the populations of the native endemic lizards, specially from Ibiza and Formentera islands which remained snake-free until recently. Sadly, it seems that *P. pityusensis* may be now at risk of following the declining trajectory that *P. lilfordi* had in the past (Montes et al. 2015; Hinckley et al. 2017). The analysis of the source areas and introduction pathways as well as the interaction with other disturbance factors such as to climate change is then essential to understand the current and future colonization dynamics of these predators, in order to establish efficient conservation measures.

Figure 1.5: Snakes introduced in Balearic Islands. From left to right: *Hemorrhois hippocrepis*, *Macroprotodon mauritanicus*, *Malpolon monspessulanus* and *Rhinechis scalaris*.

1.2.3 Podarcis sicula as a model species

Within Mediterranean reptiles, the Italian wall lizard, *Podarcis sicula*, represents a paradigmatic case study in invasion biology. This is an opportunistic lizard, able to inhabit several kinds of habitats, including humanized areas and using man-made objects or ornamental plants as refuges. Therefore, risk of its accidental transportation is high, and the availability of anthropic environments seems advantageous for its spread (Corti and Lo Cascio 2002).

The native range of this lizard includes the Italian peninsula, Sicily and the north Adriatic coast, but it has been introduced in several other places in the world, namely Greece, Turkey, United Kingdom, several Mediterranean Islands and United States of America (Fig. 1.6).

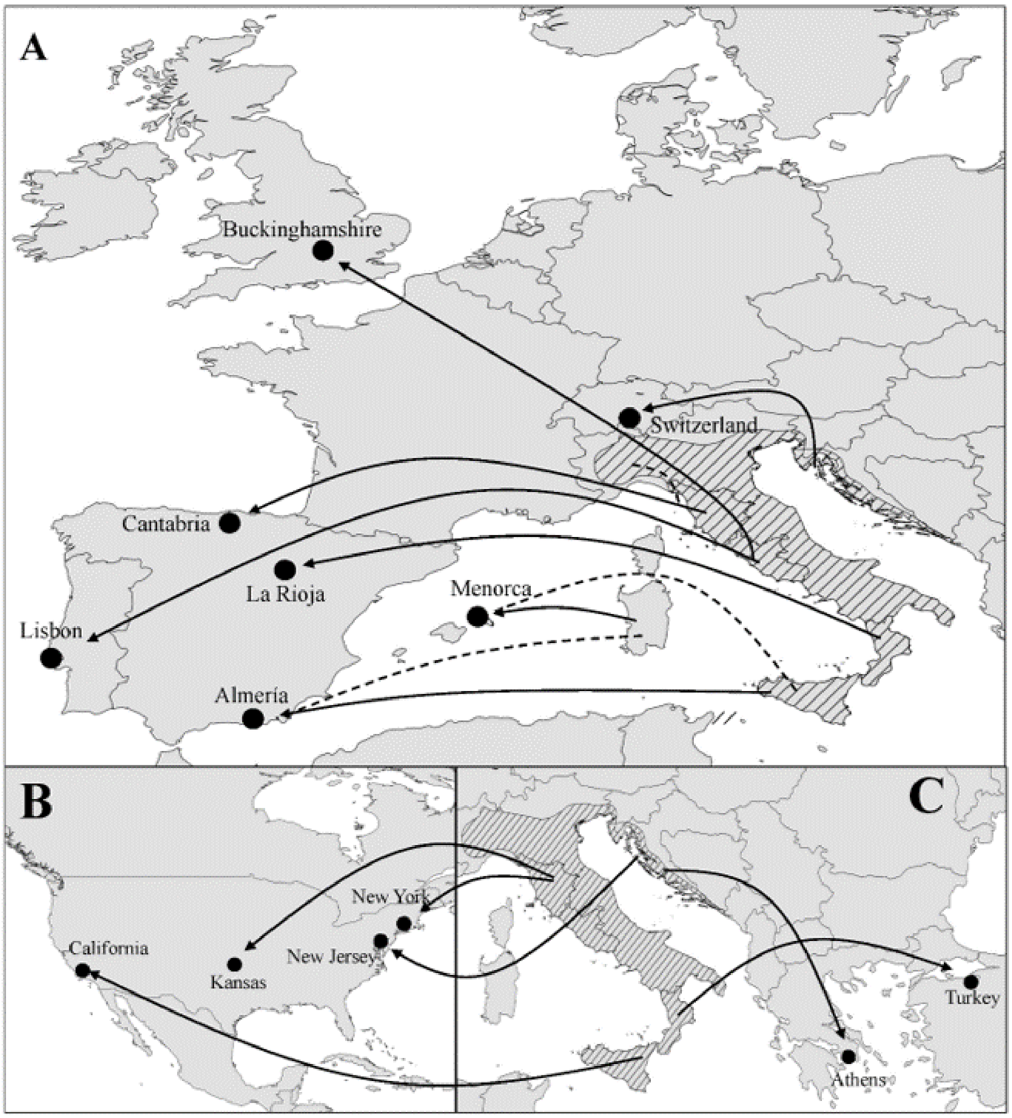

Figure 1.6: Map of introduced populations of Podarcis sicula. Source: Silva-Rocha et al. 2014.

The native or alien nature of the populations inhabiting some islands and islets surrounding the Italian Peninsula and northwest Adriatic Coast remains doubtful, due to the ancient presence of human activities (Blondel et al. 2010) and the possibility of connection with its native range during the Ice Ages. Studies from Kolbe et al., (2013) and Silva-Rocha et al. (2012, 2014) support a multiple source pattern of introductions in multiple places in the world, which makes the colonization and invasion history *P. sicula* complex and difficult to address. On the other hand, this species is able to rapidly change according to new environments requirements becoming a successful invader. For example, Vervust and coauthors (2010) performed an experiment where lizards of Pod Mrcaru (Croatia) were fed only with arthropods over the course of 15 weeks to assess if adaptation was mainly genetic or plastic. They suggest that at least some of the changes are based on plastic adaptation, since after the experiment the digestion tract length was reduced and a total loss of cecal valves was observed. The study of colonization history in the Mediterranean, as well as how this species reacts to island environments where it was introduced, are also important to understand how reptiles disperse and succeed along Mediterranean islands.

1.3 Objectives and Thesis Framework

The main aim of this dissertation was to determine the patterns and processes of biological invasions of reptiles on Mediterranean Islands and to infer the main drivers of such invasions, using biogeographical, ecological and genetic tools.

This thesis is organized in six Chapters, with **Chapter 1** as a General Introduction (the present chapter). Four chapters deal with scientific manuscripts developed with the purpose of detecting the major drivers of the biological invasion phenomena at a larger scale of the Mediterranean as well as at a finer scale focusing in two study cases.

The work developed in **Chapter 2** aimed to identify the environmental factors promoting the presence of alien reptiles on Mediterranean islands, considering human-related, geographic and species traits. Additionally, it also aimed to assess whether the use of genetic evidence to identify alien species allow a better inference on the factors determining the distribution of alien species. This chapter comprises a paper under revision in the journal Diversity and Distributions entitled “Alien reptiles on Mediterranean islands: a model for invasion biogeography”.

The work developed in **Chapter 3** aimed to identify the origin and colonization pathways of alien reptiles focusing on the snakes from Balearic Archipelago, as well as to predict their expansion under current and future climate change scenarios. This paper is composed by a paper published in PLoS One entitled “Snakes on the Balearic Islands: An Invasion Tale with Implications for Native Biodiversity".

The study from **Chapter 4** aimed to determine the population genetic structure and colonization history of the putatively introduced *Podarcis sicula*, in three independent mainland/island systems in the Mediterranean Tuscany, Aegadian and Aeolian. Analysis of an additional system (Croatia) was performed, but high level of hybridization with native species was found, hence a deeper study on the impact of inter-hybridization which is out of scope of this thesis should be made prior to comparisons with the other three systems. This chapter includes one paper currently in preparation entitled "Genetic signature of the invasion of the lizard *Podarcis sicula* on Mediterranean islands".

In **Chapter 5**, the work is addressed to recognize the phenotypic and genotypic shifts in the lizard, *Podarcis sicula*, in response to successful, recent colonization, human or natural, to novel environmental conditions in islands in different systems. This chapter comprises one paper currently in preparation entitled "Life trait shifts and genetic variation across insular systems by the opportunistic lizard *Podarcis sicula*.".

Finally, the **Chapter 6** called General Discussion, joins all the subjects addressed in the previous chapters, emphasizing the main discoveries and general achievements of this dissertation. It also suggests guidelines for further research that arose from this work.

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Chapter 2

Mediterranean Alien Reptile Biogeography

Silva-Rocha, Iolanda, Daniele Salvi, Miguel A. Carretero and G. Francesco Ficetola. under revision. “Alien reptiles on Mediterranean islands: a model for invasion biogeography.” Diversity and Distributions.

Abstract

Aim The Mediterranean basin has a long history of interactions between humans and biota, with multiple ancient and recent introductions of alien species. Such a multitude of introductions makes it difficult to distinguish between alien and native species but provides an excellent opportunity to investigate factors related to introductions and long-term persistence of alien species. In this study we combined genetic and distribution data to identify the factors promoting the presence of alien reptiles on islands, considering human-related, geographic and species traits. Furthermore, we assessed whether the use of genetic evidence to identify alien species allows a better inference on the factors determining the distribution of alien species.

Location Mediterranean islands

Methods We combined genetic data and distribution databases to obtain information on biological traits and on the native / alien status of reptiles on more than 900 Mediterranean islands, and we gathered data on geographic and human features of islands. We then used spatially-explicit generalized additive mixed models to identify the factors related to the establishment of alien reptiles.

Results Alien reptile populations are more frequent in islands far from the native range and with large human population size. Furthermore, alien populations are particularly frequent for reptiles able to feed on plants. Traditional data sources underestimate the frequency of alien reptiles in Mediterranean islands and using genetic evidence to assess the status of populations provided a more complete picture of the factors related to alien populations.

Main conclusions Humans are key drivers for the alien reptiles distribution on Mediterranean islands, but the distribution of alien reptiles is determined by the complex interplay between human activities, geographic factors and species traits. Genetic data are essential to obtain reliable biogeographic assessment of invasive species, particularly in systems with a long history of human influence.

Keywords Alien reptiles, Mediterranean, Islands, Human-mediated transport, Biological Invasions, Spatial GAMM, Island Biogeography, Genetics

Introduction

Isolation and island size limit the number of terrestrial organisms that can colonize and exploit island ecosystems. As a result, islands tend to host fewer species, often with a reduced number of populations and/or smaller population sizes compared to the mainland (MacArthur and Wilson 1967). In insular conditions, competition, predation and parasitism might be relaxed, making island biota particularly vulnerable to biological invasions (van Kleunen et al. 2015). The Theory of Island Biogeography states that species diversity on islands tends to a dynamic equilibrium, which is the result of a balance between colonization, speciation and extinction rates (MacArthur and Wilson 1967). However, with the ever-increasing global exchanges, this equilibrium is expected to be altered. Namely, human-mediated introduction of alien species into islands, might increase the colonizing species but also the extinction rate of native species. As a consequence, the current distribution of island biodiversity is expected to become strongly influenced by human factors, such as economic isolation, human population size, commercial trade and transportation (Ficetola and Padoa-Schioppa 2009; Helmus et al. 2014; Blackburn et al. 2015). In recent literature, a growing body of research is testing whether invasive species follow the same biogeographic patterns of native species, and under which circumstances human activities break the traditional biogeographic rules (Burns 2015; Capinha et al. 2015).

Assessing whether a given insular species is alien is extremely important and is crucial to guide conservation priorities and management strategies in those systems. However, the distinction between alien and native species is not always easy. In some cases, the alien status is granted, either because introductions are recent and well documented, or because species originate from very distant territories unlikely to colonize naturally. However, in many other cases invasions are more difficult to disentangle from natural colonization, particularly if introductions are ancient, if alien species originate from nearby regions, and if invaders are well integrated into the insular ecosystems. In the recent years, the development of molecular genetics and phylogeographic tools has greatly improved our understanding of invasion dynamics and demography of biological invasions, allowing a more objective identification of alien species (Le Roux and Wieczorek 2009). Nevertheless, only a small subset of putatively alien populations has been investigated in depth using genetics and it will not be feasible to assess the alien status of insular populations over broad taxonomic and geographic scales. Moreover, some patterns of low genetic diversity can be explained by either human introductions or by recent natural colonization of very small propagules (e.g. Silva-Rocha et al. 2012; Salvi et al. 2014). Integrating genetic data within eco-geographic analyses may be an effective approach to identify the drivers of island invasions, and to unravel the status of cryptic invasions. Yet to date, this approach has rarely been implemented.

The Mediterranean region is a world biodiversity hotspot (Myers et al. 2000) with one of the longest histories of interaction between humans and biodiversity, and multiple introductions of alien species occurring over the millennia (Blondel et al. 2010). As a striking example, the Balearic Islands Archipelago, in the Mediterranean sea, once harboured substantial levels of endemicity as witnessed by the rich fossil record, and now host more alien than native species of reptiles and amphibians (Pinya and Carretero 2011; Silva-Rocha et al. 2015). This is due to the historical role of the archipelago as the crossroad of several civilizations since the Neolithic, resulting in drastic shifts in land-use patterns, resource management, and biota composition. Most of studies on invasive species focus on short-term patterns, and cannot explore the long-term consequences of species introductions (Strayer 2012). Given the above, the Mediterranean is an ideal place to study human-driven introductions at large spatial and temporal scales.

Despite being terrestrial and largely sedentary, reptiles are among the most abundant vertebrates in small islands (Novosolov et al. 2016) and are often introduced by humans, during the transport of trunks, soil or cultivated plants. Thus islands often host a large number of alien reptiles (Pitt, Vice, and Pitzler 2005). In areas with long histories of human settlement and trade, ascertaining the native status of species may be challenging, and many reptiles, traditionally assumed to be native to Mediterranean islands, are now known to be the result of ancient introductions (i.e., they are cryptic introduced species; e.g. Carlton 1996; Pedall et al. 2011; Masseti and Zuffi 2011). Mediterranean reptiles have been the target of multiple phylogeographic studies that provide a rich dataset to address several unresolved questions on biological invasions and to determine the status of species where it is dubious.

In this paper, we address the following questions: (1) which are the factors related to the distribution of alien reptiles in the Mediterranean region in the long-term? (1.1) What is the relative importance of geographic and human-driven factors? (2) Which reptile groups are most frequently introduced and what are their specific traits? Finally, (3) is it possible to integrate genetic, biogeographic and life history information to infer the status of island reptiles for which accurate information is available? To address these questions, we constructed a broad-scale and multispecies database with the verified status (native/alien) of island reptiles based on genetic information. We assessed the relationships between that status and (a) island, (b) human and (c) species characteristics, to identify the eco-geographic features that increase the probability that a reptile species is alien on islands. If, in the long-term, geographic features exert the same effects on native and alien species (Whittaker and Fernandez-Palacios 2007; Whittaker et al. 2017), we expect geographic features of islands to be the dominant drivers of alien species distribution (e.g. alien species would be more frequent in large islands, as these can support a higher number of species). On the other hand, if the effects of human activities remain the strongest even in the long term, we expect that the distribution of alien species is dominated by human factors (e.g. alien species would be more frequent on islands with higher human population size). Finally, we integrated this information in a model to predict the status (native/alien) of Mediterranean reptiles for which no genetic information exist, to infer their alien status and to identify those islands where more alien reptiles might be present. Our analyses provide a baseline model for understanding the real dimension of biological invasions of reptiles in the islands of the Mediterranean and elsewhere.

Methods

Species data and Island data

We focused on the islands and species within the Mediterranean Basin (Fig. 2.1). Information on species distribution on islands and on island features was obtained from Ficetola et al. (2014). For each island, we considered ten eco-geographic factors describing geographic features and human impact on the island (Table 2.1). Furthermore, we obtained features on species life history, morphology and diet (Table 2.1) from Thuiller et al. (2015).

The calculation of the distance of each island to the native range of each species was made using *maptools*, *rgeos* and *geosphere* packages in R. We used the native species range from IUCN Red List database for reptiles and Sindaco et al. (2008) for those not present in the IUCN database (namely, *Anguis fragilis*, *Chalcides ocellatus*, *Coronella austriaca*, *Dolichophis caspius*, *Eryx jaculus*, *Hemorrhois nummifer*, *Malpolon insignitus*, *Mauremys rivulata*, *Ophiomorus punctatissimus*, *Pseudopus apodus*, *Testudo graeca*, *Testudo hermanni*, *Testudo marginata* and *Xerotyphlops vermicularis*). Taxonomic names are according to Reptile Database (http://www.reptile-database.org/). Island endemic species were not included in the analysis since these species are present only on the islands where they are endemic and so cannot be alien by definition. Likewise, species clearly originated far

Table 2.1: Composition of the dataset with a brief description of variables and their importance.

Features	Variables	Description and source	Rationale
Islands features	Area (km2)	Arnold 2008	Island biogeography theory predicts higher species richness in larger islands
	Link to mainland (yes / no)	An island was considered linked if there was a landbridge connection with the mainland during the Last Glaciation Maximum - LGM (Hijmans et al. 2005).	The link to the mainland and/or to a big island during the LGM is important to distinguish between what could be a natural colonization from a human-mediated introduction in the case of old introductions.
	Link to main island (yes / no)	A small island was considered linked if there was a landbridge connection with a main island (Sicily, Sardinia or Corsica) during the Last Glaciation Maximum - LGM (Hijmans et al. 2005).	
	Distance to species range in the mainland (km)	Distance between the island and the nearest continental area where the species is native.	Species that are far from the native range are more likely to be alien.
Human features	Ferry presence (yes / no)	Arnold 2008 and Ficetola et al. 2014	Human transports and intensity of human activities are main sources of introductions.
	Airport presence (yes / no)		
	Human population size		
Species traits	Body length (cm)	Thuiller et al. 2015	Body size can influence introduction rate and establishment both positively and negatively (Kolar and Lodge 2001, Tingley et al. 2010).
	Species fecundity (N)	Thuiller et al 2015	Invasion success often higher in species with higher fecundity (Tingley et al. 2010).
	Minimum Litter Clutch size		
	Diet (four categories: vertebrates, invertebrates, plants mostly, plants occasionally)	Thuiller et al. If 0, species does not feed on the resource, if 1 species feeds on the resource. Plants mostly diet means the species mainly feeds on vegetal material. Plants occasionally diet means species is able to feed on plants even if the main diet is not vegetarian.	The diet gives us the adaptability of the species in the new environment and contributes to the establishment likelihood.

away from the Mediterranean region (e.g. *Trachemys scripta*) were also excluded, since one of the purposes of this work is to identify long-term patterns and cryptic introductions.

Assessment of the alien status

We performed an extensive literature review for each species (see Appendix A) to check whether the status of a certain species on an island was alien or native. This status was determined based exclusively on those studies which provide compelling genetic or fossil evidence. Most of studies do not provide genetic confirmation of the alien status and rely only on information gathered from other authors expertise or suppositions. These latter studies were not considered to ascertain the alien or native status of insular populations. From the gathered information, we built two complementary datasets. The first one included cases for which the species status was confirmed by genetic and/or fossils data (Confirmed

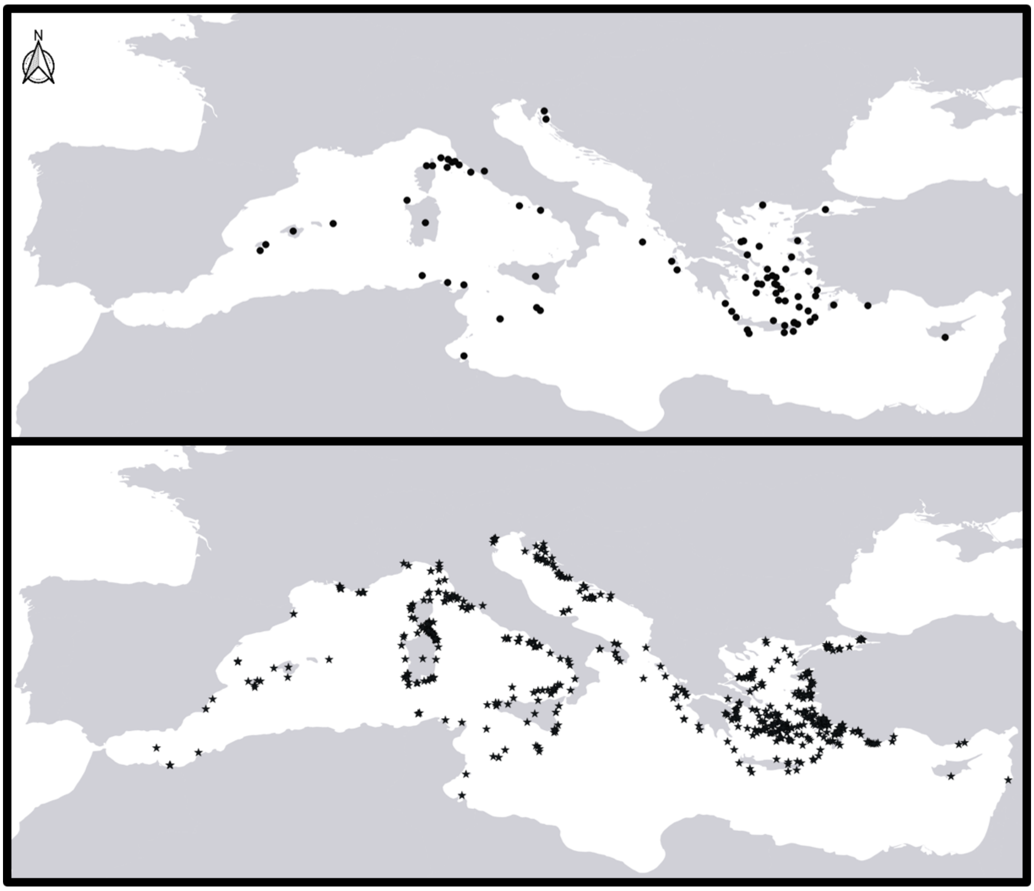

Figure 2.1: Distribution map of islands used on this study. Top: Islands from the Confirmed Dataset, for which there is a confirmation of alien/native status by genetic studies; Bottom: Islands from the Putative Dataset, for which there is no confirmation regarding the alien status on the existent bibliography.

Dataset), while the second dataset included cases for which the status was not confirmed (Putative Dataset).

Statistical analyses

First, we used the Confirmed Dataset to assess the relationships between the alien status of species on islands with eco-geographic features. Subsequently, these relationships were projected to the Putative Dataset to predict the status of island populations for which no evidence is available.

We used spatially-explicit Generalized Additive Mixed Models (GAMM) to assess the factors explaining the alien status of species (R Team 2016). GAMM are an extension of generalized additive models that allow taking into account over-dispersion and non-independence of data (Lin and Zhang 1999). The alien status of each species on each island was the dependent variable, while the eco-geographic features of islands and the species traits were independent variables. This approach allowed to simultaneously assess the role of island and species features in determining the distribution and identity of alien species.

Four variables (area, human population size, body length and distance to native range) were log-transformed to reduce skewness and ensure normality. We used a multi-model inference approach to select the most important variables. Unlike stepwise model selection, multi-model inference is based on all possible sub-models from a set of explanatory variables, which limits model selection bias and provides a relative measure of each predictors importance (weight of evidence). All possible models were then ranked according to their Akaikes Information Criterion (AIC) (Burnham and Anderson, 2002). In order to avoid collinearity, models that included variables with strong pairwise correlation ($|r| > 0.7$) were excluded from the set of candidate models (Dormann et al. 2013). Island identity and taxonomy (Family/Genus/Species) were included as random nested factors in all models, in order to take into account potential non-independence. GAMMs, were fitted using a binomial error using the gamm4 package (Wood and Scheipl 2016). The independent variables were fitted as linear terms. Spatial autocorrelation might also bias the outcome of regressions in spatial data analyses. Therefore, we incorporated the island coordinates in GAMM as tensor product smooth terms, using thin plate regression splines (Beale et al. 2010). Simulations showed that this implementation of GAMM is able to correctly estimate regression coefficients in spatially structured datasets, with relatively good performance in presence of violations of model assumptions, such as non-stationarity of autocorrelation (Beale et al. 2010). The importance of predictor variables was assessed by summing the AIC weights across all the models in the set of models where the variable occurs (Burnham and Anderson 2004; Giam and Olden 2016). The predictor variable with the largest predictor weight is estimated to be the most important, while the variable with the smallest sum is estimated to be the least important predictor. To confirm that our results are not biased by phylogenetic relationships, we used Pagels lambda to assess whether the model residuals show significant phylogenetic signal. Phylogenetic analyses were performed using cape (Tyler et al. 2013).

We tested the quality of the model by calculating the true skill statistics (TSS) on the Confirmed Dataset. A cross-validation approach was used to confirm the robustness of predictions, by randomly splitting the Confirmed Dataset into three sub-datasets (33% of data each) and then calculating TSS for each sub-dataset in which 75% of data was used to build the model and 25% to predict species status. We used the threshold maximizing TSS to predict that status (native or alien) of species in the Putative Dataset. Finally, we used the best-AIC model for predicting the most likely status (native / alien) of each species record of the Putative Dataset.

Results

The final Confirmed Dataset contained 186 island-by-species combinations, representing 41 species and 78 islands covering the whole Mediterranean (Fig. 2.1). Fifty-three cases were occurrences of alien species, mostly snakes, turtles and lizards, which were particularly frequent in Western Mediterranean Islands. A total of 133 cases were occurrences of native species, represented mostly by snakes and lizards.

The final Putative Dataset had 2045 island-by-species combinations, corresponding to 69 species and 598 islands (Fig. 2.1 bottom). A total of 82 cases were assumed to be alien and 1963 were assumed to be native by non-genetic information from literature. Geckos and snakes were the groups with more (putatively) alien cases.

Variables related to the alien status

We found a strong correlation between island area and both human population size, ferry presence and airport ($r = 0.95$, $r = 0.70$ and $r = 0.73$, respectively) and between human population size and both ferry presence and airport presence ($r = 0.80$ and $r= 0.78$, respectively). The models with only one of these variables were retained, while the models with more than one were removed to avoid collinearity (Dormann et al. 2013).

The best-AIC model suggested that alien species are particularly frequent in islands with higher human population size ($B = 5.23 \pm 0.364$; Z-value = 14.37; $P < 0.0001$). Moreover, the alien status was more frequent in species-by-island combinations that were further

Table 2.2: Models explaining the invasion status of reptiles on Mediterranean Islands, with AIC < 5. Models are ranked on the basis of Akaikes Information Criterion (AIC). K is the degree of freedom of the model of the Z-distribution. W is the Akaike weight of each model which reflects the relative likelihood of that model being the best within a set of N models.

Ranking	**Model**	**K**	**AIC**	**w**
1	Distance to Native Range + Human Population Size + Plants Occasionally - Vertebrates	15	72.24	0.46
2	Distance to Native Range + Human Population Size + Plants Occasionally	14	75.20	0.10
3	Distance to Native Range + Plants Occasionally Invertebrates Body Length	15	75.36	0.10
4	Airport + Distance to Native Range + Plants Occasionally - Vertebrates	15	76.45	0.06
5	Area + Distance to Native Range + Plants Occasionally	14	76.62	0.05
6	- Body Length + Distance to Native Range + Vertebrates	14	76.63	0.05
7	Ferry Presence + Distance to Native Range + Plants Mostly + Plants Occasionally	15	76.72	0.05
8	- Invertebrates Link to Mainland Body Length + Distance to Native Range + Plants Occasionally	16	76.96	0.04
9	Airport Link to Big Islands + Distance to Native Range + Plants Occasionally - Vertebrates	16	77.07	0.04
10	Minimum Litter Clutch Size + Distance to Native Range + Plants Occasionally - Vertebrates	16	77.14	0.04

from the continental range of the species (B = 24.83 ± 0.741; Z-value = 33.53; P $<$ 0.0001). Finally, species that can occasionally feed on plants (B = 56.35 ± 1.058; Z-value = 53.27; P $<$ 0.0001) but not depend on vertebrates (B = -39.58 ± 1.059; Z-value = -37.36; P $<$ 0.0001) were more frequently alien (Table 2.2). No phylogenetic signal was detected on model residuals (Pagels lambda = 0.452, not significantly different from 0; P = 0.29). The cross-validation approach indicated an excellent predictive power of this model, as the average TSS was 0.98 ± 0.03.

Some uncertainty in model selection existed, as several alternative models showed ΔAIC < 5, and included variables such as body length, island area, presence of airports and presence of ferry (Table 2.2). However, all the alternative models showed limited AIC weight. Overall, distance to the native range and the capacity of feeding on plants were the variables with the strongest variable importance (AIC weight $\geq$ 0.95). Vertebrate diet and human population size showed a medium importance, while the importance of all the remaining variables was very low (Fig. 2.2).

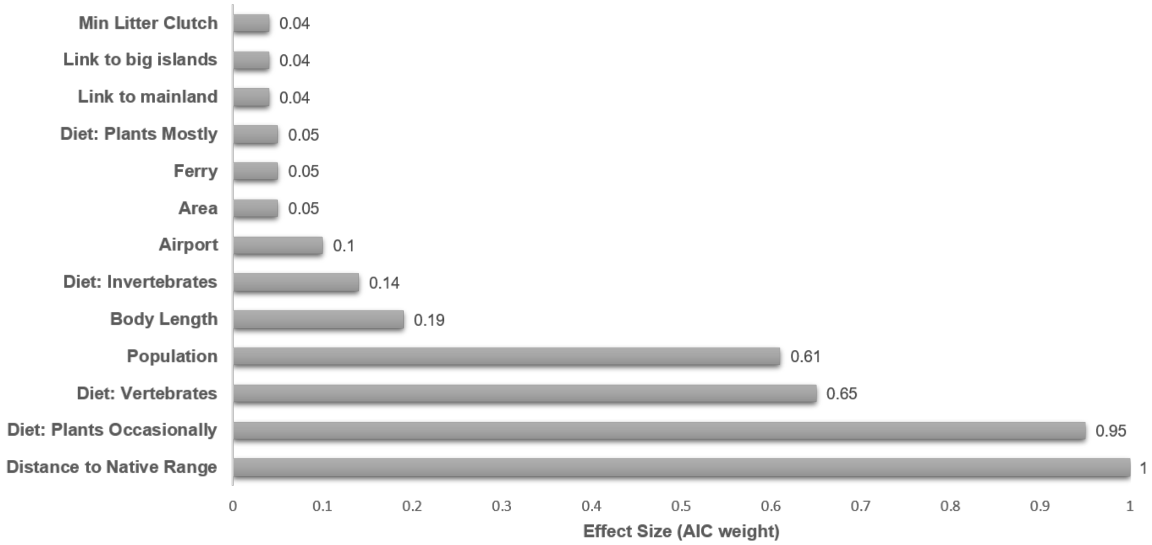

Figure 2.2: AIC weight of variables included in candidate models.

Assessing the putative status of insular populations

When we projected the best model to the Putative Dataset, 80% of occurrences were predicted to be native, and 19% of occurrences were predicted to be alien. Model projections generally matched the status proposed by the literature (likelihood ratio test: χ21 = 62.9, P $<$ 0.0001). However, in some cases model projections suggested a different species status and, out of the 1942 species records assumed to be native in the literature, 16% were spotted as potentially alien (Table 2.3). Geckos were the group with more cases of potentially alien occurrence, particularly with *Hemidactylus turcicus* (63 islands) and *Tarentola mauritanica* (69 islands). Skinks spotted as potential aliens included *Chalcides ocellatus* (56 islands) whereas, among lacertids, wall lizards of the genus *Podarcis* represent the majority of potential alien cases, especially *Podarcis sicula* (38 islands). We also detected several potential alien occurrences for snakes, such as *Hierophis viridiflavus* (42 islands) and *Natrix maura* (7 islands). Overall, if we use the predicted status instead of the status in the bibliography (Putative Dataset), the average richness of native species on Mediterranean islands decreased by 17%.

Table 2.3: Number of alien and native species from the Putative Dataset (Original Data) that kept or shifted their status in comparison with the predictions results (Predictions).

		Predictions	
		Native	Alien
Original data	Native	1614	328
	Alien	28	54

Discussion

Biological invasion is a multi-step process, characterized by (1) introduction of species in a new environment; (2) establishment; and (3) diffusion across the new area (Simberloff et al. 2013). Our study suggests that the distribution of alien reptiles on Mediterranean islands depends mostly on the processes determining introductions (stage 1), and of traits favouring success in the new environments (stage 2). Despite several studies analysing biogeographic and human factors related to the distribution of alien species, most of them relied on expertise and general knowledge to define the alien/native status of island populations, and very few of them used genetics to confirm this putative status. However, the complex history of human introductions in the Mediterranean basin makes the identification of alien species challenging, and the traditional approach prone to underestimate the occurrences of aliens. Our study shows that molecular data allow identification of a higher number of alien species than expected based on traditional evidence, thus the integration of multiple sources of information, and the validation of records using unambiguous evidence, is essential for a more accurate understanding of patterns of alien species distribution.

Alien reptile distribution: the interaction between geographic and human-related factors

McArthur and Wilsons (1967) theory states that in the shallow evolutionary time the distribution of species on islands is driven by two major factors determining the equilibrium between extinction and colonization rates: island size and distance from mainland. Immediately after the introduction, the distribution of alien species is rarely at equilibrium. However, given the temporal extent of human interactions in the Mediterranean has spanned over millennia, many reptiles have been likely introduced on islands centuries or even thousands of years ago, thus potentially allowing the native and alien island biota to reach a new equilibrium. Studies on alien species suggest that alien richness is related mainly to human population size and other human-related variables, besides the island size (e.g. Roura-Pascual et al. , 2016). Our findings suggest that human factors remain more important than the geographic ones (e.g. island area) even after very long introduction history, still the joint effect of geographic and human factors can be important. The probability that a reptile record is native decreased with distance from the native range. This finding is not unexpected, given that human-mediated dispersal can move species over much longer distances than natural processes (Wilson et al. 2009). Although reptiles can be occasionally able to long-range overseas dispersal, in several cases it is uncertain if the presence of reptiles in offshore islands is caused by natural or human-mediated processes (e.g. Kyriazi et al., 2013; Poulakakis et al., 2013). Our study confirms that the distance from the mainland range interacts with human activities in determining the island colonization and provide quantitative evidence of the relative scales of the two processes. However, the relationship between the frequency of aliens and distance from the mainland range can also be caused by additional processes related to interspecific interactions, such as those considered by the Darwins Naturalization Hypothesis (Darwin 1859) and/or by the Species-richness Hypothesis (Elton 2000). The Darwins Naturalization Hypothesis predicts that alien species phylogenetically distant from the native community are most likely to become established. Our results are potentially in agreement with these predictions, since alien species present in islands far from the native range are less likely to be closely related. Studies on reptiles either support this hypothesis (Wilgen and Richardson, 2011), or provide a weak support, and even suggest that the success of alien species might be related to the presence of congeners (i.e. pre-adaptation hypotheses alien species are likely to contain traits that pre-adapt them to the new environment) (Ferreira et al. 2012; Liu et al., 2014; Tingley et al. 2011). The Elton's species-richness hypothesis states that species-rich communities are more resistant to invasions because more of the available niche space is used by the natives. This might concur to the observed pattern, given that native richness is generally lower on islands far from the continent (Warren et al. 2015). Nevertheless, it should be remarked that several studies found weak support for this pattern, as in many cases the richest communities have more alien species (Lockwood et al., 2007; Nunez-Mir et al., 2017; Richardson, 2011; Yiming et al. 2006) On the other hand, the presence of alien reptiles is highest in islands with large human population, confirming the keystone importance of human activities on reptile invasions. The strong human signal found on this study was also found in many other studies in the Mediterranean (Ficetola and Padoa-Schioppa, 2009) and in other regions of the world (Helmus et al., 2014; Phillips, 2010; Roura-Pascual et al., 2016; Spatz et al., 2017), although studies focused on different predictors. Many analyses considered human population size or human presence as overall proxies of human impact (e.g. Ficetola and Padoa-Schioppa (2009), Roura-Pascual et al. (2016) and Spatz et. al (2017)), but other parameters can better target the key drivers, such as the frequency of tourist visitation (Phillips, 2010) or the economic isolation (Helmus et al. 2014). Overall, although the human influence on the islands could be mediated by different pressures, humans have a deep impact on islands biota and its distribution, which reinforces the importance of integrating human-related variables into biogeographic analyses.

Reptile traits and invasions

Since our study area involves ancient introductions, we could not discriminate between two separate steps of the invasion process: (i) the frequency of introductions, and (ii) the establishment success. All the alien species have been introduced and successfully established through the years. Diet was the only species trait included in highly-supported models. Alien reptiles were often able to use plants as a significant part of their diet, and generally did not depend on vertebrates as main resource. These traits are clearly not associated with colonization but instead with the success of the species to establish and persist in the new island environment. Most reptiles are generalist predators, but some of them are also able to shift trophic level incorporating plant items into their diets. Islands are resource-poor environments, and a wide dietary spectrum (i.e. the ability of eating both plant and animal resources) is important to survive in habitats with limited food availability, especially during the dry season within Mediterranean climates. In small islands alien lizards often eat more plants than conspecifics living on the mainland, and species feeding on plants can reach higher densities than the ones feeding mostly on arthropods (Herrel et al. 2008; Sagonas et al. 2014). As an outstanding example, wall lizards introduced to a small islands developed in a few decades strategies for consuming and digesting plant matter, being able to better survive and increase their abundance in these resource-poor environments (Pérez-Mellado and Corti 1993; Carretero 2004; Herrel et al. 2008; Sagonas et al. 2014). Our study shows that these processes, acting at the population level, can be also evident at the biogeographic scale, as diet spectrum of species emerged among the most important factors (Fig. 2.2).

The usefulness of genetics to understand the biogeography of invasions

Molecular data are still lacking for most species on islands. The Mediterranean is among the most-studied areas of the world, still genetic data were available for less than 10% of species-by-island combinations. However, genetic data extensively changed our vision of alien reptiles in the Mediterranean. In fact, 28% of reptile records of the confirmed dataset were alien, while just 14% of them would have been considered as such if genetic information would had been omitted. In other words, without genetic information half of alien records would be erroneously considered to be native, hampering the understanding of invasion dynamics. For instance, when we analysed the same dataset ignoring the genetic information, we obtained a completely different picture of the factor related to the distribution of alien species, and we did not identify some of the most important variables (see Appendix B). This sends a word of caution regarding the conclusion extracted from metanalyses not incorporating this source of evidence (e.g. Capinha et al., 2017) Obtaining exhaustive genetic data for all the islands and species is far from complete, yet the integration of genetic data within biogeography can be used to improve our understanding of present-day biodiversity. When projecting the model built using genetic data, to a dataset of records without genetic variable (”putative dataset”), we identified many species-by-island combinations that might actually be alien records. Model extrapolation certainly bears a degree of error and cannot be used as the sole source of information on the status of species on islands. Nevertheless, projecting the model to the unconfirmed dataset can provide preliminary information on how frequent the cryptic invaders in this system can be, and highlights species and islands that deserve specific research. Geckos, for example, have been extensively studied and yet, the origin and human role on their distributions are still being highly discussed (Rato, Carranza, and Harris 2012; Delaugerre, Ouni, and Nouira 2011; Rato, Carranza, and Harris 2011). Our predictions suggest that most geckos are indeed considered alien on the islands where they are present. We argue that the integration of different tools and methodologies could be the way to clarify this kind of problematics. In conclusion human-related factors stand up as key drivers of the current distribution of alien reptiles in the Mediterranean, in combination with species traits, such as diet, that are important for the success of invasion and persistence of alien species on islands. The integration of distribution, genetic and fossil data could allow to identify some cryptic introduced species, some of which (e.g. snakes) can potentially threaten native biota. The same framework can then be applied to other groups with low natural dispersal in human-dominated regions, providing a baseline to delineate more realistic conservation strategies and detect putative alien species.

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Supporting Information

Additional Supporting Information can be found in the Appendices section.

Appendix A - S2.1: References of the literature review to ascertain the alien status of each species-island pair

Appendix B - S2.2: Analysis with data not updated with genetics

Chapter 3

Snakes on the Balearic Islands

Silva-Rocha, I, D Salvi, N Sillero, JA Mateo and MA Carretero. 2015. "Snakes on the Balearic Islands: An Invasion Tale with Implications for Native Biodiversity Conservation". PLoS ONE 10(4): e0121026. https://doi.org/10.1371/journal.pone.0121026

Abstract

Biological invasions are a major conservation threat for biodiversity worldwide. Islands are particularly vulnerable to invasive species, and especially Mediterranean islands which suffer human pressure since ancient times. In the Balearic archipelago, reptiles represent an outstanding case with more alien than native species. Moreover, in the last decade a new wave of alien snakes landed in the main islands of the archipelago, some of which were originally snake-free. The identification of the origin and colonization pathways of alien species, as well as the prediction of their expansion, is crucial to develop effective conservation strategies. In this study, we used molecular markers to assess the allochthonous status and the putative origin of the four introduced snake species (*Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus* and *Rhinechis scalaris*) as well as ecological niche models to infer their patterns of invasion and expansion based on current and future habitat suitability. For most species, DNA sequence data suggested the Iberian Peninsula as the potential origin of the allochthonous populations; although the shallow phylogeographic structure of these species prevented the identification of a restricted source-area. For all them, the ecological niche models showed a current low habitat suitability in the Balearic, which is however predicted to increase significantly in the next few decades under climate change scenarios. Evidence from direct observations and spatial distribution of the first-occurrence records of alien snakes (but also lizards and worm lizards) suggest that nursery trade, and in particular olive tree importation from Iberian Peninsula, as the main pathway of introduction of alien reptiles in the Balearic Islands. This trend has been reported also for recent invasions in NE Spain, thus showing that olive trees transplantation may be an effective vector for bioinvasion across the Mediterranean. The combination of molecular and ecological tools used in this study reveals a promising approach for the understanding of the complex invasion process, hence, guiding conservation management actions.

Keywords Alien species, Mediterranean, islands, pathway, nursery trade, olive trees, phylogeography, ecological niche modelling

Introduction

Biological invasions are a major cause for current species extinctions, together with the destruction and fragmentation of habitats and climate change [1,2]. The rates of these processes, and in particular of biological invasion, have strongly increased during the last century due to human growth and globalization causing a parallel increase in the rate of biodiversity loss [3-5]. Thus, understanding the processes of introduction and expansion of invasive species has become a conservation priority [5].

Islands ecosystems are especially vulnerable to biological invasions since many insular native species have been evolving long time in isolation often losing their ability for competing with other species, eluding predators or defending themselves from parasites, which may arrive from nearby continents [6-9]. In particular, islands within the Mediterranean basin are distinctive due to the ancient interactions between biota and human activities which caused severe changes on the biodiversity present over time. Thus, many Mediterranean islands now share evolutionary lineages with the surrounding continents or other islands. The process is so intensive and widespread that even the distinction between allochthonous and autochthonous species may be problematic [10]. This is particularly true for reptiles, one of the groups most widely introduced in Mediterranean islands, but at the same most threatened by introductions [9,11].

In this respect, reptiles in the Balearic Islands (Mallorca, Menorca, Ibiza, and Formentera; see Fig. 3.1) represent the most paradigmatic case within the Mediterranean region and perhaps one of the outstanding cases in the world [12], with by far more alien (19) than native (2) species. Isolated from the continent since the Messinian (5.33 my BP, [13]), this archipelago currently harbours only two endemic reptiles, the Lilfords and Ibiza wall lizards (*Podarcis lilfordi* and *P. pityusensis*, respectively) [12]. By contrast, a striking number of alien species have been reported for the main islands, namely, 16 in Mallorca, two in Cabrera (south of Mallorca), 11 in Menorca, six in Ibiza, and five in Formentera.

Figure 3.1: Map of the Balearic archipelago. The inset (top right) shows the geographic position of the archipelago in the Western Mediterranean.

In this archipelago, the first reptile introductions date back to the Neolithic [12]: thus, that part of the alien species contingent of the Balearics was already naturalized and widespread in one or more islands since historical times (for a review see [12, 14]). The historical introduction of the snakes *Macroprotodon mauritanicus*, together with some introduced mammals, have been considered responsible for the extinction of native lizards *P. lilfordi* in Mallorca and Menorca [12, 15-17] and of the native Menorcan amphibians *Alytes muletensis* and an undescribed *Discoglossid* [18]. On the other hand, many other species such as the Red-eared Slider Turtle *Trachemys scripta*, the ocellated lizard *Timon lepidus*, and surprisingly four snakes, have been recorded only recently, i.e. in the last decades, in one or more Balearic islands, likely as a result of the intensification of human activity such as tourism and pet trade [12]. Among these new invaders, those that certainly deserve particular conservation attention are the introduced snakes. Indeed, while several Balearic islands such as Ibiza and Formentera has been until recently free of snakes, since 2003 the alien snakes *Hemorrhois hippocrepis*, *Malpolon monspessulanus*, and *Rhinechis scalaris* have been recorded in Ibiza, and the latter also in Mallorca and very recently in Formentera [19-21].

Alien snakes represent one of the largest threats to the native Balearic biota, since they are effective predators capable to transform the community composition and ecosystem properties [5]. Most of the introduced species are partially saurophagous and potentially very harmful for the populations of the native endemic lizard *P. pityusensis* from Ibiza and Formentera, which remained snake-free islands until recently. Moreover, some of these snakes are able to adapt their diet based on food availability on islands. For instance, Balearic populations of *Macroprotodon* seems to have shifted dietary habits from reptiles, consumed by the mainland snakes, to mammals, due to the extinction of the endemic lacertid lizards from Mallorca [22]. This reflects the ability of these snakes to adapt to island environments and to have a wider potential impact on native species than expected according to their realized niche on mainland. Additionally, snakes are able to use human manufactures and ornamental plants as a refuge [23,24] which favours both their introduction and the development of populations. Remarkably, the number of alien snake observations in the Balearics has dramatically increased during the last decade [19], suggesting a range and population size expansions and/or recruitment through new introductions. Thus, understanding the origin and pathways of introduction of these alien species as well as forecasting their future expansion is extremely urgent for setting conservation actions.

In this study, we analysed in a phylogeographic framework mitochondrial DNA sequence data of the snakes *Hemorrhois hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus* and *Rhinechis scalaris* reported for the Balearic Islands to assess their allochthonous status and putative origin and to infer pathways of introduction. Moreover, in an attempt to assess their spatial patterns of invasion and expansion in the Balearics, we estimated for each species the habitat suitability and the occurrence of environmental limiting factor at present and under future climatic scenarios using ecological niche models.

This integrated approach, joining molecular data and ecological models, represents a promising tool for the understanding of the complex invasion process, hence, guiding conservation management actions.

Materials and Methods

Study Area

The Balearic Islands have a total area of 5040 km2 and 1428 km of coastline, laying 80 to 300 km east of the Iberian Peninsula. This archipelago is composed by two islands groups: the Gymnesic (eastern) and the Pityusic (western) (see Fig. 3.1). The Gymnesic group is constituted by the main islands of Mallorca and Menorca, the small island of Cabrera (south of Mallorca) and about 30 surrounding islets. The Pityusic group includes Ibiza and Formentera islands and about 60 surrounding islets. The Balearic Islands are covered by typical Mediterranean vegetation with more diversity of ecosystems and landscapes in Mallorca due to its wider altitudinal range (highest altitude 1445 m). Introduced reptiles are currently present on the main islands (Mallorca, Menorca, Ibiza and Formentera) and not in the small islets.

Ethical statement

Tissue samples for genetic analyses were collected from road kills animals by us or by technicians of the Balearic Government, thus no live animals have been damaged or sacrificed for this study. Samples have been collected according to the permits issued by the Balearic Government (CAP 55/2011), which approved all animal procedures used in this study.

Molecular data gathering

In order to assess the allochthonous status and putative origin of the snakes *H. hippocrepis*, *Malpolon monspessulanus*, *Macroprotodon mauritanicus*, and *R. scalaris*, currently occurring in the Balearics, we compared DNA sequences generated for Balearic specimens with those published for conspecific individuals from the native range in previous phylogeographic studies: *H. hippocrepis* [25]; *Malpolon monspessulanus* [25]; *Macroprotodon mauritanicus* [26,27]; and *R. scalaris* [28]. Note that we followed the taxonomy proposed by Carranza et al. [26] and the Reptile Database (http://reptile-database.reptarium.cz/), that list four *Macroprotodon* species: *M. cuccullatus*, *M. brevis*, *M. mauritanicus and M. abubakeri*. Tissue samples for genetic analyses were collected in all the main Balearic Islands from live and road-killed animals either directly by the authors during a field session in 2011 or thought the collaboration with local environmental authorities (see acknowledgements and ethical statement paragraph). Samples were stored in pure ethanol until total genomic DNA was extracted following the standard saline method [29]. We amplified fragments of the mitochondrial gene cyt*b* from 34 Balearic specimens of all the four snake species. Additionally, for *H. hippocrepis* and *Macroprotodon* sp., a fragment of the ribosomal gene 12S was also amplified (details on gene fragments size and primers used are indicated in Table 3.1). Amplifications were conducted by the Polymerase Chain Reaction method (PCR), using conditions reported in previously published phylogeographies of these species [25-28]. PCR products were purified and sequenced by an external service (company Macrogen Korea).

Table 3.1: List of primers used for amplification and sequencing of selected gene fragments for each species, with indication of fragment size.

Species	Gene	Primer	Size (bp)
Hemorrhois hippocrepis	cyt*b*	cyt*b*1 and cyt*b*2 (Palumbi 1996)	241
	12S	12Sa and 12Sb (Kocher et al. 1989)	219
Malpolon monspessulanus	cyt*b*	cyt*b*1 and cyt*b*2 (Palumbi 1996)	254
Macroprotodon sp.	cyt*b*	GluDG and cyt*b*2 (Palumbi 1991)	250
	12S	12Sa and 12Sb (Kocher et al. 1989)	311
Rhinechis scalaris	cyt*b*	GluDG and cyt*b*2 (Palumbi 1991)	353

Sequence data generated from the native range of each species, as well as the outgroup taxa, were mainly obtained from GenBank from previous phylogeographic studies (see above). Additionally, for *R. scalaris* we generated cyt*b* sequences for 10 specimens from the native range in order to extend the preliminary phylogeographic assessment by Nulchis et al. [28] which was based on only 12 samples. A complete list of the sequences used in this study along with locality and GenBank Accession numbers is provided in Appendix C.

Molecular data analysis

The sequences generated in this study were aligned with those gathered from GenBank using CLUSTALW [30]. For those species for which cyt*b* and 12S were sequenced, we generated also a concatenated alignment cyt*b* + 12S. In order to infer the genealogical relationships between the Balearic samples and those from the native range of each species, we analysed cyt*b* and cyt*b*+12S datasets using the Maximum Likelihood (ML) and the statistical parsimony network methods. The ML analyses with the heuristic search mode, as well as the estimation of the best model of sequence evolution, were performed in MEGA5 [31]. Node support was calculated over 1000 bootstrap replicates. Statistical parsimony networks were estimated using the program TCS 1.21 [32].

Distribution and environmental data gathering

We collected occurrence data for each species and environmental data for their native range in order to assess habitat suitability using ecological niche models.

We compiled occurrence data of the global native distribution of each species from GBIF (Global Biodiversity Information Facility, www.gbif.org), and cleaned from obvious data errors. For the species with North African distribution (i.e. all species except *R. scalaris*), more occurrence points were needed to conduct a proper analysis, since the information contained on GBIF database was not enough. In these cases, we collected additional geographic records from literature review, CIBIO database, and National Atlases from Portugal, Spain and Morocco [33-35]. We collected a total of 2953 records for *H. hippocrepis*, 7164 for *Malpolon monspessulanus*, 796 for *Macroprotodon* sp., and 6152 for *R. scalaris*. For *Macroprotodon* sp., we considered the presence records from the whole genus from Morocco, Algeria and Tunisia since the distribution of the species as proposed by [36] included very few records while the ascription of many others would have remained doubtful. We are aware that modelling the genus as a whole could lead to results not adjusted to the habitat characteristics of the particular species. However, by performing a join analysis of the genus we intended to obtain the maximum expected habitat suitability, provided that there would be niche conservatism within the genus. Whatever other case (i.e. niche differences between the putative species, with more arid or mesic affinities) would result in more restricted habitat suitability and thus in a lower predicted expansion. Hence, the suitable habitat for the particular species will be always included in the suitable habitats for the whole genus. We consider that this conservative procedure is advisable when dealing with invasive species and incomplete biogeographic information.

We downloaded climatic predictors from WorldClim v. 1.4 online data (www.worldclim.org, [37]) at a resolution of 30 arc-seconds (1 km2). We selected a total of seven bioclimatic variables (from a total of 19) with a Spearman's correlation lower than 0.7 and meaningful for the species to calculate the realised niche models (sensu [38]): Bio2 Mean Diurnal Range; Bio3 Isothermality; Bio4 Temperature Seasonality; Bio8 Mean Temperature of Wettest Quarter; Bio15 Precipitation Seasonality; Bio18 Precipitation of Warmest Quarter; and Bio19 Precipitation of Coldest Quarter. For future climate scenarios, we used three coupled atmosphere-ocean general circulation models (CCCMA, HadCM3 and CSIRO) with three socio-economic emission scenarios (A1b, A2a and B2a), for three future periods: 2020, 2050 and 2080. As we only want to evaluate the tendency over the years, an average model was made with all the scenarios.

Ecological Niche Model analyses

We modelled the realised ecological niche of each native range species (hence excluding the invasive range) at present and then projected to the present and future climatic scenarios of the Balearic Islands. This is the most correct methodology to avoid relying on species distribution data for areas where a lack of equilibrium is expected due to recent introduction (i.e. the Balearic islands), since we considered that the species are in pseudo-equilibrium (i.e. species must occupy all suitable habitats available, [39]). We use the Maximum Entropy method (implemented in Maxent 3.3 software: www.cs.princeton.edu/ schapire/MAXENT) to calculate the models. Maxent is a general-purpose machine learning method that uses presence-only occurrence data and is consistently competitive with the highest performing methods [40,41]. Maxent output represents the habitat suitability, ranging from 0 to 1. We run Maxent with autofeatures, excluding the product feature, selecting at random 70% of the presence records as training data and 30% as test data for each species. The final model and projections for each species were the average of 10 different replicative models. Standard deviation was also calculated to determine the uncertainty of the models.

To maximize the predictions, we built models with an increasing regularisation parameter (β) for each species. The regularisation multiplier allows constraining the model to over-parameterization, and hence increasing values of β is expected to achieve the model with less complexity [42]. For all the species, we started with β=1 and increased β+2 until we reached consistent results (*H. hippocrepis*: β=61; *Macroprotodon* sp.: β=43; *Malpolon monspessulanus*: β=13; *R. scalaris*: β=21). To compare all the models with the different β, we calculate the AICc values with ENMTools 1.3 [42].

We tested the quality of the models by calculating the area under the curve (AUC) of the receiver operated characteristics (ROC) plots [43] in order to discriminate a species' model from a random model. The importance of each climatic variable for explaining the species distribution was determined by jack-knife resampling: (1) of the training and test gain; and (2) of AUC values. For this purpose, environmental variables were excluded in turn and a model created with the remaining variables; then a model was created using each individual variable. Finally, we obtained an average percentage contribution of each environmental factor to the models.

A MESS (Multivariate Environmental Similarity Surfaces) analysis was also performed in order to detect non-analogous environmental conditions. This analysis measures the similarity of any given point to a reference set of points, regarding the chosen predictor variables [44].

We reclassified the projected models into habitat suitability maps (sensu Sillero [38]) according to the maximum training sensitivity plus specificity logarithmic thresholds given by Maxent. Cells with values higher and lower than the threshold were considered respectively as suitable and unsuitable (i.e. the species was considered absent) for the presence of the species.

Results

Genetic analyses

We obtained sequences for a total of 34 Balearic specimens of the four snakes: 24 *H. hippocrepis* (19 from Mallorca and five from Ibiza); one *Malpolon monspessulanus* from Mallorca; five *Macroprotodon* sp. (four from Mallorca and one from Menorca); and four samples of *R. scalaris*. A total of 10 cyt*b* sequences of *R. scalaris* from the native range were also generated. These sequences were analysed together with sequences from Nulchis et al. [28] downloaded from Genbank (see S1 Table for further details).

The genetic analysis of *H. hippocrepis* revealed two different haplotypes, both in cyt*b* and 12S+cyt*b* datasets, among the Balearic samples, corresponding to those already found in the native range by Carranza et al. [25] (Fig 3.2A). Snakes from Mallorca and Ibiza are related to those most commonly found in Spain and Morocco. Therefore, the genetic data do not allow to distinguish the exact source.

We identified a total of 14 haplotypes for native and Balearic samples of *Malpolon mospessulanus*, 13 of them corresponding to haplotypes previously found in samples from Carranza et al. [25] and one corresponding to the Mallorcan sample (Fig 3.2B). The genetic analysis revealed two main clades: one including Moroccan and Iberian Peninsula samples belonging to *Malpolon m. monspessulanus*; other including Tunisian, Cyprian, Egyptian, and Greek samples corresponding to *Malpolon insignitus insignitu*s and *Malpolon m. fuscus* (see [25]). The sample from the Balearics clustered together with *Malpolon m. monspessulanus* from Morocco and Iberia.

The genetic analyses of *Macroprotodon* sp. identified a single haplotype among the Balearic samples, identical to those previously found in two Tunisian samples and one Mallorcan sample by Carranza et al. [26] and ascribed to *Macroprotodon mauritanicus* clade (Fig. 3.2C).

Finally, we found three cyt*b* haplotypes in *R. scalaris*, one corresponding to the commonest haplotype widespread across the species range identified by Nulchis et al. [28] and two occurring in two localities of the native range analysed in this study. Due to the low genetic differentiation between the three haplotypes (one or two nucleotide differences), we show their genealogical relationships by a network, since the ML tree was less informative (not shown). The Balearic samples carried the most common haplotype widespread across the native species range (Fig 3.2D).

Ecological Niche Modelling

The AUC values were higher than 0.80 for all the species *H. hippocrepis* 0.858 ± 0.004; *Macroprotodon* sp 0.902 ± 0.004 ; *Malpolon monspessulanus* 0.802 ± 0.001; and *R. scalaris* 0.814 ± 0.002 indicating a good performance of the models. The contribution of each bioclimatic variable to species models is reported in Table 3.2. For *R. scalaris*, *H. hippocrepis* and *Macroprotodon* sp., the variable explaining most of the variation in the models was the precipitation of warmest quarter (bio18). Regarding *Malpolon monspessulanus*, the temperature seasonality seems to be the variable that more contribute to the models. The second most explanatory variable varied with the species: the precipitation seasonality (bio15) for *H. hippocrepris* and *Macroprotodon* sp., the precipitation of warmest quarter (bio18) for *Malpolon monspessulanus* and the temperature seasonality for *R. scalaris*. These patterns were concordant after jack-knife resampling (Appendix D).

Regarding the MESS analysis, we obtained negative values for all the present models, which means a high degree of dissimilarity between any given point and the distribution of reference points. However, the variables showing dissimilarity are also those that less contribute to the model (bio3 and bio15, Appendix F), with exception of *Macroprotodon* sp. for which bio15 is the second most explanatory variable, however it contributes much less than bio18. For the future models, we obtained positive values for all the species with none dissimilar variable (Appendix F).

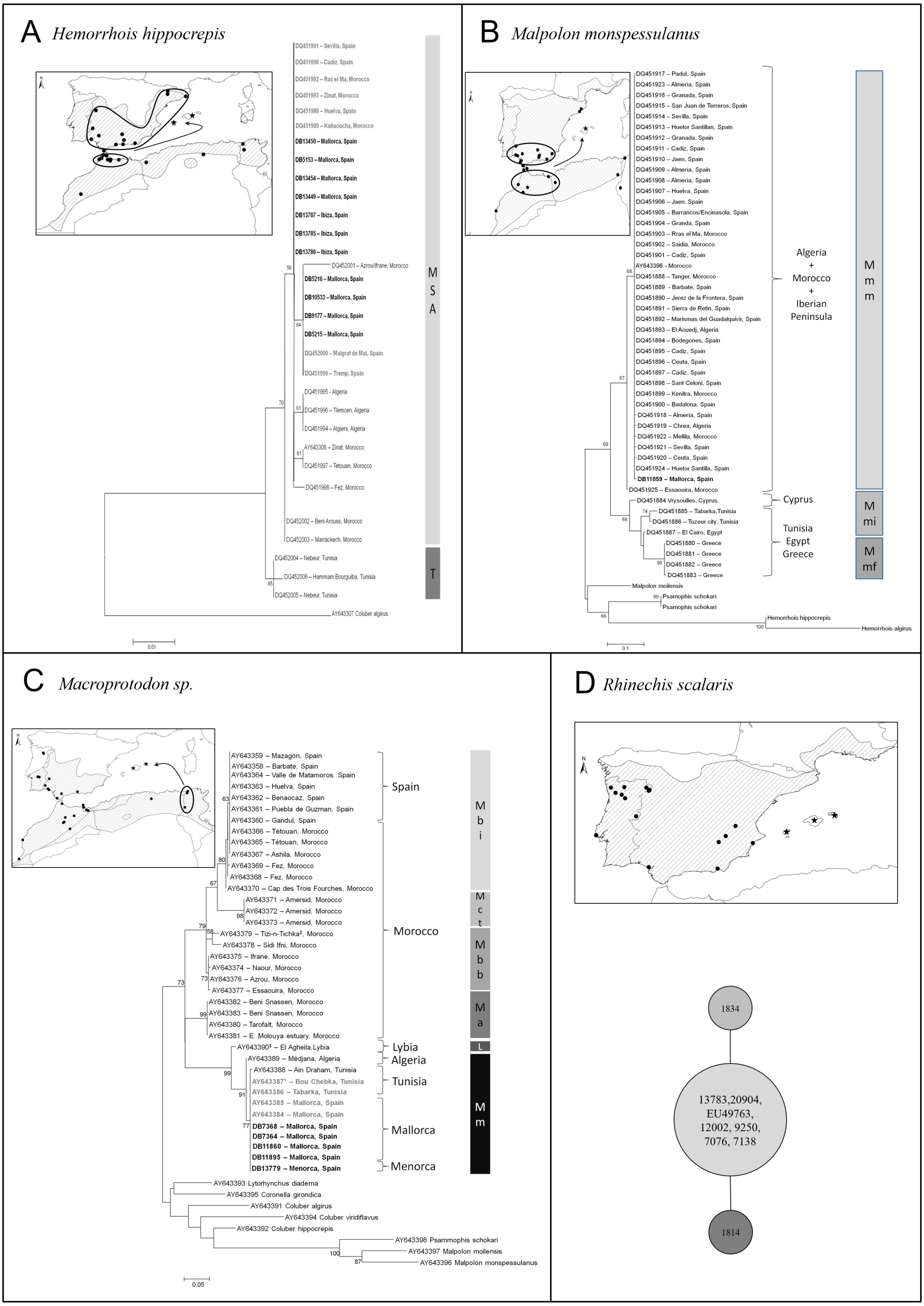
A Hemorrhois hippocrepis
M S A
T
DQ451991 – Sevilla, Spain
DQ451990 – Cadiz, Spain
DQ451992 – Ras el Ma, Morocco
DQ451993 – Zinat, Morocco
DQ451988 – Huelva, Spain
DQ451989 – Kahaoucha, Morocco
DB13450 – Mallorca, Spain
DB5153 – Mallorca, Spain
DB13454 – Mallorca, Spain
DB13449 – Mallorca, Spain
DB13787 – Ibiza, Spain
DB13785 – Ibiza, Spain
DB13786 – Ibiza, Spain
DQ452001 – Azrou/Ifrane, Morocco
DB5216 – Mallorca, Spain
DB10533 – Mallorca, Spain
DB9177 – Mallorca, Spain
DB5215 – Mallorca, Spain
DQ452000 – Malgrat de Mal, Spain
DQ451999 – Tremp, Spain
DQ451995 – Algeria
DQ451996 – Tlemcen, Algeria
DQ451994 – Algiers, Algeria
AY643308 – Zinat, Morocco
DQ451997 – Tetouan, Morocco
DQ451998 – Fez, Morocco
DQ452002 – Beni Arouss, Morocco
DQ452003 – Marrackech, Morocco
DQ452004 – Nebeur, Tunisia
DQ452006 – Hammam Bourguiba, Tunisia
DQ452005 – Nebeur, Tunisia
AY643307 Coluber algirus
0.01
B Malpolon monspessulanus
Algeria + Morocco + Iberian Peninsula
Cyprus
Tunisia Egypt Greece
M m m
M mi
M mf
DB11859 – Mallorca, Spain
DQ451925 – Essaouira, Morocco
DQ451884 Vrysoulles, Cyprus
DQ451885 – Tabarka,Tunisia
DQ451886 – Tozeur city, Tunisia
DQ451887 – El Cairo, Egypt
DQ451880 – Greece
DQ451881 – Greece
DQ451882 – Greece
DQ451883 – Greece
Malpolon moilensis
Psamophis schokari
Hemorrhois hippocrepis
Hemorrhois algirus
0.1
C Macroprotodon sp.
Spain
Morocco
Lybia
Algeria
Tunisia
Mallorca
Menorca
M b i
M c t
M b b
M a
L
M m
AY643359 – Mazagón, Spain
AY643358 – Barbate, Spain
AY643364 – Valle de Matamoros, Spain
AY643363 – Huelva, Spain
AY643362 – Benaocaz, Spain
AY643361 – Puebla de Guzman, Spain
AY643360 – Gandul, Spain
AY643366 – Tétouan, Morocco
AY643365 – Tétouan, Morocco
AY643367 – Ashila, Morocco
AY643369 – Fez, Morocco
AY643368 – Fez, Morocco
AY643370 – Cap des Trois Fourches, Morocco
AY643371 – Amersid, Morocco
AY643372 – Amersid, Morocco
AY643373 – Amersid, Morocco
AY643379 – Tizi-n-Tichka², Morocco
AY643378 – Sidi Ifni, Morocco
AY643375 – Ifrane, Morocco
AY643374 – Naour, Morocco
AY643376 – Azrou, Morocco
AY643377 – Essaouira, Morocco
AY643382 – Beni Snassen, Morocco
AY643383 – Beni Snassen, Morocco
AY643380 – Tarofalt, Morocco
AY643381 – E. Molouya estuary, Morocco
AY643390³ – El Agheila,Lybia
AY643389 – Médjana, Algeria
AY643388 – Ain Draham, Tunisia
AY643387¹ – Bou Chebka, Tunisia
AY643386 – Tabarka, Tunisia
AY643385 – Mallorca, Spain
AY643384 – Mallorca, Spain
DB7368 – Mallorca, Spain
DB7364 – Mallorca, Spain
DB11860 – Mallorca, Spain
DB11895 – Mallorca, Spain
DB13779 – Menorca, Spain
AY643393 Lytorhynchus diadema
AY643395 Coronella girondica
AY643391 Coluber algirus
AY643394 Coluber viridiflavus
AY643392 Coluber hippocrepis
AY643398 Psammophis schokari
AY643397 Malpolon moilensis
AY643396 Malpolon monspessulanus
0.05
D Rhinechis scalaris
1834
13783,20904, EU49763, 12002, 9250, 7076, 7138
1814

Figure 3.2 *(previous page)*: Results from genetic analysis and geographic origin of the introduced populations. Numbers on branches indicate ML bootstrap values (BP) over 1000 replicates (BP<50 are not reported). (2A): ML tree based on of the combined data set cyt b depicting the relationships between haplotypes of the native range of *Hemorrhois hippocrepis* from Carranza et al. [23] and those from the introduced populations from Mallorca and Ibiza Islands. MSA: Morocco+Iberia+Algeria; T: Tunisia. (2B) ML tree based on of the cyt b data set depicting the relationships between haplotypes of native range of *Malpolon monspessulanus* from Carranza et al. [23] and those from the introduced populations from Mallorca Island. Mmm: *Malpolon m. monspessulanus*; Mmi: *Malpolon m. insignatus*; Mmf: *Malpolon m. fuscus*. (2C) ML tree based on of the cytochrome b data set depicting the relationships between haplotypes of native range of *Macroprotodon sp.* from Carranza et al. [24] and those from the introduced populations from Mallorca and Menorca Islands. Mbi: *Macroprotodon brevis ibericus*; Mct: *Macroprotodon cucullatus textilis;* Mbb: *Macroprotodon brevis brevis;* Ma: *Macroprotodon abubakeri*; L Libyan clade; Mm: *Macroprotodon mauritanicus*. (2D) Statistical parsimony network depicting the genealogical relationships between haplotypes from the native range and from Balearic individuals (Ibiza: 12002; Mallorca: 9250; Menorca: 7076 and 7138) of *R. scalaris*.

Table 3.2: Contribution of each bioclimatic variable for each species model. Two main explanatory variables for each species are in italic. PC: Percentage contribution; PI: Permutation Importance. Bio2: Mean Diurnal Range; Bio3: Isothermality; Bio4: Temperature Seasonality; Bio8: Mean Temperature of Wettest Quarter; Bio15: Precipitation Seasonality; Bio18: Precipitation of Warmest Quarter; Bio19: Precipitation of Coldest Quarter.

Variable	***Hemorrhois hippocrepis***		***Malpolon monspessulanus***		***Macroprotodon sp.***		***Rhinechis scalaris***	
	PC	PI	PC	PI	PC	PI	PC	PI
Bio2	2.5	6.7	9.6	15	12.6	12.6	18.4	21.2
Bio3	0	0	3.5	4.3	0	0.1	2.7	2
Bio4	6.9	2.7	28.8	13.7	2	0.5	28.4	15.3
Bio8	0	0	0.5	3.8	0.9	2.5	0.1	1.9
Bio15	22.8	0.6	8.3	6.7	17.2	0	12.3	5.3
Bio18	39.3	71.5	29.8	36.3	57.2	81	37.6	44.5
Bio19	28.6	18.5	19.6	20.2	15.7	3.4	0.5	9.8

Suitability of Balearic Islands for invasive snakes

In general, the models projected to the Balearic Islands showed a limited suitability in the present for the four snakes at the given occurrence threshold (see below; Fig 3.3). Nevertheless, it is worth noting that known occurrence points from each species fall within unsuitable areas. Moreover, in the projections for the future, Balearic Islands become more suitable for the all the species, with exception of the smooth false snake (*Macroprotodon* sp.) which only might occupy Formentera, south of Mallorca and Cabrera (Fig. 3.3). Both

R. scalaris and *Malpolon monspessulanus* attained the highest suitability probabilities and might occupy almost all the extension of all the islands. The horseshoe whip snake (*H. hippocrepis*) might be widespread on the island with exception of the Serra Tramuntana mountain range. It is important to remark that the habitat suitability index was relatively high in the future models (with exception of *Macroprotodon* sp.), being the maximum values 0.57 in 2020 for *H. hippocrepis*, 0.34 in 2080 for *Macroprotodon* sp., 0.60 in 2080 for *R. scalaris* and 0.59 in all the three years for *Malpolon monspessulanus*.

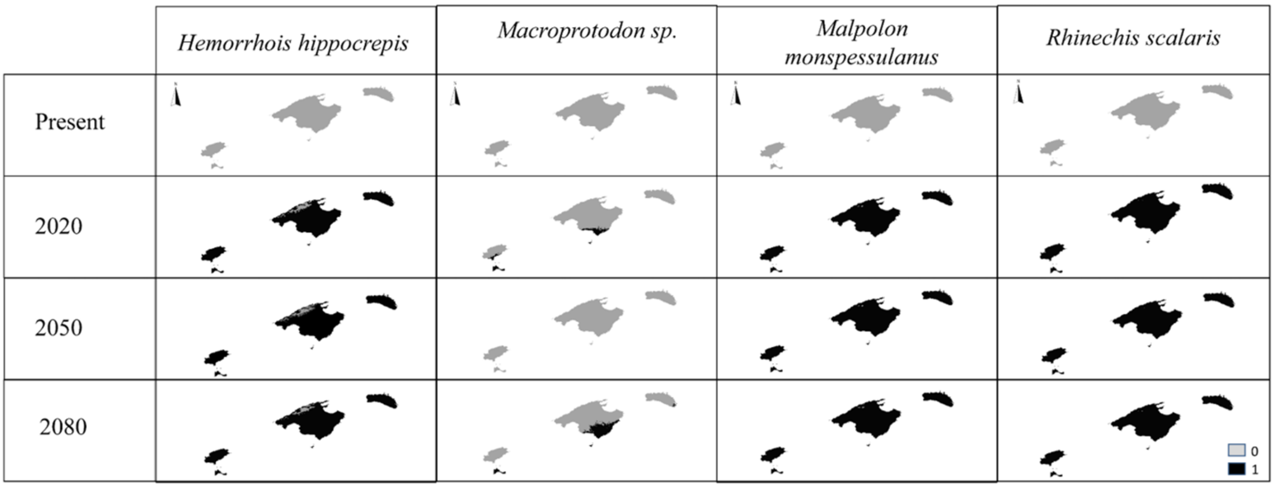

Figure 3.3: Habitat suitability models for the present and for the future (2020, 2050 and 2080) of all the species in study.

Discussion

To protect Balearic endemic biota from contemporary biological invasions we have first to understand the current dynamic of this process and its possible future development. In particular, to plan effective measures aimed to prevent further introductions it is crucial to know, first, where are these aliens from, and, second, which is the pathway through which they arrive in the Balearic Islands. On the other hand, regarding those snakes that are already established in the archipelago, management measures rely on the knowledge on their current and future spatial patterns of distribution. In the following sections we discuss the results of this study following these three main points and, finally, addressing their conservation implications.

Timing and origin of the snake introductions

The occurrence records and previous literature allow distinguishing between species historically present in the archipelago and those arrived only very recently (last decade). The first group (historical introductions) includes the populations of *Macroprotodon* sp. from Mallorca and Menorca, and *R. scalaris* from Menorca. Previous studies suggest introduction during Roman times for both the species, however it is important to stress that this is just a hypothesis [34,45-47]. The second group (recent introductions) includes *H. hippocrepis* and *Malpolon monspessulanus* from Ibiza and Mallorca and *R. scalaris* from Ibiza, Mallorca and Formentera. In fact, for *H. hippocrepis* and *Malpolon monspessulanus*, the first observations in Ibiza only date from 2003 [19]; for *R. scalaris*, observations date from 2003 in Ibiza, 2004 in Mallorca, and 2006 in Formentera [19-21].

Phylogeographic analyses of all the species sampled in the Balearic Islands confirm their allochthonous status, suggesting an origin from North African or Iberian populations. Genetic evidence supports a Tunisian origin for Mallorcan and Menorcan population of *Macroprotodon* sp. (historical introduction; Fig 2C), which therefore were assigned to *Macroprotodon mauritanicus* following [36].This result is in agreement with the morphological identification of 168 *Macroprotodon* individuals recorded in the Balearic Snakes Database of the Servei de Protecció d'Especies of Govern Balear (124 records from Mallorca, 44 from Menorca). Moreover, a Tunisian origin for Mallorcan *Macroprotodon* snakes was already proposed by Busack and McCoy [46], Wade [36], and Carranza et al. [26], on the basis of the morphological and genetic similarity between Mallorcan and Tunisian specimens. In the case of *H. hippocrepis* and *Malpolon monspessulanus* (recent introductions), genetic data do not unambiguously differentiate between North-western African and European origins (Fig 2A and 2B, respectively), given the extreme genetic similarity between native populations from these two regions [25]. Indeed, for both species, a recent expansion during Late Pleistocene from North-eastern Africa to Iberia has been suggested to explain the slightly higher genetic diversity observed in the North African populations of these species and their overall similarity with Iberian populations [25]. However, after analysing the phylogenetic tree of *H. hippocrepis*, Mallorcan samples clustered in a sub-haploclade including haplotypes found only in the Iberian Peninsula. This suggests an Iberian origin for *H. hippocrepis* from Mallorca, yet a North African origin cannot be completely discarded. Besides the phylogenetic evidence, the finding of this species in Mallorca near nurseries of olive trees [19] (J. A. Mateo pers. obs.) that arrived from southern Spain further supports an Iberian origin of Mallorcan populations (see the section Pathways of introduction for further details).

Phylogenetic network analyses show that the haplotype of *R. scalaris* found in Menorca (historical introduction), Mallorca, and Ibiza (recent introductions) is identical to the commonest haplotype found across the species range, suggesting a recent arrival to the Balearic archipelago from the mainland (Fig 2D). Yet it was not possible to point a restricted source-area given the lack of phylogeographic structure of this species [28], while the genetic identity between all the Balearic populations and the continent allows discarding an ancient (i.e. Pleistocenic) colonization of the archipelago. Therefore, summarizing an allocthonous status of Balearic snakes was confirmed for both historical and recent introductions, being the latter most likely originated from Iberia. The high genetic homogeneity of Balearic individuals of *H. hippocrepis* (two similar haplotypes), *Macroprotodon mauritanicus* and *R. scalaris* (one single haplotype) would suggest that each species invaded the Balearic Islands from a single origin. However, the very shallow mitochondrial phylogeographies of these species hampered the identification of restricted source-areas and therefore of multiple-origin patterns. For this purpose, it will be necessary the use of more variable markers (e.g. microsatellites) across the native and the Balearic ranges of these species, possibly with additional Balearic samples.

Pathways of introduction

The introduction pathway for all the species is clearly human-mediated. In the case of the older introductions, such as the Menorcan population of *R. scalaris*, and the Mallorcan and Menorcan populations of *Macroprotodon mauritanicus*, the anthropogenic origin is supported by the absence of fossil record and the low genetic differentiation between the islands and the mainland populations [12,19,24,47]. The main pathways by which these species arrived to the Balearic Islands in historical times can be either voluntary or accidental. Among the voluntary factors, ancient Greek and Roman religious purposes cannot be discarded, since snakes appear to have a wide range of religious associations (i.e. Asklepios/Aesculapius cult), namely as guardians of a place, to bring luck, and to reveal if a place was sacred or not [48,49]. Moreover, snakes were also used by ancient Greeks, Carthaginians and Romans to frighten enemies during assaults [50]. Therefore, a probable voluntary introduction is favoured, since the humans likely obtained direct benefits from the translocation of snakes in these islands. Among the accidental factors, one possible way of introduction may have been the involuntary transport via shipments during an intense maritime trade along commercial routes established since long times between Balearic Island and the mainland. Many species use the human manufactures as a refuge, making more probable its accidental translocation from the native range toward the introduced range.

While historical pathways of introduction are difficult to disentangle, the inference of recent introductions can be based on the large amount of information we collected joining bibliographical and Government entities data. Commercial shipments by sea is most likely the main pathway for the recent biological invasions observed in the Balearic Islands. Mounting evidence strongly indicates that several alien reptiles (not only snakes) arrive in the Balearic islands through the nursery trade, which refers to the trade in live plants for ornamental purposes [11,19]. Most of the records of *H. hippocrepis*, *Malpolon monspessulanus*, and *R. scalaris*, and significantly their first appearance in the Balearic Islands, have been recorded by environmental authorities inside trunks or root balls of olive trees deposited in the nursery centres of Capdepera (Mallorca) and Sant Lloren de Baláfia (Ibiza) [19] (Balearic Snakes Database of the Servei de Protecció d'Especies del Goberno Balear, BSD-SPEGB). In the former centre, an adult individual of *H. hippocrepis* was found by one of us (J. A. Mateo) inside the trunk of an old olive tree just arrived from Córdoba province (Andalusia, southern Spain). Moreover, nursery centres have been the places where several alien reptiles have been recorded for the first time in the Balearic during the last decade, namely the amphisbaenian *Blanus cinereus* (BSD-SPEGB; J. A. Mateo, personal observation), the blind snake *Indotyphlops braminus* (BSD-SPEGB) and the ocellated lizard *Timon lepidus* (Mallorca Reptile Database of COFIB, MRD-COFIB).

The nursery trade has increased in importance since the 1970s, and accordingly this trade has increased its importance as vector of biological invasions [11]. This is particularly true for olive trees translocation across the Mediterranean [14,19,51-53] (this study). While formerly olive trees were moved across this region due to olive oil production, nowadays old trees are being transplanted also as decorative plants for gardens. Paradoxically, this trend is somehow in line with the European Agricultural Policies which encourage the removal of old, less productive, trees [54]. It is worth noting that only continental trees from Spain (Córdoba, Sevilla, Jaen, and Valencia provinces) and in small amounts from Southern Portugal (E. Ayllón com. pers.) are used to fulfil the gardening demands of the Balearics. No olive tree importations from North Africa are known. For instance, one of the nurseries in Mallorca imported 4000 specimens of different sizes since 1992 [54], which reflects the big market associated with these plants.

Several factors make the olive trade a powerful vector for biological invasions across the Mediterranean. First, olive trees are normally uprooted and transported during the winter because these practices require the trees to be in the latency phase. Second, the uprooting procedure implies the removal of a very large root ball about 2 m3 around the olive tree. Third, both the plant nurseries that import these olive trees and the gardens where they are implanted are generally located in areas with Mediterranean conditions, i.e. with a similar climate to the place of origin. These three factors make the time, the mode, and the destination of olive tree translocations, particularly suitable for the transport, survival, and naturalization in the new environment of the entire ark of organisms that use the olive tree roots and cavities as a winter refuge (see also [11]). Indeed, old olive trees are hollow and able to carry several species of reptiles including snakes, lizards, and geckos [51,52,55], but likely also toads, small mammals and several arthropods, which increases the probability of introductions of multiple species even with few trees. Evidence for an anthropogenic introduction of the Italian wall lizard *P. sicula* from southern Italy to Spain through the olive trees trade has been reported by Valdeón et al. [51] and Rivera et al. [52] for La Rioja and Sant Celoni populations respectively. The finding of numerous species of alien reptiles in the nursery gardens of the Balearic Islands provides a further evidence for this scenario.

Thus olive tree trade emerges as a modern vector for bioinvasions across the Mediterranean of a wide spectrum of alien species. Further studies covering all the Mediterranean Basin are need to uncover the magnitude and pattern of this invasion pathway, its conservation impact, and the eventual management strategies at transnational level.

Current and future suitability of Balearic Islands for aliens snakes

As inferred from the current occupation of their native continental ranges, the Balearic

Island seems of limited habitat suitability for all the four introduced species. The fact that a more relaxed threshold would predict these snake occurrences (Appendix E), further suggests that most of Balearic territory is still climatically suboptimal for these snake species. Nevertheless, when comparing the models projection with the known presence points, it becomes evident that despite this limited habitat suitability all the study species are widely distributed. Moreover, all these recently introduced species are currently spreading in the Archipelago [19].

The lack of complete congruence between the predictions and the observed records recommends a cautious interpretation of modelling results. There are three possible, not mutually exclusive, causes for the currently wider distribution of invasive snakes than predicted by the models: (i) a low performance of the models due to the use of only climatic variables and coarse spatial resolution; (ii) the favourable conditions found by these species on islands respect to the continent due to the absence of predators, parasites and competitors, or the availability of different food resources and empty niches in islands ecosystems; and (iii) finally the establishment of a source-sink patterns [56,57]. According to this latter hypothesis, the wide distribution of invasive species can be simply due to the constant release of individuals in different islands localities (sink) from the native range (source).

Certainly, the number of adult individuals of alien snakes observed in the Balearic islands has been increasing year by year (BSD-SPEGB; MRD-COFIB; J. A. Mateo personal observation) suggesting a continual importation of snakes. However, for all the introduced species, Álvarez et al. [19] reported evidence of reproduction in the Balearic Islands- i.e. the observation of pregnant females, hatchings, and immature snakes. Therefore, the large number of snakes observed in the Balearic and their wide distribution is not merely due to continuous importation of snakes, but also to in loco recruitment of individuals and perhaps enhanced by the tendency of young individuals to disperse (e.g. [58]). The evidence of reproduction suggests that factors such as available habitat and food resources may be important for the future distribution and expansion of the alien snakes in these islands (see also [59-64]).

Forecasting the future, all the scenarios tested predicted an increased suitability for most of the island territory for the alien snakes (with the exception of *Macroprotodon mauritanicus*). These results suggest that climate change might improve the environmental conditions for these invasive species. Indeed, recent studies demonstrated that climate change is already increasing the activity period and benefiting the survival and reproduction of *Malpolon monspessulanus* [65-67] and *H. hippocrepis* [68] in the Iberian Peninsula. Therefore, the invasiveness of all snake species is expected to increase and an increase of their impact on native biota can be the anticipated.

Conservation implications

The introduction of snakes in the Balearic Islands is expected to have negative impacts on the native biota. The four introduced species are effective predators and, according to the foraging ecology of these species in their native areas, the main impact is presumed on native lizards, amphibians, and birds (many of which are endemic and already endangered by other factors) [12]. Among other potential impacts on native fauna, alien snakes may be the vector for the introduction of new parasites. This could be particularly straightforward in the case of blood parasites since those carried by Mediterranean snakes seems to have generally low host specificity [69]. Conservation measures should be taken with a maximum urgency.

The Convention on Biological Diversity (2002) adopted the principle that the prevention of biological invasions is the priority. Only when prevention fails, early detection, rapid response, and possible eradication of invasive species should be applied [5]. The long-term management would be only the last option. In the case of Balearic Islands, suitable measures to apply depend on the timing and pathways of arrival of alien species.

Species introduced historically, like M. mauritanicus and *R. scalaris* in Menorca, are already widespread and naturalized during several centuries with stable reproductive contingents. Thus, the eradication for these species is not possible. However, the prevention of secondary dispersal of these species to new areas of the archipelago where they are still not present is essential. It is especially important to prevent the introduction of the snakes in the surrounding islets of Ibiza, Menorca and Mallorca and Cabrera, where Balearic endemic species such as *Podarcis lilfordi* and *P. pityusensis* still persist [12]. Of course this guidance apply also for those species recently introduced in the main Balearic Islands (Ibiza, Menorca, and Mallorca). Several measures can be applied to prevent further introductions within and across the Balearic archipelago: (i) inspection and quarantine of arriving goods, containers, baggage and vessels; (ii) control and monitoring of importation of trees or other plants; (iii) methodical inspection on maritime cargo. Preventive actions may also benefit from studies aimed at evaluating the risk of invasion across the Balearic Islands associated with current touristic and commercial activities.

In cases where prevention would reveal ineffective, the early detection and the rapid eradication of invasive species should be pursued. For such programs to be successful, a specific educational effort for the general public is needed. Currently, Balearic regional authorities have designed a snake eradication program which they are already implementing in Ibiza and Formentera. These islands deserve the maximum priority since they have never harboured snakes. The first actions of eradication in Ibiza have included the involvement of the general public and snake collection combining traps and dogs search in the main areas of snake sightings [70]. Despite the considerable amount of economic and human resources devoted to the eradication, the outcome of this program is still highly uncertain, because in this case the snake invasion already moved forward from the earlier stages as indicated by the occurrence of cases of reproduction [70]. On the other hand, these actions would certainly help to restrict the spread of the invasion within the islands and to keep the density of the alien snakes as low as possible, thus minimizing their impact and their probability of spread across the archipelago.

In conclusion, in this study the combination of molecular and ecological tools with chronogeonemic data on the introduced snakes allowed dissecting the main pathways and dynamic of the process of biological invasions. This knowledge will be crucial for implementing preventive strategies to avoid further introductions within the main islands and the arrival of the alien snakes to the satellite islets which still host much native biodiversity. Moreover, the identification of the olive trees trade as a powerful vector of alien species claims for the attention of conservationist to apply the required preventive measures across the Mediterranean areas potentially threatened by such new waves of biological invasions.

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Supporting Information

Additional Supporting Information can be found in the Appendices section.

Appendix C - S3.1: Genetic Data. Information on locality, gene fragment and GenBank accession number of each sequence used in the genetic analysis.

Appendix D - S3.2: Jackknife analyses. Jackknife re-sampling results of the training and test gain and of AUC values for all the four species used in the study.

Appendix E - S3.3: Habitat suitability models with logistic threshold. Habitat suitability models for the present and for the future (2020, 2050 and 2080) of all the species in study, with the balance training omission, predicted area and threshold value logistic threshold (a lower threshold compared to the one used on the principal study).

Appendix F - S3.4: MESS analysis. (Left) Year and scenario corresponding to the results. (Centre) MESS results: areas in red have one or more environmental variables outside the present range in the training data. (B) MoD results, showing the most dissimilar variable.

Chapter 4

Genetic signature of *P. sicula*

Silva-Rocha, I, GF Ficetola, MA Carretero and D Salvi. in prep. “Genetic signature of the invasion of the lizard *Podarcis sicula* on Mediterranean islands”.

Abstract

Biological invasions are one the major causes of biodiversity loss, but at the same time, they can provide an interesting window to study ecological and evolutionary processes. The biota from Mediterranean region has a long-term interaction with humans. Such interaction leads to a combination of ancient and recent introductions within the basin and, consequently, a complex invasion history for the Mediterranean biotic communities. The main aim of this work is to understand how genetic of an alien population respond to the invasion process and what is the role of humans on the invasion. The Italian wall lizard, *Podarcis sicula*, has been introduced and able to succeed in several places, namely in many archipelagos within the Mediterranean basin. This provides a study design with replicates, important to understand the invasion patterns and their consistency across different systems. Three different systems composed by islands (alien) and mainland (native) populations, with different colonization times, were chosen: Tuscany, Aeolian and Aegadian. We used ddRAD data to infer genetic diversity and structure, by performing population genetic, PCA and NJ tree analysis. Results suggest a process of either natural colonization (Tuscany) or human-mediated invasion (Aeolian and Aegadian), with a different pattern for each of the systems. The human influence is different on Aeolian and Aegadian, although humans represent a key driver of invasion on both systems. Additionally, a homogeneous genetic diversity is observed on Aegadian and Aeolian systems, suggesting a multiple invasion scenario and/or ongoing invasion. Overall, our results illustrate the intrinsic complexity of colonization dynamics in insular systems with complex biogeography and long history of human influence. For invasion science, it also provides a stronger support on the genetic implications and processes for alien species

Keywords Biological invasions, population diversity, ddRAD-seq, human-role, colonization

Introduction

Biological invasions are a major component of current global change. Humans have been disturbing, intentionally or unintentionally, species distribution by introducing them into new environments. These movements can impact human health and agriculture, can accelerate biodiversity loss and determine changes on ecosystem services (Lockwood, Hoopes, and Marchetti 2007). On the other hand, biological invasions also represent natural experiments which allow to study ecological and evolutionary processes occurring in real-time (Miura 2007; Fitzpatrick et al. 2012; Chown et al. 2015).

The Mediterranean region is considered one of the world hotspots of biodiversity (Myers et al. 2000). It also represents a region with a long history of human-biota interactions which caused severe changes on the biodiversity present over time and on the composition of the Mediterranean biotic communities. Humans have been, indeed, translocating species across the whole Mediterranean region, homogenizing the biotic communities which now share evolutionary lineages with surrounding continents or/and islands. Mediterranean islands are especially vulnerable to these changes, since many organisms native to islands have been evolving long time in isolation, losing their capacity for competing with other species, eluding predators and defending themselves from parasites and diseases. Indeed, the invasion process in the Mediterranean is so intensive and widespread that even the distinction between allochthonous and autochthonous species may be very difficult to assess in practice (Blondel et al. 2010).

In this context, molecular tools are now particularly useful to unveil the human contribution to biogeographic patterns and processes , namely to assess species status regarding its origin (alien/native) (e.g. Silva-Rocha et al. 2012, 2015), to identify cryptic invasions (e.g. Roman 2006), to reconstruct colonization history and to evidence population bottlenecks and founder effects (e.g. Konen et al. 2013). Nowadays, genomic tools, such as reduced-representation sequencing approaches, are increasing popularity to study non-model organisms with no prior genome available (da Fonseca et al. 2016). They provide an accessible way to study population genetics, to identify parts of the genome involved in adaptation, to infer phylogeographic and phylogenetic patterns, and to deep other questions of evolutionary ecology (Ekblom and Galindo 2010; McCormack et al. 2012). One first step to begin to understand how the human impacted the populations of a given species in the Mediterranean region is the determination of the genetic diversity and structure.

Genetic diversity provides the substrate for natural selection acting on populations undergoing new conditions in its allochthonous range. This will determine if it is able to acclimatize to the new environment and, therefore, it is an important issue in determining the potential of the species to establish and to become invasive (Dlugosch et al. 2015; Bock et al. 2015). Alien populations are often thought to have reduced genetic variability relative to their source conspecific populations, because of genetic founder effects associated with small population size during the introduction and establishment stages. This low genetic diversity should limit the invasion success since it will limit the population capacity to respond to the new selective pressures. Furthermore, the small population size can also lead to other problems, such as inbreeding which can result in exposure of deleterious recessive mutations in homozygous individuals. However, several studies have been demonstrated that invasions are not necessarily characterized by a significant loss in genetic diversity. In fact, in some cases high genetic diversity in alien populations have been observed, generally explained by multiple introductions, particularly when source populations are genetically divergent (Bock et al. 2015; Dlugosch et al. 2015; Kolbe et al. 2007). In Mediterranean region, multiple source and invasions scenarios cannot be discarded, and indeed, can be the most probable ones.

Inferring the genetic structure of invasive species can also help to understand the population history, namely the invasion route. The reconstruction of invasions routes can allow setting and testing different hypotheses concerning ecological and evolutionary factors related to biological invasions process (Estoup and Guillemaud 2010; Cristescu 2015) and can help designing strategies for preventing future invasions. Molecular data have a predominant role on inferring invasion routes, since historical records and direct observations on the spread of alien species are often sparse.

Most studies analyzing genetic signature of invasive species focus on a single study case and take conclusion about that specific study case. In this work, we have different systems, each one composed by alien (islands) and native (mainland) populations, which are replicates of different invasions by the same species. This study design allows to compare patterns and understand if they are consistent across study systems. For invasion science provides a stronger support on the genetic behavior of alien species.

The Italian wall lizard *Podacis sicula* is an excellent model species to understand how invasion history determines genetic diversity of invasive species. This lizard is native of mainland Italy, Sicily and mainland Croatia (Podnar, Mayer, and Tvrtkovi 2005; Senczuk et al. 2017), and has been introduced since historical times within the Mediterranean Basin and lately also outside the Basin and in different continents (Silva-Rocha, Salvi, and Carretero 2012). Although there is limited information on the approximate dates of introduction across the Mediterranean islands and the detailed pathways of dispersal, the most common pathway was probably accidental by cargo due to maritime connections and trade between mainland and islands. Nowadays, besides the cargo, the nursery trade (i.e. trade of ornamental plants) is also an important vector of artificial dispersal of this species (Silva-Rocha, Salvi, and Carretero 2012; Valdeón et al. 2010). This lizard has been introduced in a large number of archipelagos, furnishing several replicated systems to assess how introduction patterns determine the genetic diversity of invasive species, and the evolutionary consequences of invasions (Herrel et al. 2008).

We analyzed the genetics of the invasion by *P. sicula* in three different mainland-islands systems in the Mediterranean basin, which are likely to differ on colonization times and history of *P. sicula* (Fig. 4.1): (1) Tuscany system Talamone, Capalbio, Argentario, Giglio, Formica and Giannutri; (2) Aegadian system Erice, Birgi, Favignana, Marettimo, Levanzo; (3) Aeolian system Milazzo, Vulcano, Salina, Stromboli and Filicudi.

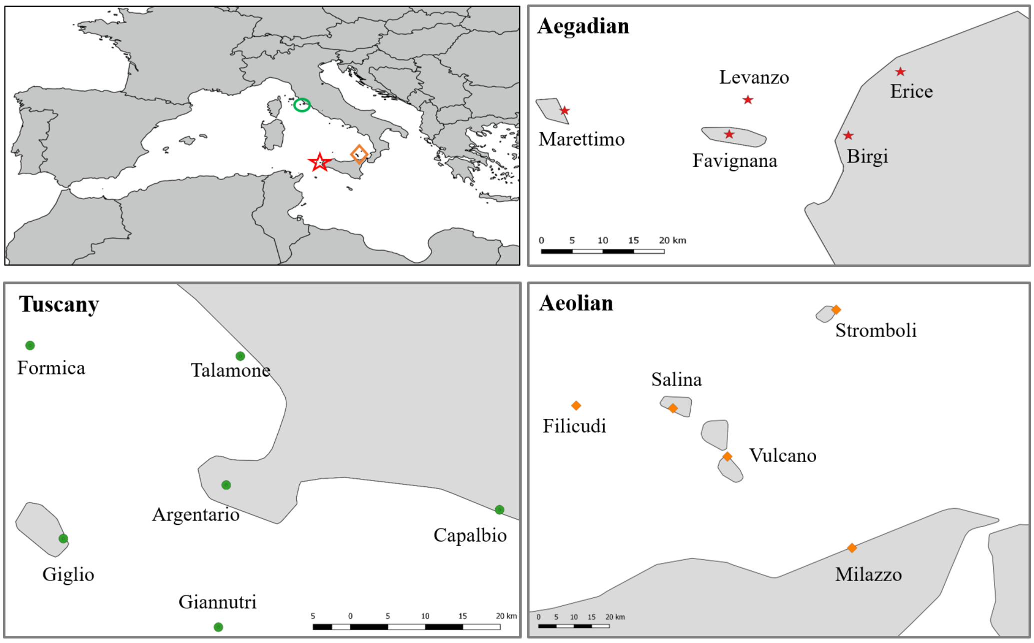

Figure 4.1: Map of the study systems where is potentially introduced. Top left: Location of the three systems within the Mediterranean basin; Bottom Left: Tuscany system; Top Right: Aegadian system; Bottom right: Aeolian system.

The main aim of this work is to understand the genetic signature of the invasion of the Italian wall lizard, across different insular systems. One of the hypotheses underlying this work is that alien insular populations follow a multiple introductions scenario. If that is the case, the genetic diversity on alien populations (islands) can be the same or higher than in the mainland native populations. However, if a single introduction happened, the mainland populations will have a higher genetic diversity than islands. Another parallel hypothesis is that studied insular populations were introduced recently and, therefore, they will have a weaker genetic structure if compared with older introductions or even natural colonization patterns. Finally, a last hypothesis is that insular populations far from the mainland will probably be human-mediated colonization, as suggested by the results obtained on Chapter 2.

This study will provide insights about the alien status and potential colonization history of alien insular populations of *P. sicula*, giving the invader capacity of this species in Mediterranean islands. Furthermore, it will add a layer of knowledge on the invasion process itself, by supporting the tendency for single/multiple introductions scenario and by clarifying the importance of genetics and human help to the success of an alien species.

Material and Methods

Study systems and sampling

The Tuscany system comprises three islands and three mainland populations (Fig. 4.1 - Tuscany). Both Giannutri and Formica were connected to mainland during Last Glacial Maximum (LGM), while Giglio was never connected to mainland or other islands, as suggested by the presence of endemic taxa only in this island (Fattorini 2009). In these Tuscan islands, the presence of *P. sicula* could be either due to transmarine natural or human-mediated colonization (Fattorini 2010). For the other two systems, Aegadian and Aeolian, Sicily is considered to be the mainland source. In the Aegadian system (Fig. 4.1 - Aegadian), three insular populations and two mainland populations are included. These islands were inhabited by humans since the occupation by Phoenicians (Bradford 2000). The origin of the islands is different for the three islands: Marettimo was never connected to Sicily during the Last Glacial Maximum (LGM), Levanzo was connected until 10000 years ago and Favignana was the last island to disconnect from Sicily (Dawson 2005). The colonization on Marettimo by *P. sicula* is assumed to be human-mediated in historical times as suggested by the low genetic variability and differentiation, while for Favignana and Levanzo it is not clear if it was natural colonization or historical introduction (Capula 1994). The Aeolian system has a volcanic origin, the four islands represented were never connected to the population from mainland (Fig. 4.1 - Aeolian) or between each other. The first human settlements is recorded 7000 BP (Lo Cascio and Corti 2006). The colonization by *P. sicula* is thought to take place during historical times, due to low genetic diversity compared to Sicily and to the proneness to be introduced (Capula et al. 2002). Moreover, the absence of *P. sicula* from unexploited or inaccessible islets surrounding the main Aeolian islands where the native species *P. raffonei* is/was present, it also suggests a human-mediated introduction. A total of 108 *P. sicula* individuals were sampled across the three systems: 48 individuals on Tuscany system (September 2014), 30 individuals on Aeolian and 30 individuals on Aegadian (August 2015; see Table 4.1). Tail tips were collected from lizards and then stored in pure ethanol.

Table 4.1: Sample size and genetic diversity results for each population.

System	M/I	Population	Sample size	SNPs	Number alleles	Hexp	Hobs	Private alleles	HWE
Aegadian	Mainland	Birgi	6	14087	6506	0.31	0.26	169	0.97
Aegadian	Mainland	Erice	6	14087	7113	0.29	0.28	220	0.96
Aegadian	Island	Levanzo	6	14087	6906	0.26	0.24	220	0.95
Aegadian	Island	Favignana	6	14087	7597	0.28	0.28	373	0.95
Aegadian	Island	Marettimo	6	14087	7321	0.3	0.28	288	0.95
Aeolian	Mainland	Milazzo	6	15232	12303	0.33	0.25	377	0.97
Aeolian	Island	Filicudi	6	15232	10319	0.28	0.24	584	0.97
Aeolian	Island	Salina	6	15232	12234	0.27	0.24	412	0.94
Aeolian	Island	Stromboli	6	15232	10019	0.31	0.24	450	0.95
Aeolian	Island	Vulcano	6	15232	12832	0.34	0.26	645	0.92
Tuscany	Mainland	Talamone	8	21020	22886	0.23	0.16	296	0.93
Tuscany	Mainland	Argentario	8	21020	23291	0.23	0.16	356	0.93
Tuscany	Mainland	Capalbio	8	21020	22936	0.23	0.16	278	0.94
Tuscany	Island	Giannutri	8	21020	19082	0.15	0.11	902	0.96
Tuscany	Island	Giglio	8	21020	17931	0.13	0.11	2150	0.97
Tuscany	Island	Formica	8	21020	16941	0.1	0.09	495	0.97

ddRAD laboratory and bioinformatics

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturers protocol. DNA was eluted with 100 μL of Buffer AE and then concentrated up to values required by library preparation procedures. DNA quantification was performed using a Qubit Fluorometric Quantitation (ThermoFisher Scientific).

A double-digested RAD (restriction site-associated DNA) experiment was conducted on the 108 individuals using a modified version of the protocol described in Capblancq et al. (2015). The modification comprised the use of 200 ng of DNA template from each individual which was double-digested with 10 units each of SbfI-HF and MspI (New England Biolabs Inc.) at 37C during two hours in a final volume of 34 μL using the CutSmart buffer provided with the enzymes. The amplified ddRAD library was purified with QIAgen MinElute PCR Purification Kit (Qiagen) and sequenced on an Illumina Hi-Seq 2500 at Fasteris SA, Switzerland.

The bioinformatic analysis was made using the Stacks pipeline (Catchen et al. 2013). This pipeline assembles identical reads into stacks which are subsequently broken into k-mers and compared among stacks to form clusters. These clusters are then separated into alleles or loci based on a similarity threshold. A stack is defined as a set of identical sequences. Adapter sequences were identified and removed from the analysis prior to subsequent steps. Raw data was demultiplexed and sorted by barcode and filtered for read quality using default settings in *process_radtags* module of Stacks. Since we do not have a reference genome, we performed a de novo analysis with the following parameters: (1) *ustacks* module: maximum distance allowed between stacks (M) = 6; and, (2) *cstacks* module: number of mismatches allowed between sample loci when build the catalog (n) = 8; all other parameters were kept at default values. Preliminary tests were carried out to identify those optimal parameters, important to achieve true alleles. Finally, we ran *populations* to retain only loci that were successfully genotyped in at least 10% of the individuals and with a frequency higher than 5%, to be used in downstream analysis. Only one random polymorphic site was kept for each RAD-tag. The full dataset was then split by each study system on VCF Tools (Danecek et al. 2011) with a minimum allele frequency parameter of 5%.

General population parameters, such as number of alleles, number of private alleles, expected and observed heterozygosity, and Hardy-Weinberg equilibrium, were calculated with functions from *poppr* R package (Kamvar, Tabima, and Grnwald 2014). The average pairwise F_{ST} was calculated with *StAMPP* R package (Pembleton, Cogan, and Forster 2013), with a bootstrap of 100 000 to generate p-values, to check the relatedness between populations within each system. A Principal Component Analysis (PCA) was performed in *adegenet* R package (Jombart and Ahmed 2011) to identify the major structure present in our dataset. A neighbor-joining network was built in Tassel v. 5.0 (Bradbury et al. 2007) to check for structure and possible invasion route. A Mantel test was performed to test if there is a connection between geographic distance and genetic diversity, in order to infer the human role on the colonization of the alien lizard. We used the results of the pairwise F_{ST}

as the genetic diversity and the distances were drawn from GoogleEarth.

Results

We obtained an average of 21500 fragments (RAD-tags) across the 108 individuals analyzed. The total polymorphic sites (SNPs) obtained was different for each system. Tuscany has the higher number of SNPs (21020 SNPs), followed by Aeolian (15232 SNPs) and Aegadian (14087 SNPs) (Table 4.1).

Tuscan Archipelago

The PCA clearly separated the three island populations from the three mainland populations (Fig.4.2a). The NJ network supported the structure given by PCA, with island populations clearly differentiated from mainland (Fig.4.3a). The pairwise population F_{ST} differences ranged from 0.042 to 0.776. These results indicated a significant genetic differentiation between island-island and island-mainland population pairs and weak differentiation among mainland populations (Table 4.2). The genetic diversity results showed a higher number of alleles for mainland populations, a higher number of private alleles for island populations, as expected for a single invasion scenario and an independent evolution of the islanders (Table 4.1). The Mantel test between genetic distance and geographic distance was not significant (P = 0.20), hence there is no direct relationship between distance of islands with human-mediated introductions.

Aegadian Archipelago

For the Aegadian, the PCA also separated the island populations from the mainland populations, although with a less clear pattern. Moreover, the PCA revealed a closer relationship between Marettimo and the mainland populations (Fig.4.2b). The NJ network supported the closeness of Marettimo to the mainland compared to the other islands and spotted an individual from Erice and another from Levanzo closer to Favignana cluster rather than the respective clusters (Fig. 4.3b). The pairwise population F_{ST} differences ranged from 0.089 to 0.245, which revealed a moderate genetic differentiation between mainland-island and island-island pairs and low genetic differentiation among mainland populations (Table 4.2). Regarding genetic diversity results, the number of alleles was homogeneous, and a

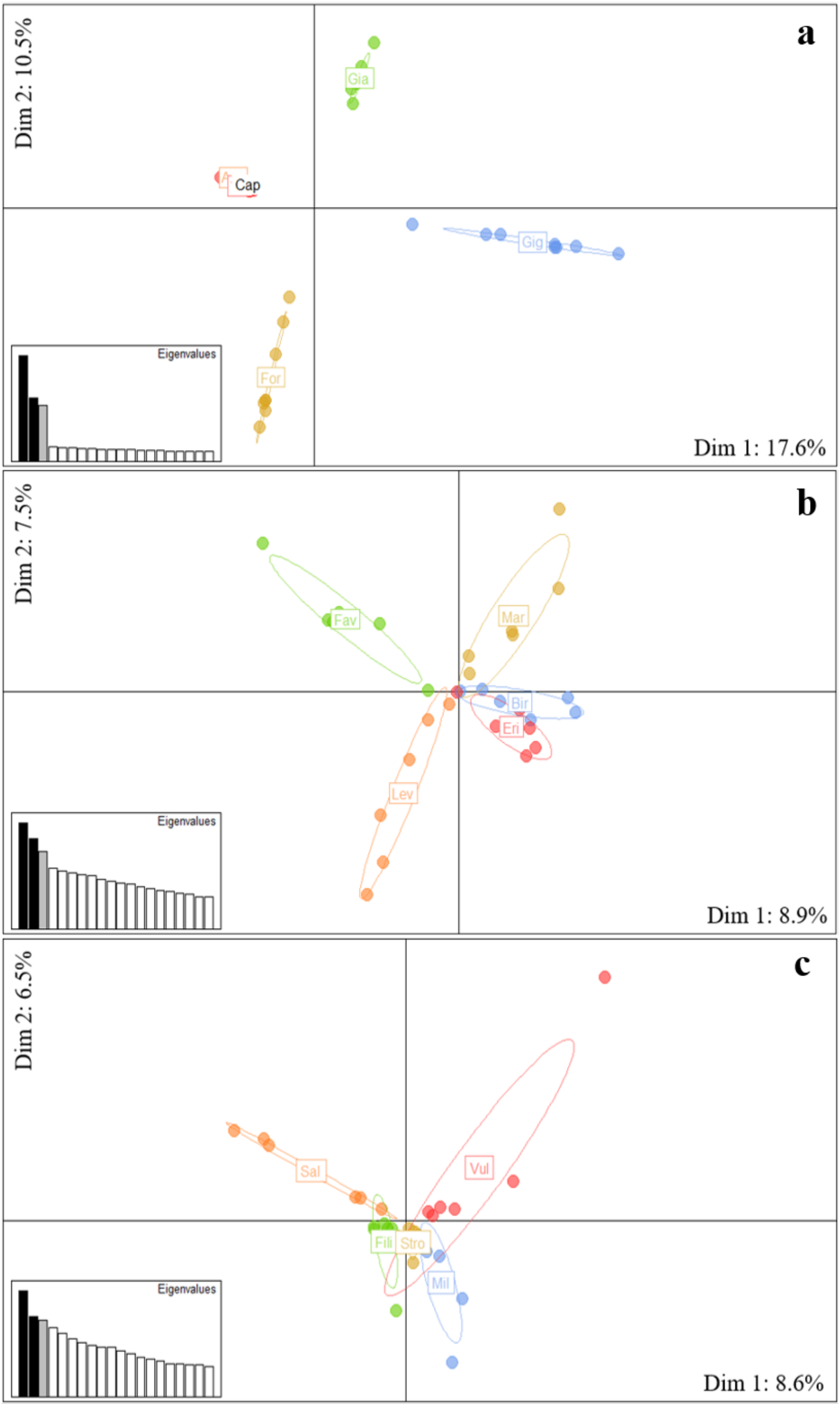

Figure 4.2: Principal Component Analysis (PCA) plots of each the systems, representing PCA1 and PCA2, and respective explanation percentage. (a) Tuscany system, (b) Aegadian system, (c) Aeolian system.

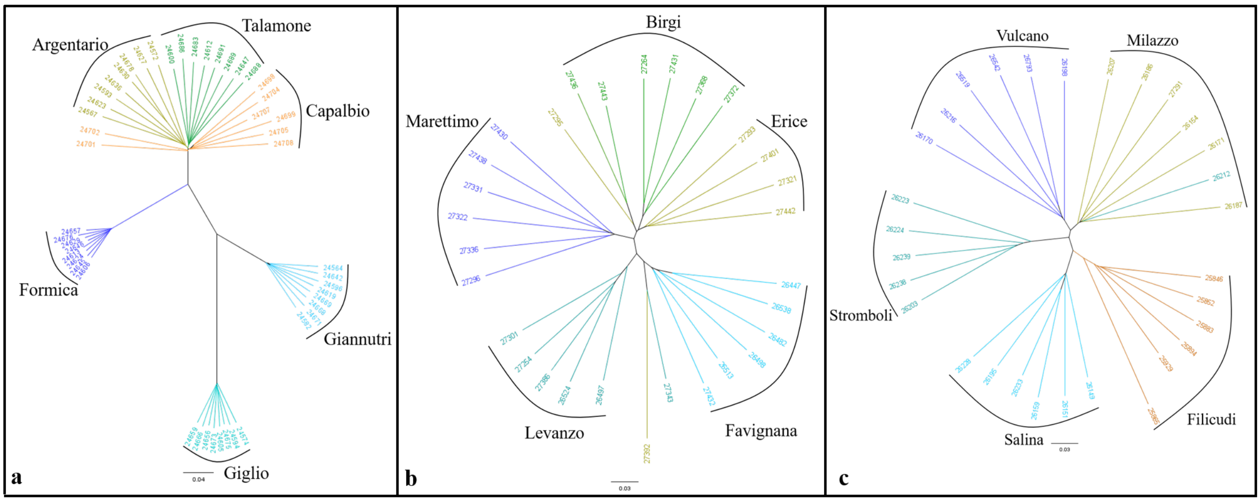

Figure 4.3: Neighbour-Joining Trees of each study system. (a) Tuscany system, (b) Aegadian system, (c) Aeolian system.

higher number of private alleles was registered on islands (Table 4.1). The Mantel test, as for the previous system, was not significant (P = 0.65).

Aeolian Archipelago

The PCA revealed a lack of differentiation between all the populations (Fig.4.2c), with more overlap than in the other two systems. The NJ tree showed a well differentiated cluster for Salina and Filicudi populations, while the Stromboli and Vulcano populations are closer to the Milazzo cluster (Fig. 4.3c). The pairwise population F_{ST} differences ranged from 0.085 to 0.245. A low differentiation was detected between Milazzo-Vulcano and Milazzo-Stromboli populations pairs, and moderate differentiation in the other population pairs. Remarkably, the population of Vulcano shows a lower genetic distance to Stromboli in comparison with genetic differentiation among other closer islands (Table 4.2). The Aegadian system shows a homogeneous number of alleles across all the populations and values of expected heterozygosity higher than the observed heterozygosity for all the populations. As for the other two systems, a higher number of private alleles was registered on islands (Table 4.1). The Mantel test between genetic distance and geographic distance was not significant (P = 0.64).

Discussion

The Mediterranean biota has a complex geological and biogeographic history which contributed to the rich biodiversity and regional heterogeneity. Since the Neolithic, humans

Table 4.2: Pairwise F_{ST} values (below diagonal) and P-values from bootstrapping (above diagonal) between populations of each system.

	Islands	*Giannutri*	*Argentario*	*Giglio*	*Talamone*	*Formica*	*Capalbio*
Tuscany	*Giannutri*		0	0	0	0	0
	Argentario	0.521		0	0	0	0
	Giglio	0.676	0.664		0	0	0
	Talamone	0.524	0.061	0.668		0	0
	Formica	0.712	0.535	0.776	0.528		0
	Capalbio	0.512	0.053	0.661	0.042	0.532	
	Islands	*Favignana*	*Levanzo*	*Birgi*	*Erice*	*Marettimo*	
Aegadian	*Favignana*		0	0	0	0	
	Levanzo	0.159		0	0	0	
	Birgi	0.241	0.246		0	0	
	Erice	0.193	0.169	0.089		0	
	Marettimo	0.175	0.214	0.139	0.131		
	Islands	*Filicudi*	*Salina*	*Milazzo*	*Vulcano*	*Stromboli*	
Aeolian	*Filicudi*		0	0	0	0	
	Salina	0.211		0	0	0	
	Milazzo	0.221	0.191		0	0	
	Vulcano	0.228	0.194	0.085		0	
	Stromboli	0.198	0.245	0.143	0.176		

also started to play a role both as vector of dispersion and as homogenizers of the environment, leading to a decrease of ecological differences between two distinct areas (i.e. native and invaded areas). Therefore, the different biogeographic contexts and patterns of humanization are expected to result in different genetic diversity structures in alien insular populations. As such, our results in the three island-mainland systems of *P. sicula* reflect different roles of humans in lizard distributions, which apparently have promoted homogeneous genetic diversity on two of them.

In Tuscany, all islands, Giglio, Giannutri and Formica, are very well differentiated from mainland populations, Talamone, Capalbio and Argentario, and among each other. This suggests a very old colonization of *P. sicula*, perhaps by natural means, when the islands were still connected to the mainland and between each other, at least in the cases of Giannutri and Formica. Furthermore, the clear separation also suggests that if it was an introduction, such event has not been continuous over time and there is no current gene flow homogenizing the genetic diversity. Here the human role seems disposable, since each island population followed independent evolution from each other and relative to mainland populations.

On the other hand, on the Aegadian system the population structure recovered is weaker and the number of alleles and private alleles is homogeneous between all the populations (either island or mainland). This pattern suggests some level of current geneflow, and so, a recent and ongoing introduction of *P. sicula* on Favignana, Levanzo and Marettimo islands. Another support for this hypothesis is the presence of one individual from Levanzo and another from Erice closer to Favignana cluster, which suggests a recent introduction by humans. Furthermore, the close genetic affinity between Marettimo and mainland populations, also suggest a human role on the arrival of the species to this island, as pointed by Capula (1994).

Finally, for the Aeolian system, a volcanic archipelago whose islands were never connected to main Sicily or to other islands (Corti and Lo Cascio 2002), there is no clear separation of the populations, but the number of private alleles is higher on islands. This suggests an ongoing invasion, although the immigrants are not so frequent. Here we hypothesize that there was an historical introduction of *P. sicula* on Vulcano, Salina, Stromboli and Filicudi islands, which has been followed by some occasional translocations. The close genetic relationship between Stromboli and Vulcano and mainland populations rather than to Salina island, which is geographically closer, gives insights on the active role humans could have been playing on this system.

Remarkably, levels of genetic diversity are not so different between mainland and islands, with exception of Tuscany. Predictions regarding alien species propose that introduced populations will have lower overall levels of genetic diversity in relation to native range (Michaelides et al. 2017). However, in our study systems, island populations of *P. sicula* do not show signature of strong bottleneck as we might have expected. The same genetic diversity of island populations compared to mainland populations could be due to the recurrent introductions from multiple sources, which would counterbalance founder effects and promote higher levels of genetic diversity. Alternatively, hybridization with native species may have added new genetic background to face the new environment (Lombaert et al. 2014), at least for Marettimo and Vulcano islands where are supported evidences from allozyme data of hybridization between *P. wagleriana* and *P. raffonei* (Capula 1993).

A look over the three systems shows us three different colonization stories but these probably are related to each other in a continuum from natural colonization to human-mediated introductions. Tuscany may represent what can be seen from a natural colonization, Aegadian a recent invasion and the Aeolian somewhere between the two an historical introduction with punctual events of invasion occurred more recently. The most recent phylogeographic study of *P. sicula* (Senczuk et al. 2017) shows a monophyletic relationship between Marettimo and Favignana and a polyphyletic relation between all the Aeolian islands (this study: Vulcano, Salina, Stromboli, Filicudi), which suggests independent invasion events on Aeolian system, but not in the Aegadian where Marettimo and Favignana are more closely related. The Aeolian islands, as referred before, were never connected between each other, and the same applies to Marettimo and Favignana.

Regarding Aeolian systems, the human colonization followed the resource availability (i.e. food, obsidian, fertile soil) and so, the islands were colonized on different events (Dawson 2005 and references within). Salina and Filicudi were colonized first and in the same wave by early Neolithic Stentinello, while the others were colonized in independent events, namely Stromboli during Early Copper Age/Middle of 4th millennium BCE and Vulcano was visited during Bronze Ages, but was only occupied by the 18th century, when the volcanic activity decreased, and the island became more suitable for living of men. These differences in colonization by humans in timing can be also precepted as genetic signature in *P. sicula*. In fact, we can recover this same colonization pattern in our genetic data: Salina and Filicudi populations of *P. sicula* seemed to be introduced much earlier than Stromboli and Vulcano islands which are more closely related to mainland, Milazzo. An indication that probably *P. sicula* took a ride from first settlers during their occupation of Aeolian Archipelago.

For Egadi islands, the history seems to be different. The human colonization was made in one wave for all the three islands by the 4th millennium BCE (Dawson 2005). Therefore, there is no congruence with our results. If *P. sicula* individuals colonized the islands at the same time of humans, it would be expected three independent clusters corresponding to the three islands. However, Marettimo cluster is closer to mainland populations than to the other islands, and a couple of individuals from other populations were found in Favignana. All together supports a more recent invasion of the Aegadian than of the Aeolian archipelago. It is difficult to infer the first invasion time, however since 1995, a ferry boat is linking frequently the islands to mainland and to each other, which certainly increase the hitchhiking probability of *P. sicula* and, maybe, justifies the Marettimo pattern as well as the individuals out of their cluster.

In summary, our study allowed disentangling the signals of natural and human-mediated colonization in Tuscany and suggests a recurrent colonization scenario for the other two systems with humans playing a key role on the distribution of *P. sicula*. The vectors by which the lizards were introduced in the islands in historical times and nowadays would more probably be hitchhiking with cargo or material from the native range. As referred before, this species inhabits human altered environments and therefore is prone to be transported in human-manufactures.

This case study reflects the complexity of the history of the Mediterranean biota. As proposed by our other study (Chapter 2), the biogeography of the Mediterranean is a mix between human actions, geographic and species traits factors. Humans have been influencing species distribution since ancient times, introducing and re-introducing species repeatedly, blurring their genetic signature, and consequently, their invasion history. In this case, we can infer that we have a mixed signal of human introductions and the ability of the lizard to colonize the islands naturally, due to the lower sea levels during the Last Glacial Maximum (LGM). Overall, our results illustrate the intrinsic complexity of colonization dynamics in insular systems with complex biogeography and long history of human influence (Chapter 2). Analysis on the invasion routes, such ABC or Site Frequency Spectrum approaches, are needed to clarify introduction time as well as the invasion routes. Complementarily, analysis on genes under selection would be important to distinguish between demographic and selection processes and, so, clearly elucidate the genetic signature of the alien lizard.

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Chapter 5

Shifts on alien systems of *Podarcis sicula*

Silva-Rocha, I, GF Ficetola, D Salvi, G Llorente and MA Carretero. in prep. “Life trait shifts and genetic variation across insular systems by the opportunistic lizard *Podarcis sicula*.”.

Abstract

The introduction of species outside their native range provides an opportunity to study how those species are able to change and be successful on the new environment. The nature of those shifts can be either plastic (phenotypic plasticity) or adaptive (local adaptation), and the discernment among them is important to understand the alien species success. In the Mediterranean region, the introduction of species occurred from ancient times until nowadays, determining a mix pattern of ancient and recent introduction within the basin. Such a complex history and the different environmental conditions make the Mediterranean basin a unique system to study these issues. In this study, we investigate the shifts in phenotype and genotype associated to biological invasions on islands by mainland populations of the Italian wall lizard, *Podarcis sicula*. This species has been introduced in different insular systems of the Mediterranean region and is also able to change rapidly its morphology and ecological habits when introduced in a novel environment. Three different systems composed by alien (islands) and native (mainland) populations, with different colonization times, were chosen: Tuscany, Aeolian and Aegadian. A combination of three different approaches was made to understand the different shifts and the connection among them. A stable isotope and morphologic (SVL) analysis were used to infer shifts on trophic niches and on body size, respectively, from native to alien insular populations. Additionally, an outlier loci analysis was also performed to detect signs of selection on the genome of insular populations. Tuscany system reveal a different pattern relative to the Aeolian and Aegadian systems. Islanders of Tuscan archipelago show the broadest trophic niche, the highest size shifts, and strong signal of selective pressure at multiple loci. These results are consistent with the hypothesis that lizards from Tuscany have been evolving longer time in insular conditions (time-for-evolution hypothesis), and the other two seem to be historical or recent introductions with some ongoing geneflow. This work confirms the *P. sicula*'s ability to change under different conditions, but also demonstrate that such ability is constrained by the evolutionary time and gene flow from source populations.

Keywords Alien species, diet, trophic niche, selection, stable isotopes, ddRAD, Mediterranean islands

Introduction

The introduction of a species in a new environment will expose it to new environmental and biotic conditions. Introduced species can successfully acclimate or adapt and successfully spread in this new environment (García-Ramos and Rodríguez 2002). This provides scientific community the opportunity to study evolution on live time, by questioning what changes occurred and how.

Alien species may either have all needed in their genotype to withstand in the new environment and can express different phenotypes according to the new requirements (i.e. phenotypic plasticity). Alternatively, natural selection can determine genotypic shifts that will allow alien species to acquire new phenotypes needed to survive and succeed under the new conditions (Dlugosch & Parker, 2008; Ghalambor, McKay, Carroll, & Reznick, 2007). If the alien species have already phenotypic traits allowing to deal with new conditions, the invasion and colonization of new environments will be probably facilitated and faster (i.e.

phenotypic plasticity is more immediate than natural selection processes) (Richards et al. 2006), even if genetic diversity is low. On the other hand, a genetic paradox often occurs during biological invasions, since species overcome bottleneck and founder effects, and they are forced to respond to new environmental pressures in the novel area despite their limited genetic variation (Dlugosch et al. 2015). Other authors suggest, however, that loss on neutral genetic diversity is not the main issue, instead the loss on adaptive genetic variation will be limitative since natural selection requires genetic variation to act on (Lee 2002). Nevertheless, biological invasions are a complex phenomenon and multiple introductions can contribute with new sources of diversity and increase standing genetic variation, as shown by several studies (Kolbe et al. 2007; Rius and Darling 2014; Wagner et al. 2016). Hybridization is also a process that would also facilitate the establishment and success of the alien species, by incorporating parts of the native species genotype, which is already locally adapted, within the alien genotype (Mesgaran et al. 2016; Roy et al. 2015).

The Mediterranean region is one of the global biodiversity hotspots (Myers et al. 2000), with Mediterranean islands sharing common species with the mainland but also with high levels of endemism due to its complex history and isolation periods. The Messinian Salinity Crisis (5.9-5.3 Myr ago) with the consequent desiccation of the Mediterranean Sea, and the Pleistocene climatic oscillations with glacial and interglacial episodes were the main geological and climatic events with a huge impact on the Mediterranean biota (Blondel et al. 2010a). However, since the Neolithic and probably earlier, humans also had a great influence on Mediterranean biota, with multiple impacts on land-use, resource availability and biota composition. This region was the meeting point of several civilizations which used the islands as hubs for their commercial networks and nowadays is an appealing touristic destination which attracts a lot of people and moves a lot of boats around. All this movement started and increased the biological invasions phenomena, and species are being introduced either on purpose (e.g. as a food resource) or by accident (e.g. as stowaways among cargos), endangering the endemic and native species already present in the islands (Ficetola and Padoa-Schioppa, 2009; Kraus, 2009). The long interaction history between humans and biota causes a mix of ancient and recent introductions which turns difficult to disentangle which are the alien species from the native ones (Blondel et al. 2010b), but also it may have given time enough for a number of alien species to adapt to different systems.

The wide array of environmental conditions and colonization times within the Mediterranean basin provide a unique system which deserves to be analysed in deep.

In this study, we investigate the deterministic or reliant nature of the shifts in phenotype and genotype associated to biological invasions of islands by mainland populations. To this purpose, we focus on a representative species which has been repeatedly introduced in different insular systems and is abundant enough to be sampled and studied. The Italian wall lizard is an opportunistic species native of the Italian peninsula, Sicily and North Adriatic coast, and it has been introduced in several islands of the Mediterranean basin (Senczuk et al. 2017). It is still not clear whether the presence of this species in some islands and islets surrounding the Italian Peninsula and northwest Adriatic Coast is due to natural colonization or human-mediated colonization, due to the ancient presence of human activities in these islands (Blondel, Aronson, Bodiou, and Boeuf, 2010; Chapter 4 of this thesis) and the temporary connection between these islands and the mainland during Ice Ages. It is well-established that this species is very prone to be accidently introduced because of its the closeness to humanized areas and the use of human-manufactures and ornamental trees as a refuge (Corti and Lo Cascio 2002). Studies by Kolbe et al., (2013) and Silva-Rocha et al. (2012, 2014) support a multiple introductions source pattern, which reflects the ability of *P. sicula* to establish in new areas. This species displays phenotypic shifts when successfully invading new environments. For example, Herrel et al. (2008) and Vervust et al. (2010) took advantage of an experimental introduction in the 70s (Nevo et al. 1972) on two islands of Adriatic coast (Pod Mrcaru and Pod Kopiste) to study morphological and physiological variation between insular and mainland lizards and concluded that those in Pod Mrcaru islet display a number of phenotypic changes associated to feeding on greater proportion of plant material. However, Vervust et al. (2010) experiments suggested that some of the changes are based on phenotypic plasticity, and not on local adaptation. These studies provide an excellent understanding on the ability of *P. sicula* to change in new environments and has revealed a phenotypic flexibility that can be advantageous to its establishment and colonization success.

The aim of this study was to understand how *P. sicula* populations changes in genotype and phenotype as a result of introduction to different Mediterranean islands, as well as on whether changes are consistent across islands or are intrinsic of each insular system. Insular ecosystems are known to drive specific changes in lizard populations (Novosolov, Raia, and Meiri 2013). Therefore, some of our hypothesis fall in the natural shifts that species would experience after a natural island colonization. To understand whether these shift are observed in the phenotype or genotype of *P. sicula*, we: 1) used stable isotope analysis to infer the trophic niche changes both in the native (mainland) and in the alien (islands) populations; 2) analyzed morphology (overall size) to check for differences among native and alien populations; and 3) we used next generation sequencing (ddRAD) to test for the presence of loci under selection in insular populations. The integration of these data will allow to clarify hypothesis, such: (i) alien populations will have a wider trophic niche due to increased herbivory and cannibalism on islands; (ii) alien populations will be tendentially bigger; (iii) on systems with lower selective pressure, the shift will be more based on phenotypic plasticity, since adaptation will take more generations to evolve; (iv) genes under selection will be similar in all systems. Hypotheses (i) and (ii) are intrinsically connected to the island syndrome (Raia et al. 2010; Monti et al. 2013; Carretero 2004; Novosolov, Raia, and Meiri 2013; Damme 1999) that states that the stable environmental conditions, the low predation pressure and high population densities will decrease fecundity, aggressiveness and sexual dimorphism, and, consequently, lizards will intake the food resources available and allocate more energy in body growth. It is expected that an alien species when introduced in an island will also find a predictable environment and low predation pressure, and so, it will respond in the same way. Hypothesis (iii) and (iv) are in concordance with the theory that once the species colonizes a new environment, a rapid initial increase in phenotypic plasticity accelerates evolution of a new optimal phenotype, followed by slow genetic assimilation of the new phenotype, reduction of plasticity and increase of local adaptation (Lande 2015).

Material and Methods

Study systems

We sampled lizards from three independent populations systems composed of islands and closest native range on mainland Italy and Sicily (Sicily will be from here on referred also as mainland due to its size) (Fig. 4.1 Chapter 4): (1) Tuscany system: three islands

(Giannutri, Formica and Giglio) and three mainland populations (Talamone, Capalbio and Argentario); (2) Aegadian system: three islands (Favignana, Levanzo and Marettimo) and two mainland populations (Erice and Birgi); and (3) Aeolian system: four islands (Filicudi, Salina, Stromboli and Vulcano) and one mainland population (Milazzo).

Some of these islands were connected to mainland during Last Glacial Maximum (LGM), namely Giannutri and Formica from Tuscan archipelago, and Favignana and Levanzo from Aegadian. Therefore, on these islands, the presence of *P. sicula* can be the result either of a natural colonization or of human-mediated introduction (Fattorini 2010). The other islands, especially Aeolian Islands which have a volcanic origin, were never connected to mainland or other islands, hence the existence of *P. sicula* on these islands is assumed to be the results of historical human-mediated introductions (Capula, 1994; Capula, Luiselli, Bologna, & Ceccarelli, 2002). Having in account the results obtained on the Chapter 4 of this thesis, Tuscany insular populations seem to be a result of natural colonization while the insular populations of the other two systems seem to be introduced by humans, in historical and/or recent times.

The climate on all these islands is typical Mediterranean, with hot and dry summers, and mild and wet winters. In Tuscany, colder temperatures and higher precipitation frequencies are expected, compared to in Sicilian archipelagos (Fick and Hijmans 2017).

Sampling

For genetic analyses, a total of 108 tail tips of *P. sicula* individuals were sampled across the three systems: 48 individuals on Tuscany system (September 2014), 30 individuals on Aeolian and 30 individuals on Aegadian (August 2015). For diet analyses, we sampled a total of 319 lizards in the three systems: 107 individuals on Tuscany, 103 individuals on Aegadian and 109 individuals on Aeolian. Tail tips collected from these 319 lizards were also stored in pure ethanol. Additionally, in each system we simultaneously collected potential food sources (plant material and arthropods) and stored them in pure ethanol. All lizards were measured for snout-vent length (SVL) using a caliper (0.01 mm of precision) and released after examination. The sampling was made on the late summer, since it is the part of the season when lizards have more scarce food resources, allowing us to more accurately verify dietary shifts. Detailed information on sampling size for each population for each analysis is reported in Table 5.1.

Table 5.1: Sampling size for each population and for each analysis performed on this study. SVL: Snout-Vent Length; ddRAD: Double-digested Restriction Associated DNA; M/F: Males/Females - Number of males and females on SVL analysis.

System	Ecosystem	Alien status	Population	Stable Isotopes and SVL	M/F	ddRAD
Tuscany	Mainland	Native	Talamone	19	11/8	8
	Mainland	Native	Argentario	11	7/4	8
	Mainland	Native	Capalbio	19	10/9	8
	Island	Alien	Formica	18	8/10	8
	Island	Alien	Giglio	16	10/6	8
	Island	Alien	Giannutri	24	18/6	8
Aegadian	Mainland	Native	Erice	20	15/5	6
	Mainland	Native	Birgi	24	12/12	6
	Island	Alien	Favignana	20	12/8	6
	Island	Alien	Marettimo	21	12/9	6
	Island	Alien	Levanzo	18	11/7	6
Aeolian	Mainland	Native	Milazzo	19	13/6	6
	Island	Alien	Vulcano	25	15/10	6
	Island	Alien	Salina	19	13/6	6
	Island	Alien	Stromboli	24	16/8	6
	Island	Alien	Filicudi	22	18/4	6

Stable Isotopes laboratory and analysis

Stable isotope values of Nitrogen and Carbon were measured for each lizard tail tip and food source. In general, stable isotopes reflect the diet over the period during which the tissue analyzed was formed. Stable isotope values of Nitrogen (δ15N) provide information regarding the trophic level of the organism and Carbon (δ13C) refers to the primary sources (Fry 2006).

All samples (tail tips and potential food samples) were dried and homogenized before isotope analysis. The homogenization was manual by crushing to a fine powder. Isotope analysis was conducted at the Serveis Cientifico-Tècnics (University of Barcelona, Spain). Weighed subsamples of the powered tail tips and food sources were placed in tin capsules. N and C isotope analysis was carried out using an elemental analyzer (Flash EA 1112) coupled to stable isotope ratio mass spectrometry equipment (CF-IRMS). The laboratory uses international standards, which are generally run after every 12 samples: IAEA CH7 (87% C), IAEA CH6 (42% C) and USGS 24 (100% C) for 13C; IAEA N1 and IAEA N2 (21% N) and IAEA NO3 (13.8% N) for 15N. The international standards for N and C are atmospheric nitrogen (AIR) and Pee Dee Belemnite (PDB), respectively. Precision was 0.1%

and 0.2% for δ13C and δ15N, respectively.

Trophic niche width was estimated using a Bayesian approach based on multivariate ellipse-based metrics. In particular, we calculated standard ellipse areas (SEAc) for each population within each system following methods from Jackson, Inger, Parnell, & Bearhop (2011) which provides a bivariate measure of mean core isotopic nice, using the R package *SIBER*. This Bayesian approach accounts for uncertainty in data sampling and propagates the error through to derived metrics. This software estimates trophic niche widths of species from variable and small sample sizes by measuring a sample-size corrected standard ellipse for each species. Furthermore, we used Layman metrics: Nitrogen (dN) and Carbon (dC) ranges which provide a univariate measure of the total nitrogen and carbon ranges exploited by a species, and the mean distance to the centroid (CD) which provides a description of trophic diversity (Layman et al. 2012). A t-test was performed to test the differences significance for the standard ellipse areas (SEAc) obtained and for each Layman metrics, using the function *ttest* in R. For the SEAc a t-test was made between each pair of populations within the study system.

For diet inference, we used the Bayesian mixing model software packager *MixSIAR* (Stock et al., 2018) which gives us the contribution of different sources of food to the isotopic composition of *P. sicula*. The Markov Chain Monte Carlo parameters were set at the very long test length (chain = 1,000,000; burn = 700,000; thin = 300; chains = 3). We used the Gelmen-Rubin and Geweke diagnostics to assess convergence of all mixing models (Stock and Semmens 2016). We used generalist (non-informative) priors which assume that all combinations of proportions of diet items are equally likely. In short, MixSIAR models use isotopic values and discrimination factors to account for the organisms assimilated diet and thus estimate what portions of the diet compose its separate or combined tissues (Parnell et al. 2010).

Morphological analysis

Snout-vent length (SVL) is one of the most common measure of lizard size, since total length includes tail length which may change in response to selective pressures or just due to tail loss behavior (Meiri, 2010). To test for differences on SVL among ecosystems (Eco:

mainland/island), Populations (Pop) and lizards gender (Sex: male/female), we used permutational multivariate analysis of variance (perMANOVA) (Anderson 2001) with the *adonis* function in the Vegan package in R (Oksanen et al. 2017), with a total permutations number of 9999. PerMANOVA, is a non- parametric method for multivariate analysis of variance based on a multivariate analogue to Fishers F-Ratio and using subsequent permutations to calculate the P-value (Anderson 2001). A nested hierarchical design was used with Populations within Ecosystems.

ddRAD analysis

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturers protocol. DNA was eluted with 100 μL of Buffer AE and then concentrated up to values required by library preparation procedures. DNA quantification was performed using a Qubit Fluorometric Quantitation (ThermoFisher Scientific). A double-digested RAD (Restriction site-associated DNA) experiment was conducted on the 108 individuals sampled for genomic purposes, using a modified version of the protocol as described in Chapter 4.

The bioinformatic analysis of demultiplexing and filtering the sequencing results was the same used in Chapter 4. In summary, we used Stacks pipeline (Catchen et al. 2013) to demultiplex and sort by barcode, to perform a de novo analysis to get RAD-tags and to filter the Single Nucleotide Polymorphisms (SNPs). VCFtools was then used to split the dataset into each system and in each ecosystem (island/mainland) within each system, with a minimum allele frequency of 0.05.

The approach used to test the selective pressure in each system was that one implemented in PCAdapt (Luu, Bazin, and Blum 2017) which uses PCA to control for population structure without priori population definition. Simulations showed that PCAdapt is less prone to type 2 errors and is less affected by the presence of admixed individuals in the samples (Capblancq et al. 2018). The optimal number of principal components necessary to describe population structure was determined using the graphical method described in Luu, Bazin, & Blum (2017) and varying K from 1 to 10. First, a test on each system was made to find the total loci under potentially under selection and, afterwards, a comparison was made between islands outlier loci of each system, in order to find common outlier loci among them.

These tests will give further support to the other phenotype results, allowing to infer the role of genetics on the success of the alien lizard.

Results

Stable Isotopes

In the Tuscany system, lizards from Formica and Giannutri islands are those with the widest Standard Ellipse Area (SEA) (Fig. 5.1), followed by mainland populations and Giglio (Table 5.2). Regarding Layman metrics, the results from island lizards showed a higher N range and mean distance do centroid (CD), and a lower C range (Table 5.3). Concerning diet proportion of each population, lizards from islands showed an isotope profile corresponding to a diet more based on arthropods and lizards from mainland showed a generalist diet (Appendix G).

In the Aegadian system, SEA was significantly higher in the mainland population of Birgi, followed by the insular populations of Levanzo, Marettimo and Favignana and the mainland population of Erice (Fig. 5.1, Table 5.2). Layman metrics suggested a lower N range and mean distance to centroid, and a higher C range for the islanders (Table 5.3). The diet proportion analysis revealed a similar diet between islands and mainland lizards, more plant based on both cases (Appendix G).

Finally, the results from Aeolian system showed a wider SEA for all the island populations when compared to mainland population (Fig. 5.1, Table 5.2). The Layman metrics were only possible to calculate for the islands since we only have one mainland population (Table 5.3). Regarding diet composition, the island lizards from Salina and Stromboli seemed to feed more on arthropods while the lizards from Filicudi and Vulcano fed more on plant material (Appendix G).

Body size

On Tuscany, mainland lizards have smaller body size (SVL) of 6.47 ± 0.91 cm than the islanders with 7.08 ± 0.68 cm (Fig. 5.2). The same can be observed on Aegadian system, with mainland populations with 7.07 ± 0.76 cm and island populations with 7.19 ± 0.67 cm

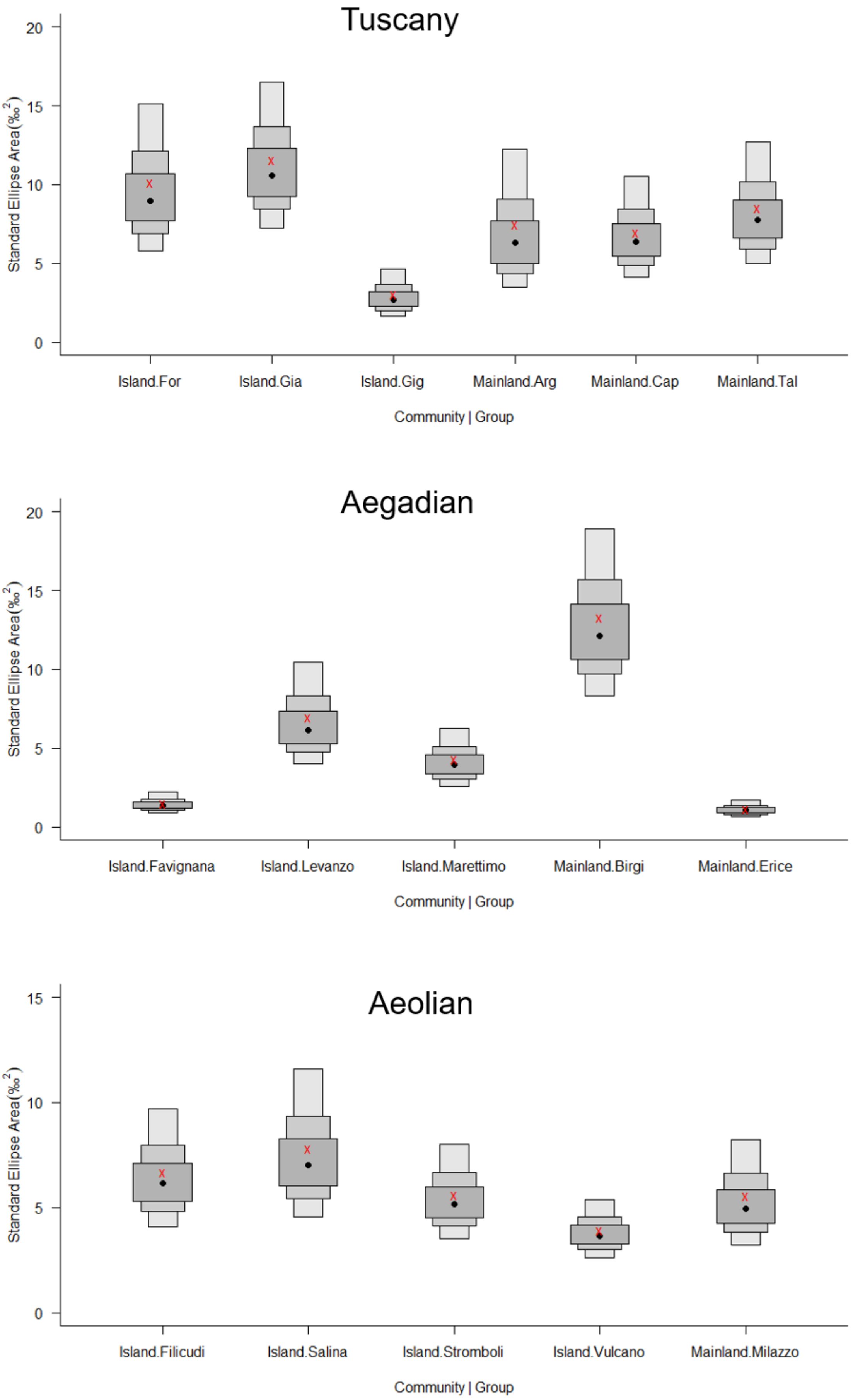

Figure 5.1: Standard Ellipses Area (SEAc) for each population within each system analysed on this study.

Table 5.2: T-test results on Standard Ellipse Areas (SEAc) for each pair of populations within each system. Tuscany: For Formica, Gia Giannutri, Gig Giglio, Arg Argentario, Cap Capalbio, Tal Talamone. Aegadian: Fav Favignana, Lev Levanzo, Mar Marettimo, Bir Bigi, Eri Erice. Aeolian: Fil Filicudi, Sal Salina, Str Stromboli, Vul Vulcano, Mil Milazzo.

System	Pop1	Pop2	Mean Pop1	Mean Pop2	t value	p value
Tuscany	For	Gia	10.045	11.53	-26.644	<2.2e-16
	For	Gig	10.045	3.03	168.81	<2.2e-16
	For	Arg	10.045	7.363	49.075	<2.2e-16
	For	Cap	10.045	7.105	61.23	<2.2e-16
	For	Tal	10.045	8.492	30.525	<2.2e-16
	Gia	Gig	11.53	3.03	205.34	<2.2e-16
	Gia	Arg	11.53	7.363	76.427	<2.2e-16
	Gia	Cap	11.53	7.105	92.433	<2.2e-16
	Gia	Tal	11.53	8.492	59.888	<2.2e-16
	Gig	Arg	3.03	7.363	-108.62	<2.2e-16
	Gig	Cap	3.03	7.105	-135.07	<2.2e-16
	Gig	Tal	3.03	8.492	-158.28	<2.2e-16
	Arg	Cap	7.363	7.105	5.5393	3.14E-08
	Arg	Tal	7.363	8.492	-22.817	<2.2e-16
	Cap	Tal	7.105	8.492	-32.985	<2.2e-16
Aegadian	Fav	Lev	1.504	6.952	-194.67	<2.2e-16
	Fav	Mar	1.504	4.316	-165.64	<2.2e-16
	Fav	Bir	1.504	13.259	-262.92	<2.2e-16
	Fav	Eri	1.504	1.157	49.598	<2.2e-16
	Lev	Mar	6.952	4.316	82.934	<2.2e-16
	Lev	Bir	6.952	13.259	-120.92	<2.2e-16
	Lev	Eri	6.952	1.157	208.76	<2.2e-16
	Mar	Bir	4.316	13.259	-189.55	<2.2e-16
	Mar	Eri	4.316	1.157	190.3	<2.2e-16
	Bir	Eri	13.259	1.157	271.54	<2.2e-16
Aeolian	Fil	Sal	-26.621	6.769	7.804	<2.2e-16
	Fil	Str	36.69	6.769	5.639	<2.2e-16
	Fil	Vul	104.52	6.769	3.939	<2.2e-16
	Fil	Mil	38.969	6.769	5.514	<2.2e-16
	Sal	Str	60.505	7.804	5.639	<2.2e-16
	Sal	Vul	118.78	7.804	3.939	<2.2e-16
	Sal	Mil	62.035	7.804	5.514	<2.2e-16
	Str	Vul	75.919	5.639	3.939	<2.2e-16
	Str	Mil	4.406	5.639	5.514	<2.2e-16
	Vul	Mil	-65.184	3.939	5.514	<2.2e-16

Table 5.3: Results of SIBER analysis and correspondent t-test p value on Layman metrics for each system. There is only one mainland population in Aeolian, therefore it was not possible to calculate Layman metrics. N: N range - trophic level; C: C range - basal diversity; CD: Centroid distance - trophic diversity.

System	Ecosystem	N	p value	C	p value	CD	p value
Tuscany	Island	9.14	<2.2e-16	1.06	<2.2e-16	3.84	<2.2e-16
	Mainland	2.61		1.55		1.23	
Aegadian	Island	2.42	<2.2e-16	1.75	<2.2e-16	1.21	<2.2e-16
	Mainland	9.02		0.50		4.51	
Aeolian	Island	4.62		1.64		1.86	

of SVL (Fig. 5.2). These differences are all significant (Table 5.4). For Aeolian, the same SVL was found for both ecosystems, 7.06 cm (Fig. 5.2). For all the islands, the population with higher SVL is insular and in the same order of length across systems, namely Formica (Tuscany) with 7.39 ± 0.76 cm, Favignana (Aegadian) with 7.37 ± 0.74 cm and Vulcano (Aeolian) with 7.33 ± 0.81 cm. While the populations with lower SVL are from the mainland if we look to Tuscany (Talamone 5.87 0.85 cm) and to Aegadian (Birgi 6.89 ± 0.82 cm), and from another island in the Aeolian system (Salina 6.65 ± 0.76 cm) (Fig. 5.2). Only on Tuscany and on Aeolian was possible to identify a significant relationship between SVL and Populations (Table 5.4). Regarding sexual dimorphism across body size, male lizards are consistently bigger than females in all the systems, but sexual size dimorphism remains similar across populations and systems (Appendix H, 5.4).

Outlier loci

Results revealed a higher number of outlier loci along the genome, and so a higher selective pressure, for Tuscany system, followed by Aeolian and Aegadian systems. Although Aegadian and Aeolian have some outlier loci, both systems have much lower pressure when compared to Tuscany. Additionally, if we compare the number of shared outlier loci, only Tuscany share outlier loci with the other two systems, while neither Aegadian nor Aeolian share between each other (Table 5.5).

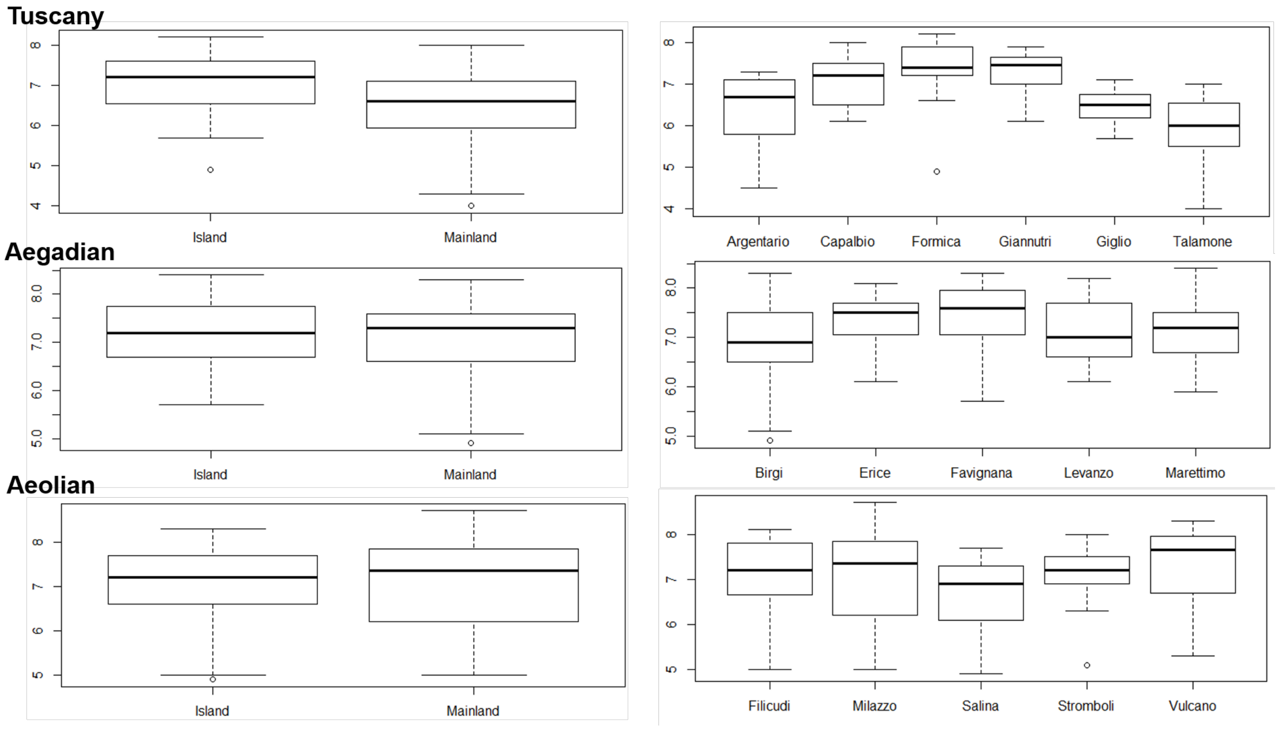

Figure 5.2: Snout-Vent Length (SVL) of each system comparing ecosystems (island/mainland) and populations.

Table 5.4: PerMANOVA results of Snout-Vent Length (SVL) for each study system. Formula: SVL Eco/Pop * Sex. Number of permutations: 9999. * Significant under α = 0.05.

System	Variables	Df	F model	R2	Pr(>F)
Tuscany	Eco	1	26.2042	0.1242	0.0001*
	Sex	1	18.4317	0.0873	0.0001*
	Eco:Pop	4	15.7249	0.2981	0.0001*
	Eco:Sex	1	0.9550	0.0045	0.3375
	Eco:Pop:Sex	4	1.1337	0.0215	0.3451
	Residuals	98		0.4644	
	Total	109		1.0000	
Aegadian	Eco	1	1.290	0.0080	0.2625
	Sex	1	60.442	0.3770	0.0001*
	Eco:Pop	3	1.024	0.0192	0.3860
	Eco:Sex	1	0.038	0.0002	0.8422
	Eco:Pop:Sex	3	0.830	0.0155	0.4840
	Residuals	93		0.5800	
	Total	102		1.0000	
Aeolian	Eco	1	0.074	0.0005	0.7863
	Sex	1	39.154	0.2709	0.0001*
	Eco:Pop	3	2.848	0.0591	0.0392*
	Eco:Sex	1	0.250	0.0017	0.6184
	Eco:Pop:Sex	3	0.833	0.0173	0.4840
	Residuals	94		0.6504	
	Total	103		1.0000	

Table 5.5: Number of total outliers for each system and comparison of number of outliers among different systems.

System	Total outliers	Comparison with other systems	Common outliers
Tuscany	918	Aegadian/Aeolian	7/15
Aegadian	42	Aeolian	0
Aeolian	106	Aegadian	0

Discussion

Understanding how the phenotype of an alien species changes in response to a new environment and which is the subjacent genotypic shifts, if any, gives important insights on the short-term adaptive processes of species and on the ecological dimensions involved. The lizard *P. sicula* is a demonstrated successful invader and this work is one more evidence of the ability to change and flourish in a wide array of environment conditions across multiple island systems.

One of our hypotheses is that alien (insular) populations will have wider trophic niches, due to increased herbivory and cannibalism (Damme 1999; Carretero 2004). This hypothesis was only partially confirmed, since Aegadian system lizards showed a narrower trophic niche on alien populations (i.e. islands). In this system, introduced lizards have a lower trophic level, feeding on a higher diversity of plant-sources, and lower trophic diversity. For the other two systems, the opposite pattern arises. A wider trophic niche is observable on Tuscany and Aeolian introduced populations (i.e. islands). Lizards on these populations have a higher trophic level and feed on a higher diversity of resources, not only the plant-based ones. In the case of the Aeolian archipelago, *P. sicula* arrival seems to follow the colonization of the archipelago by humans (Dawson, 2005; see Discussion of Chapter 4). According to this hypothesis , the lizards were introduced first in Filicudi and Salina islands, and after in Vulcano and Stromboli. Hence, the lizards with higher trophic niche correspond to the islands where lizards were presumably introduced first (Filicudi and Salina), while the populations from the islands where *P. sicula* was introduced more recently (Vulcano and Stromboli) have a narrower trophic niche. This supports the idea that the trophic niche is narrow in the beginning of the introduction and after a while the alien species is able to explore the new environment and find and use novel resources. An alternative explanation can be that on Tuscany the environmental conditions are not so harsh, namely the summer is not so dry, providing more food resources to lizards other than plant-resources.

The results of the diet analysis also do not clearly support the studies that lizard populations which become established on islands shift their diet toward a more plant-based diet (Damme 1999; Carretero 2004; Prez-Mellado and Corti 1993). Our results are a mix of patterns, with some island populations feeding more on plants as hypothesized, and with some other island populations feeding more on non-plant resources than expected. This unclear result could be because of missing resources which lizards could feed on. When in the field, we had the chance to observe that some animals are opportunistic and feed even on human-based food, such as bread or fish remains. Cannibalism and small vertebrates are also possible prey (Capula and Aloise, 2011) that were not considered in this study. On the other hand, some authors suggest that diet of lizards is a continuum from carnivorous to herbivorous diet, being the species able to find an equilibrium along this continuum according to environmental pressures, namely scarcity of arthropods on summer or other insular conditions (see Carretero, 2004 and references within). Additionally, this heterogeneity of observed patterns is likely associated with a heterogeneity of environmental conditions across the studied islands.

Body size was another characteristic we used to understand possible variation of *P. sicula* across alien insular populations and native continental populations. The body size is one of the most identifiable traits of an organism and it is related to its functional ecology, life history, morphology and physiology (Slavenko et al. 2016). Our prediction is that lizards from alien insular populations will tend to be bigger than on native range (mainland). This prediction is only supported by Tuscany system, where lizards from alien insular populations are significantly bigger than the lizards from mainland populations. Nevertheless, this trend does not fit the prediction that bigger animals would be more herbivorous, given that on Tuscany alien insular populations lizards seem to feed on a diversity of food sources, from plant-material to possibly cannibalism, due to the high trophic level detected. For Aegadian system only sexual dimorphism was significant, while for Aeolian we have differences between sexes and between populations. Sexual dimorphism seems to be the same in all the systems with larger males than females. Our results do not entirely confirm that body size increase in the lizards is related to the colonization of a new insular environment, and if so, it is the result of a long-term change in the new insular environment and not a short-term reaction by the alien species. Only in the system where *P. sicula* colonized earlier, the Tuscany islands (see Chapter 4), the differences are notable. Furthermore, a recent study on difference on body sizes in islands in general (Itescu et al. 2018), not taking into account any genetic evidence, showed that lizards do not follow any specific rule; in fact body size seem to be more affected by species identity than by island conditions. In the case of *P. sicula* in Aegadian and Aeolian alien populations (islands), we cannot rule out multiple and/or introductions from the mainland (Sicily) or other islands, which will probably emphasize the species identity, and decrease the effect of island conditions.

Finally, our two last hypotheses predict that on systems with lower selective pressure, the shift will be probably more based on phenotypic plasticity, and that genes under selection will be similar in all the systems. Tuscany shows a higher number of outlier loci which might be related to a wider environmental heterogeneity, and thus a wider array of selective pressure. Tuscany lizards likely colonized the islands a long time before the other two systems (Chapter 4), which can reflect predominant adaptive shifts for lizards from Tuscany islands and predominant phenotypic plasticity shifts on the case of Aegadian and Aeolian lizards. While some of the outlier loci are shared between Tuscany and the other two islands systems (Aeolian and Aegadian populations), overall each island system shows a different panel of loci showing sign of selection. This suggests that adaptation to new environments in *P. sicula* is determined by different loci across introduction events.

This study is one of the first ones comparing different systems where the same species was introduced to investigate trends in phenotypic and genetic changes following the colonization of new environments by the lizard *P. sicula*. One pattern that arises is that *P. sicula* can overcome environmental differences and differential resources availability and be successful in all the three systems. Our results suggest that this species can strongly shift diet and body size according to the selective pressures they suffer in new environments and the time they have to evolve. Another pattern that our results suggest is that Tuscany system is somewhat different from the other two in all the features analyzed. The changes and trends detected suggest that lizards from Tuscany island had more time to develop adaptations to the insular environment when compared to Aegadian and Aeolian lizards. This seems to support the hypothesis of Chapter 4 of a natural colonization of Tuscany islands during the Last Glacial Maximum, while for the other two the introduction would be historical or even recent. All the studied shits respond slowly to insular conditions, and time is required so differences can be detected (time-for-evolution hypothesis). Therefore, the expectation is that those differences will be more accentuated in natural colonized or ancient introduced systems, and more indistinct in systems with recent or repeated introductions. On those systems, the populations are still not in equilibrium and ongoing geneflow may be blurring the adaptation signature.

Overall, the results support the success of *P. sicula* and its ability to change in both phenotype and genotype in response to new conditions in small islands but also illustrate that such ability is constrained by the evolutionary pace and geneflow from source populations. Further investigation, namely on other phenotypic traits such us internal anatomy, behavior and physiology and mainly on the complex links between phenotype and genotype in island systems are still needed to elucidate the ecological and evolutionary processes are underlying this success.

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Supporting Information

Additional Supporting Information can be found in the Appendices section.

Appendix G - S5.1: Isoplots for each group of islands (alien) and group of mainland (native) populations, within each system studied.

Appendix H - S5.2: Snout-Vent Length (SVL) of females and males of each system.

Chapter 6

General Discussion

The invasion science is a crossroad where ecology, social sciences, policy and general community meet. The consensus over the best definitions, the best management strategies and the way invasion science should evolve in the future is still under debate (Crowley et al. 2017; Russell and Blackburn 2017; Simberloff et al. 2013; Courchamp et al. 2017; Ricciardi et al. 2017). There is also some debate on whether human-mediated colonization is the same as the natural colonization (Hoffmann and Courchamp 2016; Wilson et al. 2016). In our humble opinion, human-mediated invasions differ in rates and mechanisms when compared to natural colonization processes. The scientific disagreement is partly due to the complexity of the biological invasions phenomena. A clarification and translation of the scientific evidence for all the parties involved (scientist, politicians and society) is nowadays of extreme importance to handle invasions and protect the biodiversity. The main focus of former studies on biological invasions was mainly the origin and impacts of alien species on the native communities, from an ecological point of view (Richardson 2011). Through the years and with the development of new technologies, biological invasions are now considered natural experiments, and scientists are taking advantage of them to solve questions on evolutionary and ecological processes (Meyerson and Simberloff 2017; Sakai et al. 2001; Bock et al. 2015). Nowadays, all the parties involved on invasion science also acknowledge that biological invasions imply a mix of priorities set by moral values, ethics and scientific knowledge, and so, answers to processes of biological invasion are not always black and white. An integrative point of view over the invasion phenomenon is needed, accumulating all the evidences and facts in the table and compromising with moral values and ethics. This thesis born from the need for more knowledge and understanding of biological invasions phenomena in the Mediterranean basin.

The Mediterranean basin has been a meeting point of several civilizations through the years and nowadays is a desired touristic destination. This noticeably makes this region rich for biological invasions, with a mix of ancient and recent introductions (Blondel et al. 2010). The knowledge of the status of the phenomena within the basin is then crucial either to understand invaders or the invasion process, either to define better conservation strategies or prevent multiple invasions.

One distinctive point of this thesis is that we performed studies at multiple scale, allowing the identification of broad patterns that can also be observed on a more narrow spatial scale and by providing different layers of evidence that complement each other and discriminate the invasion process. At the broad Mediterranean scale (Chapter 2), we identified three main factors explaining the distribution of alien reptiles in Mediterranean islands: geographical (distance to native range), species traits (plant-based diet) and human-related (human population size) factors. The information gathered reflect the complexity of the Mediterranean with the mixed signal between own species ability to survive and the human role on shaping species distribution. Additionally, we showed how genetic data is essential to obtain a reliable biogeographic assessment of invasive species, particularly in systems with a long history of human influence. These results gave us some hints on the archipelagos more affected by biological invasions, namely the Balearic archipelago, and one of the species more prone to be alien within the basin, such is the Italian wall lizard *P. sicula*. In Chapter 3, we narrow the scale down to an archipelago scale. This work allowed to assess the origin and potential distribution of the introduced snakes on the Balearic Islands. The Balearic Islands represent an outstanding case within the Mediterranean with more alien reptiles than native (Pinya and Carretero 2011) ones. Moreover, this archipelago has received multiple invasion waves, and we focused on the most recent one involving snakes species. The inferred origin, joined with information from other sources, allowed understanding the main pathway of introduction of alien reptiles in the Balearics namely the nursery trade, in particular of olive trees from SE Spain (Rivera et al, 2011). Nowadays old olive trees are being transplanted as a decorative plant for gardens. Furthermore, the translocation of olive trees is somehow in line with the European Agricultural Policies which encourage the removal of old, less productive, trees (Mateo and Aylln 2012). This trend has been reported also for recent invasions in NE Spain (Rivera et al., 2011). Hence our work supports that ornamental trees transplantation may be an effective vector for biological invasions across the Mediterranean. Moreover, snakes are predators and alien ones represent a great threat for native biota. The pattern found in this work also reveals a serious invasion affecting multiple species at high trophic levels which will be potentially be boosted by the climate change. Finally, on Chapters 4 and 5, we direct our focus on one species, *P. sicula*, studying how a remarkably good invader arrives and evolves in new environments represented by multiple Mediterranean islands in three different island-mainland systems. Both studies confirmed that invasion history of *P. sicula* is complex and tell different histories across the systems. The complexity and differences in invasion history among systems seem to be associated with time and duration of the invasion, reflecting a wide spectrum from a potential natural colonization to a more recent and ongoing introduction. Looking at neutral markers (Chapter 4), we found evidence of ongoing introductions in the Aegadian islands (Levanzo, Favignana and Marettimo) and historical and punctual introductions in the Aeolian islands (Vulcano, Stromboli, Salina and Filicudi), while for Tuscany our results rise doubts on the alien status of the species in Tuscany islands. The outlier loci and phenotypic shifts analyses (Chapter 5) indicates that changes in alien populations are due to a mix of phenotypic plasticity and adaptation, dependent on the time and duration of the invasion as found on the Chapter 4. For Tuscany, a reinforcement of the natural colonization is found, with lizards shifts and higher selective pressure reflecting a time-to-evolve outline. Overall, the different scales of these studies allow to have different perspectives about the same phenomena, and a common pattern arises: the difficulty to clearly understand the processes underlying biological invasions due to the long-term relationship among humans and biota present on Mediterranean islands. All the scales, from Mediterranean basin to species level, reflect humans as a key factor to understand the distribution and the success of alien reptiles. However, species itself can take opportunity of new environmental conditions and the time to evolve and succeed in that new environment, with different degrees of human involvement.

Another distinctive point of this thesis is the integrative approach to understand a phenomenon. Biological invasions, as known, are a very complex phenomenon which involves many biological layers from genome to ecosystems. Each layer has growing properties that cannot be deduced directly by the other. For example, analyzing only the genomic effects in the alien populations of an invasive species is not enough to confirm the impact in the native ecosystems, although it can give insights and have complement information (Simberloff et al. 2013; Sousa et al. 2011; Heger and Trepl 2003).Therefore, the use of different tools and disciplines could allow a better clarification of the integration of those layers in the invasion process. On Chapter 2, a combination of literature review to assess alien status and a modelling approach to determine relevant factors was used to get a clear picture on the alien reptiles of the Mediterranean. The literature review involved the search of manuscripts on genetic data of a determined species and/or island herpetofauna, even on the grey literature, so we could get a database with solid knowledge on the alien status of the species. Additionally, we also complement with a database on biological traits of the species, which is crucial to understand their distribution through the basin. The models alone, without having into account the genetic information provided by phylogeographic studies, would not allow to correctly infer the main drivers and to predict more accurately the islands richer on alien species. This advices against uncritically incorporating data on alien species or ignoring evolutionary patterns in invasion studies. On Chapter 3, we used genetic data to understand from where snakes are coming or came and modelling approaches to predict to where they are going to expand in the future. The origin of the species is essential to prevent repeated introductions by identifying possible sources and the potential expansion is important to implement control measure of population growth. The study of Chapter 5 combines different kinds of data, such stable isotopes signal (diet), morphology (body size) and genomics (selection). The use of the different analysis allows to understand how *P. sicula* colonizes new environment and how it changes in response to that new environment. This information is crucial to prevent new invasions, as well as the expansion of the alien species already introduced. For example, the risk of predation of native fauna by *Rhinechis scalaris* on the Balearic Islands was considered reduced compared to other snakes, since this species on its native range feeds mainly on mammals and birds which are also alien in the archipelago (Carretero and Silva-Rocha, 2015). However, a recent study demonstrated that 40% of the *R. scalaris* diet at Formentera island is composed by the endemic *Podarcis* *pityusensis* (Pinya et al. 2017), a reptile species. This reveals an unexpected potentiality for niche shift during the invasion process, but that could be predicted if having in account other reptile alien species such *P. sicula*. This thesis, and the work of other authors (Vervust et al. 2010; Herrel et al. 2008) demonstrated the ability of *P. sicula* to shift diet and associated phenotypic traits in new environments. Besides, as referred, this species has been introduced in several places, several times, which increases the potential of this species as a model to study this problematic and serve as guideline for other alien species with less information available. The integration of all the approaches on the different studies enable a better support on the results obtained and a better understanding of the factors and processes involved on the invasion, providing a baseline for other scientific studies or even to guide conservation measures to be implemented in the field.

This thesis provides a different perspective over the biological invasions in the Mediterranean while providing some novelties to invasion science. Here are the points to support our statement. First, the biogeographic history of alien reptiles on Mediterranean islands apparently has a weaker human signal when compared to other areas of the world. Classic biogeographic rules affirm that species richness on islands depends on the equilibrium between species extinction, species colonization and speciation. Nowadays, humans are increasing both colonization and extinction rates on islands (Capinha et al. 2015), but also following the general trend of island biogeography by carrying the species to the biggest and closest islands (higher human population densities too). Alien species are conquering and establishing in new islands with human help, while native species still exist on those islands, promoting a co-existence between each other. If we compare the case of reptiles in Mediterranean and of *Anolis* lizards of Caribbean Islands (Helmus, Mahler, and Losos 2014), for example, we have the former with a mix signal among geography, species traits and human-related factors, and the latter a strong human signal as explanatory of *Anolis* distribution. This difference could be due to the human role on each of the cases, as well as the time and source of introductions. In the Mediterranean, human role started in ancient times, and introductions are old and mostly from mainland. While in the Caribbean, humans are playing a role more recently, promoting inter-island introductions in the Caribbean. This has a broader influence for invasion science and how the predictions are made. It suggests that, in the future, the human signal will probably be also camouflaged in the Caribbean, when species reach a kind of equilibrium between human actions and adaptation to the new environment.

Second, the confirmation that the integration of different data allows a clear picture of the processes underlying invasion. As referred before, the use of genetic information on the model formulation on Chapter 2 permits higher predictive power from those models. Most broad-scale analysis studies use unconfirmed information (e.g. Capinha et al., 2017), namely regarding the alien status of a determined species, which can biased the results and conclusions obtained. In Chapter 3, the integration of genetic data and other information sources, such literature and personal observations, allowed to link the snakes invasion to the nursery trade. The nursery trade across the Mediterranean islands is a trending pathway of introduction of reptile alien species, as confirmed by our work and others cited in the Chapter. However, it seems a quite recent trend, when compared to North America and the Caribbean, where the nursery trade is the main pathway of introduction of snakes and lizards (Kraus 2009). The accumulation of lines of evidence, in this case of pathways and vectors of introduction, is of extreme importance to delineate preventive measures. On Chapter 5, the combination of different types of data (morphology, ecology and genomics) revealed phenotypic shifts on the alien populations analyzed and provided the distinction between adaptation and phenotypic plasticity. This information is important, not only to understand the invasion mechanisms, but also to design effective management measures and define priorities.

Finally, this thesis provides specific knowledge on the case studies that can be used to frame better conservation strategies. The study developed on Chapter 3 had a great impact on the media (Dirio Digital 2015; Ultima Hora 2015; El Confidencial 2015) and the main conclusions were forwarded to the Balearic administration. The information was incorporated into conservation actions, namely for snake eradication, monitoring and prevention of new introductions. The most evident conservation project where the work served as baseline is a project on native lizards in Ibiza. The global aim of the project is to control and evaluate the possibility of eradication of snakes populations in Ibiza, by controlling different core populations and avoid spatial connectivity among them (Montes et al. 2015; Hinckley et al. 2017). This project involves not only the scientific community, but also a collaboration with local authorities and the local people, which has been revealed has an effective way to engage local people to protect their native species. The evidences collected in this thesis provide the indication of some core populations which support the project directly and the probable origin of the imported olive trees which will allow to define a prevention plan in the future. Additionally, the ecological niche models give indication on how snakes will expand in the islands, promoting a fast action since climate change will increase islands suitability and informing about potential places to search for snakes' populations. Finally, as a final output and with a public awareness aim, the study presented here and the project developed in Ibiza joined in a book chapter of the book Histories of Bioinvasions in the Mediterranean (Silva-Rocha et al. 2018).

In conclusion, the thesis reveals the complexity of biological invasions phenomena. All the studies presented in this thesis brought new questions still to be answered. Regarding Chapter 2, a validation of the model with fieldwork and genetic analysis should be conducted on the islands where species are predicted to be alien by our model, in order to confirm if our inference is correct and if it can be applied to other groups. For Chapter 4, a deeper analysis should be done, for example using analytical approaches such as the ABC (Csilléry et al. 2010; Boissin et al. 2012), to understand the colonization history; how many lizards were introduced and following which colonization scenario. For this kind of analyses additional samples would be necessary, namely an additional population on Sicily close to Milazzo and additional samples from then Tuscany archipelago (e.g. Elba and Montecristo islands). Finally, for Chapter 5, since trophic niche is already inferred by stable isotope analysis, food assimilation can be performed to test the efficiency in digestion on the alien and native ranges, and how much food lizards would need to be successful in the alien ranges (Vervust et al. 2010; Karameta et al. 2017; Pafilis et al. 2016). Sampling is needed on native range of the Aeolian system (Sicily), both for trophic niche inference and food assimilation testing. A relationship between phenotype data and genomic data is also needed to explore and to get a better definition of which are the main factors contributing to the survival and success of a reptile alien species. A clarification if the outlier loci detected are linked to the phenotypic variation found, and in case if they represent adaptive variations, which are the correspondent genes underlying these variations (Kratochwil and Meyer 2015) is needed. Furthermore, the role of hybridization on Aegadian and Aeolian systems should be also studied, since the hybridization with native species can promote a faster change in the alien species and contribute to their success (Seehausen 2004; Mesgaran et al. 2016). Other aspects of ecology and life history of *P. sicula* in the study systems can also be explored, namely thermal ecology (unpublished results of this thesis), activity behavior (Ortega and Pérez-Mellado 2017), habitat use (Siliceo-Cantero et al. 2016), phenology and reproduction (Meiri, Brown, and Sibly 2012; Siliceo and Díaz 2010), and host-parasite interactions (Roca 1996). All the information together will certainly enrich the knowledge on the biological invasions of alien reptiles on Mediterranean islands.

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Appendix A

S2.1 - Literature Review

References of the literature review to ascertain the alien status of each species-island pair

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Appendix B

S2.2 - Analysis with data not updated with genetics

An assessment of the usefulness of the use of genetic data was made by performing the same statistical analysis as described on the Methods section of the main manuscript. The dataset used to identify the relevant factors and the best model was the old one with no updates from genetics. This Old Dataset comprises 150 records in which 129 are considered to be native and 21 considered to be alien.

Best Model: alloctono - vertebrates; TSS: 0.70; R2 = 0.098

Variables importance:

Variable	**AIC Weight**
Vertebrates	1
Plants_Mostly	0.466536
Minimum Litter Clutch Size	0.34413
Link to Mainland	0.272694
Distance to Native Range	0.183556
Invertebrates	0.155066
Plants_Occasional	0.144768
Body Length	0.120531
Human Population Size	0.114995
Ferry	0.103863
Airport	0.097388
Area	0.0953
Link to Big Islands	0.094719

Predictions results:

		Predictions	
		Native	Alien
Original data	Native	1919	45
	Alien	64	18

Appendix C

S3.1 - Genetic Data

Information on locality, gene fragment and GenBank accession number of each sequence used in the genetic analysis.

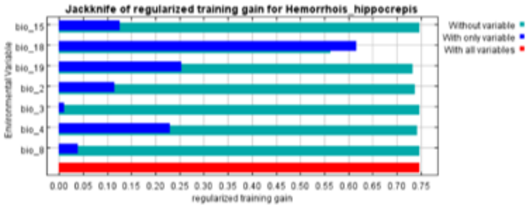
Species	Sample code	Locality	Gene Fragment	GenBank Accession number
Hemorrhois hippocrepis	DB9177	Capdepera, Mallorca	Cytb, 12S	KP036572, KP036559
Hemorrhois hippocrepis	DB9240	Capdepera, Mallorca	Cytb	KP036585
Hemorrhois hippocrepis	DB9285	Capdepera, Mallorca	Cytb	KP036573
Hemorrhois hippocrepis	DB10533	Capdepera, Mallorca	Cytb,12S	KP036574, KP036558
Hemorrhois hippocrepis	DB11104	Capdepera, Mallorca	Cytb	KP036580
Hemorrhois hippocrepis	DB5209	Capdepera, Mallorca	Cytb	KP036567
Hemorrhois hippocrepis	DB5214	Capdepera, Mallorca	Cyb	KP036569
Hemorrhois hippocrepis	DB5213	Capdepera, Mallorca	Cytb	KP036587
Hemorrhois hippocrepis	DB5215	Capdepera, Mallorca	Cytb,12S	KP036570, KP036560
Hemorrhois hippocrepis	DB11869	Capdepera, Mallorca	Cytb	KP036583
Hemorrhois hippocrepis	DB13450	Capdepera, Mallorca	Cytb,12S	KP036581, KP036557
Hemorrhois hippocrepis	DB13453	Capdepera, Mallorca	Cytb	KP036564
Hemorrhois hippocrepis	DB9164	Art, Mallorca	Cytb	KP036571
Hemorrhois hippocrepis	DB5210	Art, Mallorca	Cytb	KP036568
Hemorrhois hippocrepis	DB13454	Magaluf,Mallorca	Cytb,12S	KP036565, KP036554
Hemorrhois hippocrepis	DB13449	Calvia (Magaluf), Mallorca	Cytb,12S	KP036582, KP036553
Hemorrhois hippocrepis	DB10546	Sineu, Mallorca	Cytb	KP036584
Hemorrhois hippocrepis	DB5153	Mallorca	Cytb,12S	KP036566, KP036555
Hemorrhois hippocrepis	DB5216	Mallorca	Cytb,12S	KP036586, KP036556
Hemorrhois hippocrepis	DB13784	Can Toni Sa Rota,Ibiza	Cytb	KP036577
Hemorrhois hippocrepis	DB13785	Santa Eularia, Ibiza	Cytb,12S	KP036575, KP036551
Hemorrhois hippocrepis	DB13786	Santa Eularia, Ibiza	Cytb,12S	KP036576, KP036550
Hemorrhois hippocrepis	DB13787	Ibiza	Cytb,12S	KP036579, KP036552
Hemorrhois hippocrepis	DB13788	Sant Joan,Ibiza	Cytb	KP036578
Hemorrhois hippocrepis	DQ451988/DQ451960	Huelva, Spain	12S/Cytb	DQ451988/DQ451960
Hemorrhois hippocrepis	DQ451990/DQ451961	Cadiz, Spain	12S/Cytb	DQ451990/DQ451961
Hemorrhois hippocrepis	DQ451991/DQ451962	Sevilla, Spain	12S/Cytb	DQ451991/DQ451962
Hemorrhois hippocrepis	DQ451999/DQ451977	Tremp, Spain	12S/Cytb	DQ451999/DQ451977
Hemorrhois hippocrepis	DQ452000/DQ451978	Malgrat del Mar, Spain	12S/Cytb	DQ452000/DQ451978
Hemorrhois hippocrepis	DQ451989/DQ451954	Kahaoucha, Morocco	12S/Cytb	DQ451989/DQ451954
Hemorrhois hippocrepis	DQ451992/DQ451963	Ras el Ma, Morocco	12S/Cytb	DQ451992/DQ451963
Hemorrhois hippocrepis	DQ451993/DQ451965	Zinat, Morocco	12S/Cytb	DQ451993/DQ451965
Hemorrhois hippocrepis	DQ451997/DQ451974	Tetouan, Morocco	12S/Cytb	DQ451997/DQ451974
Hemorrhois hippocrepis	DQ451998/DQ451970	Fez, Morocco	12S/Cytb	DQ451998/DQ451970
Hemorrhois hippocrepis	DQ452001/DQ451984	Azrou/Ifrane, Morocco	12S/Cytb	DQ452001/DQ451984
Hemorrhois hippocrepis	DQ452002/DQ451964	Beni Arouss, Morocco	12S/Cytb	DQ452002/DQ451964
Hemorrhois hippocrepis	DQ452003/DQ451975	Marrackech, Morocco	12S/Cytb	DQ452003/DQ451975
Hemorrhois hippocrepis	DQ451994/DQ451971	Algiers, Algeria	12S/Cytb	DQ451994/DQ451971
Hemorrhois hippocrepis	DQ451995/DQ451972	Algeria	12S/Cytb	DQ451995/DQ451972
Hemorrhois hippocrepis	DQ451996/DQ451973	Tlemcen, Algeria	12S/Cytb	DQ451996/DQ451973
Hemorrhois hippocrepis	DQ452005/DQ451987	Nebeur, Tunisia	12S/Cytb	DQ452005/DQ451987
Hemorrhois hippocrepis	DQ452004/DQ451985	Nebeur, Tunisia	12S/Cytb	DQ452004/DQ451985
Hemorrhois hippocrepis	DQ452006/DQ451986	Hammam Bourguiba, Tunisia	12S/Cytb	DQ452006/DQ451986
Malpolon monspessulanus	DB11859	Acudia, Mallorca	Cytb	KP036593
Malpolon monspessulanus	DQ451880	Greece	Cytb	DQ451880
Malpolon monspessulanus	DQ451881	Greece	Cytb	DQ451881
Malpolon monspessulanus	DQ451882	Greece	Cytb	DQ451882
Malpolon monspessulanus	DQ451883	Greece	Cytb	DQ451883
Malpolon monspessulanus	DQ451884	Vrysoulles, Cyprus	Cytb	DQ451884
Malpolon monspessulanus	DQ451885	Tabarka, Tunisia	Cytb	DQ451885
Malpolon monspessulanus	DQ451886	Tozeur city, Tunisia	Cytb	DQ451886
Malpolon monspessulanus	DQ451887	El Cairo, Egypt	Cytb	DQ451887
Malpolon monspessulanus	DQ451888	Tanger, Morocco	Cytb	DQ451888
Malpolon monspessulanus	DQ451899	Kenitra, Morocco	Cytb	DQ451899
Malpolon monspessulanus	DQ451902	Saidia, Morocco	Cytb	DQ451902
Malpolon monspessulanus	DQ451903	Ras el Ma, Morocco	Cytb	DQ451903
Malpolon monspessulanus	DQ451922	Mellila, Morocco	Cytb	DQ451922
Malpolon monspessulanus	DQ451925	Essaouira, Morocco	Cytb	DQ451925
Malpolon monspessulanus	AY643396	Morocco	Cytb	AY643396
Malpolon monspessulanus	DQ451893	El Aouedj, Algeria	Cytb	DQ451893
Malpolon monspessulanus	DQ451919	Chrea, Algeria	Cytb	DQ451919
Malpolon monspessulanus	DQ451889	Barbate, Spain	Cytb	DQ451889
Malpolon monspessulanus	DQ451890	Jerez de la Frontera, Spain	Cytb	DQ451890
Malpolon monspessulanus	DQ451891	Sierra de Retin, Spain	Cytb	DQ451891
Malpolon monspessulanus	DQ451892	Marismas del Guadalquivir, Spain	Cytb	DQ451892
Malpolon monspessulanus	DQ451894	Bodegones, Spain	Cytb	DQ451894
Malpolon monspessulanus	DQ451895	Cadiz, Spain	Cytb	DQ451895
Malpolon monspessulanus	DQ451897	Cadiz, Spain	Cytb	DQ451897
Malpolon monspessulanus	DQ451901	Cadiz, Spain	Cytb	DQ451901
Malpolon monspessulanus	DQ451911	Cadiz, Spain	Cytb	DQ451911
Malpolon monspessulanus	DQ451896	Ceuta, Spain	Cytb	DQ451896
Malpolon monspessulanus	DQ451920	Ceuta, Spain	Cytb	DQ451920
Malpolon monspessulanus	DQ451898	Sant Celoni, Spain	Cytb	DQ451898
Malpolon monspessulanus	DQ451900	Badalona,Spain	Cytb	DQ451900
Malpolon monspessulanus	DQ451904	Granada, Spain	Cytb	DQ451904
Malpolon monspessulanus	DQ451912	Granada, Spain	Cytb	DQ451912
Malpolon monspessulanus	DQ451916	Granada, Spain	Cytb	DQ451916
Malpolon monspessulanus	DQ451905	Barrancos/Encinasola, Spain	Cytb	DQ451905
Malpolon monspessulanus	DQ451906	Jaen, Spain	Cytb	DQ451906
Malpolon monspessulanus	DQ451910	Jaen, Spain	Cytb	DQ451910
Malpolon monspessulanus	DQ451907	Huelva, Spain	Cytb	DQ451907
Malpolon monspessulanus	DQ451908	Almeria, Spain	Cytb	DQ451908
Malpolon monspessulanus	DQ451909	Almeria, Spain	Cytb	DQ451909
Malpolon monspessulanus	DQ451918	Almeria, Spain	Cytb	DQ451918
Malpolon monspessulanus	DQ451923	Almeria, Spain	Cytb	DQ451923
Malpolon monspessulanus	DQ451913	Huetor Santillan, Spain	Cytb	DQ451913
Malpolon monspessulanus	DQ451924	Huetor Santillan, Spain	Cytb	DQ451924
Malpolon monspessulanus	DQ451914	Sevilla, Spain	Cytb	DQ451914
Malpolon monspessulanus	DQ451921	Sevilla, Spain	Cytb	DQ451921
Malpolon monspessulanus	DQ451915	San Juan de Terreros, Spain	Cytb	DQ451915
Malpolon monspessulanus	DQ451917	Padul, Spain	Cytb	DQ451917
Macroprotodon mauritanicus	DB7364	Santa Eugenia, Mallorca	Cytb, 12S	KP036592, KP036563
Macroprotodon mauritanicus	DB7368	Palma, Mallorca	Cytb	KP036591
Macroprotodon mauritanicus	DB11860	Mallorca	Cytb, 12S	KP036590, KP036562
Macroprotodon mauritanicus	DB11895	Mallorca	Cytb, 12S	KP036588, KP036561
Macroprotodon mauritanicus	DB13779	Menorca	Cytb	KP036589
Macroprotodon mauritanicus	AY643359/AY643276	Mazagn, Spain	Cytb, 12S	AY643359/AY643276
Macroprotodon mauritanicus	AY643358/AY643275	Barbate, Spain	Cytb, 12S	AY643358/AY643275
Macroprotodon mauritanicus	AY643364/AY643281	Valle de Matamoros, Spain	Cytb, 12S	AY643364/AY643281
Macroprotodon mauritanicus	AY643363/AY643280	Huelva, Spain	Cytb, 12S	AY643363/AY643280
Macroprotodon mauritanicus	AY643362/AY643279	Benaocaz, Spain	Cytb, 12S	AY643362/AY643279

Species	Sample code	Locality	Gene Fragment	GenBank Accession number
Macroprotodon mauritanicus	AY643361/AY643278	Puebla de Guzman, Spain	Cytb, 12S	AY643361/AY643278
Macroprotodon mauritanicus	AY643360/AY643277	Gandul, Spain	Cytb, 12S	AY643360/AY643277
Macroprotodon mauritanicus	AY643385/AY643302	Mallorca, Spain	Cytb, 12S	AY643385/AY643302
Macroprotodon mauritanicus	AY643384/AY643301	Mallorca, Spain	Cytb, 12S	AY643384/AY643301
Macroprotodon mauritanicus	AY643366/AY643283	Tetouan, Morocco	Cytb, 12S	AY643366/AY643283
Macroprotodon mauritanicus	AY643365/AY643281	Tetouan, Morocco	Cytb, 12S	AY643365/AY643281
Macroprotodon mauritanicus	AY643367/AY643284	Ashila, Morocco	Cytb, 12S	AY643367/AY643284
Macroprotodon mauritanicus	AY643369/AY643286	Fez, Morocco	Cytb, 12S	AY643369/AY643286
Macroprotodon mauritanicus	AY643368/AY643285	Fez, Morocco	Cytb, 12S	AY643368/AY643285
Macroprotodon mauritanicus	AY643370/AY643287	Cap des Trois Fourches, Morocco	Cytb, 12S	AY643370/AY643287
Macroprotodon mauritanicus	AY643371/AY643288	Amersid, Morocco	Cytb, 12S	AY643371/AY643288
Macroprotodon mauritanicus	AY643372/AY643289	Amersid, Morocco	Cytb, 12S	AY643372/AY643289
Macroprotodon mauritanicus	AY643373/AY643290	Amersid, Morocco	Cytb, 12S	AY643373/AY643290
Macroprotodon mauritanicus	AY643379/AY643296	Tizi-n-Tichka, Morocco	Cytb, 12S	AY643379/AY643296
Macroprotodon mauritanicus	AY643378/AY643295	Sidi Ifni, Morocco	Cytb, 12S	AY643378/AY643295
Macroprotodon mauritanicus	AY643375/AY643292	Ifrane, Morocco	Cytb, 12S	AY643375/AY643292
Macroprotodon mauritanicus	AY643374/AY643291	Naour, Morocco	Cytb, 12S	AY643374/AY643291
Macroprotodon mauritanicus	AY643376/AY643293	Azrou, Morocco	Cytb, 12S	AY643376/AY643293
Macroprotodon mauritanicus	AY643377/AY643294	Essaouira, Morocco	Cytb, 12S	AY643377/AY643294
Macroprotodon mauritanicus	AY643382/AY643299	Beni Snassen, Morocco	Cytb, 12S	AY643382/AY643299
Macroprotodon mauritanicus	AY643383/AY643300	Beni Snassen, Morocco	Cytb, 12S	AY643383/AY643300
Macroprotodon mauritanicus	AY643380/AY643297	Tarofalt, Morocco	Cytb, 12S	AY643380/AY643297
Macroprotodon mauritanicus	AY643381/AY643298	E. Molouya estuary, Morocco	Cytb, 12S	AY643381/AY643298
Macroprotodon mauritanicus	AY643390	El Agheila, Lybia	Cytb	AY643390
Macroprotodon mauritanicus	AY643389/AY643306	Mdjana, Algeria	Cytb, 12S	AY643389/AY643306
Macroprotodon mauritanicus	AY643388/AY643305	Ain Draham, Tunisia	Cytb, 12S	AY643388/AY643305
Macroprotodon mauritanicus	AY643387/AY643304	Bou Chebka, Tunisia	Cytb, 12S	AY643387/AY643304
Macroprotodon mauritanicus	AY643386/AY643303	Tabarka, Tunisia	Cytb, 12S	AY643386/AY643303
Rhinechis scalaris	20904	Alicante	Cytb	KP036600
Rhinechis scalaris	EU497634	Barcelos, Braga	Cytb	EU497634
Rhinechis scalaris	EU497634	Castelo Branco	Cytb	EU497634
Rhinechis scalaris	EU497634	Resende, Viseu	Cytb	EU497634
Rhinechis scalaris	EU497634	Sintra, Lisboa	Cytb	EU497634
Rhinechis scalaris	EU497634	Motilla del Palancar, Cuenca	Cytb	EU497634
Rhinechis scalaris	EU497634	Vila Nova de Paiva, Viseu	Cytb	EU497634
Rhinechis scalaris	EU497634	Laroya, Almeria	Cytb	EU497634
Rhinechis scalaris	EU497634	Ribeira da Pena, Vila Real	Cytb	EU497634
Rhinechis scalaris	EU497634	Fermoselle, Zamora	Cytb	EU497634
Rhinechis scalaris	EU497634	Vimioso, Bragana	Cytb	EU497634
Rhinechis scalaris	EU497634	Fornillos, Zamora	Cytb	EU497634
Rhinechis scalaris	EU497634	Monte La Algaida, Seville	Cytb	EU497634
Rhinechis scalaris	1814	Peas de San Pedro, Zamora	Cytb	KP036597
Rhinechis scalaris	1834	El Carrascal, Avila	Cytb	KP036599
Rhinechis scalaris	12002	Ibiza	Cytb	KP036598
Rhinechis scalaris	9250	Sant Joan, Mallorca	Cytb	KP036594
Rhinechis scalaris	7076	Menorca	Cytb	KP036596
Rhinechis scalaris	7138	Menorca	Cytb	KP036595

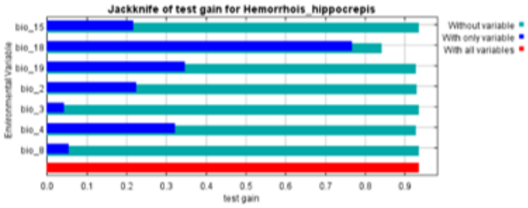
Appendix D

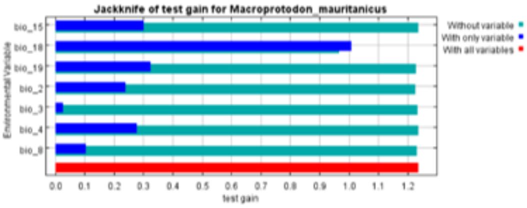
S3.2 - Jackknife analysis

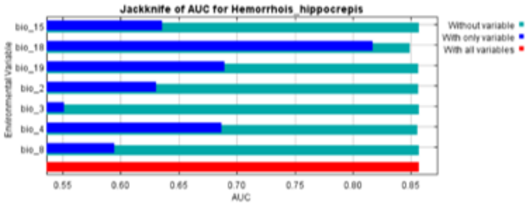
Jackknife re-sampling results of the training and test gain and of AUC values for all the four species used in the study.

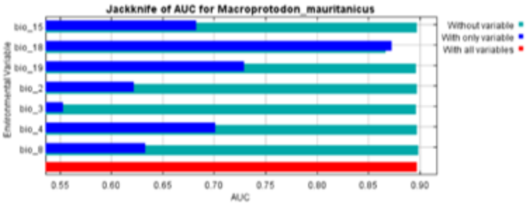

Jackknife of regularized training gain for Hemorrhois_hippocrepis
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75
regularized training gain
Without variable
With only variable
With all variables


Jackknife of regularized training gain for Macroprotodon_mauritanicus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
regularized training gain
Without variable
With only variable
With all variables

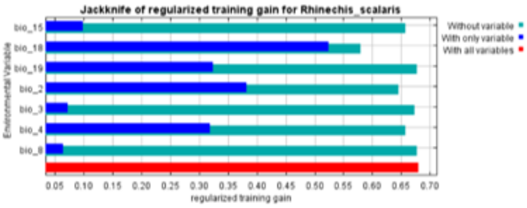

Jackknife of test gain for Hemorrhois_hippocrepis
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
test gain
Without variable
With only variable
With all variables

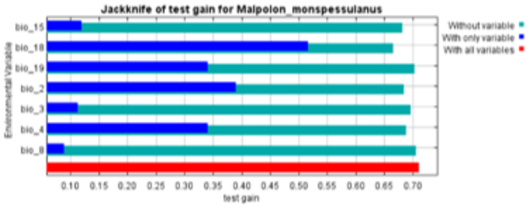

Jackknife of test gain for Macroprotodon_mauritanicus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2
test gain
Without variable
With only variable
With all variables

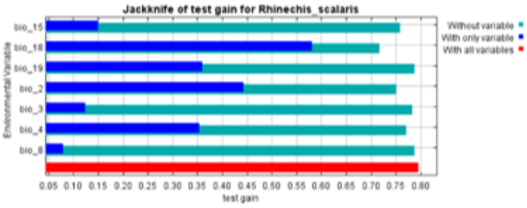

Jackknife of AUC for Hemorrhois_hippocrepis
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.55 0.60 0.65 0.70 0.75 0.80 0.85
AUC
Without variable
With only variable
With all variables

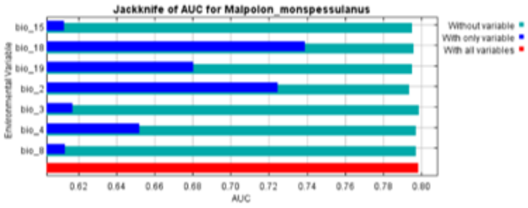

Jackknife of AUC for Macroprotodon_mauritanicus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90
AUC
Without variable
With only variable
With all variables

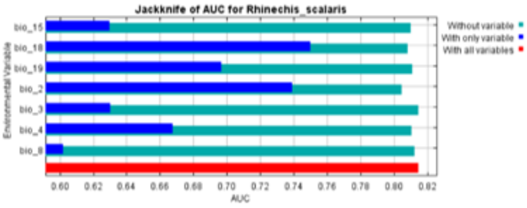

Jackknife of regularized training gain for Malpolon_monspessulanus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65
regularized training gain
Without variable
With only variable
With all variables


Jackknife of regularized training gain for Rhinechis_scalaris
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70
regularized training gain
Without variable
With only variable
With all variables


Jackknife of test gain for Malpolon_monspessulanus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70
test gain
Without variable
With only variable
With all variables


Jackknife of test gain for Rhinechis_scalaris
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80
test gain
Without variable
With only variable
With all variables


Jackknife of AUC for Malpolon_monspessulanus
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.62 0.64 0.66 0.68 0.70 0.72 0.74 0.76 0.78 0.80
AUC
Without variable
With only variable
With all variables


Jackknife of AUC for Rhinechis_scalaris
bio_15
bio_18
bio_19
bio_2
bio_3
bio_4
bio_8
Environmental Variable
0.60 0.62 0.64 0.66 0.68 0.70 0.72 0.74 0.76 0.78 0.80 0.82
AUC
Without variable
With only variable
With all variables

Appendix E

S3.3 - Habitat suitability models with logistic threshold

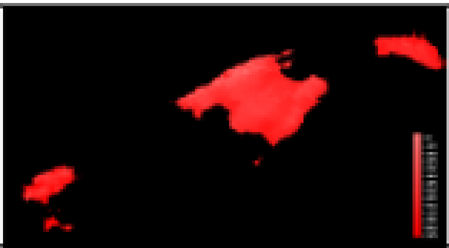
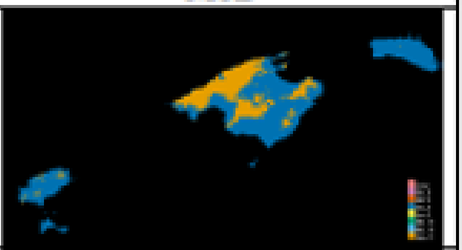
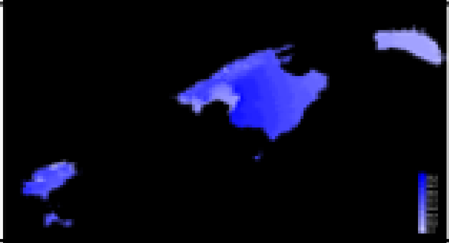
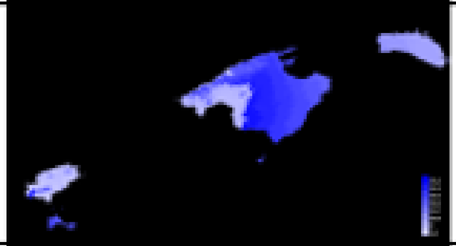
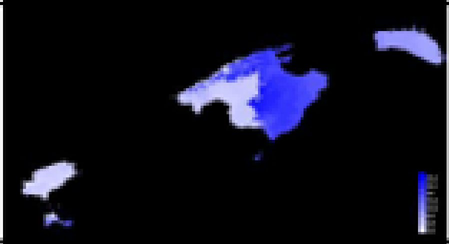
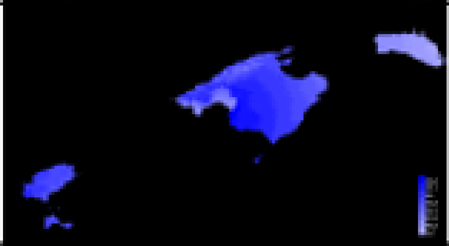
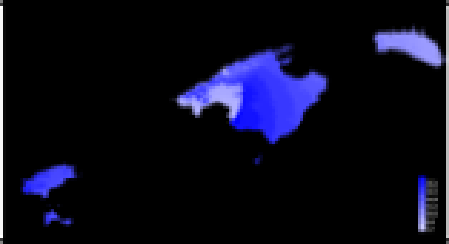
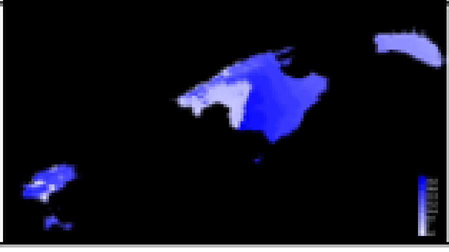
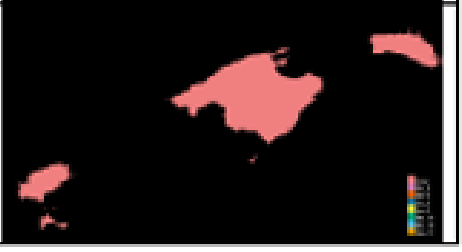
Habitat suitability models for the present and for the future (2020, 2050 and 2080) of all the species in study, with the balance training omission, predicted area and threshold value logistic threshold (a lower threshold compared to the one used on the principal study).

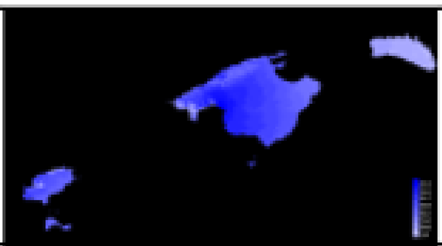
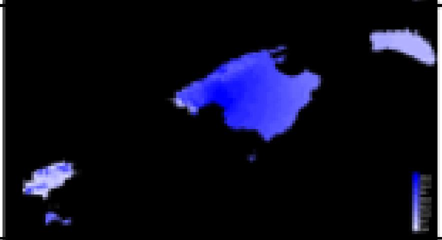
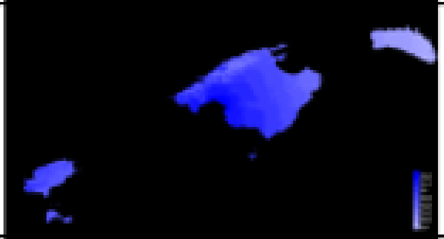
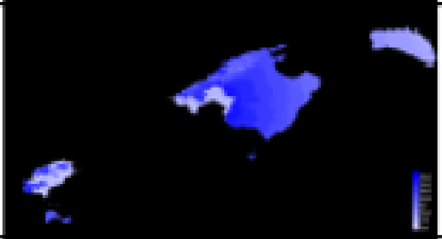
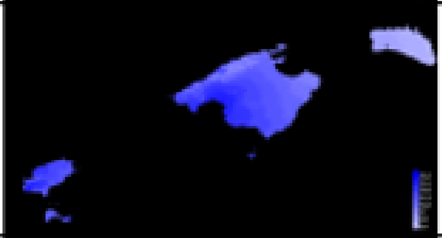
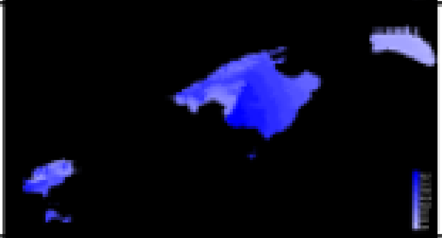
	Hemorrhois hippocrepis	*Macroprotodon mauritanicus*	*Malpolon monspessulanus*	*Rhinechis scalaris*
Present				
2020				
2050				
2080				

Appendix F

S3.4 - MESS analysis

(Left) Year and scenario corresponding to the results. (Centre) MESS results: areas in red have one or more environmental variables outside the present range in the training data. (B) MoD results, showing the most dissimilar variable.

Hemorrhois hippocrepis		
Year/Scenario	MESS	MoD
Present		
Alb_cccma_2020		
Alb_cccma_2050		
Alb_cccma_2080		
Alb_csiro_2020		
Alb_csiro_2050		
Alb_csiro_2080		

A1b_hadcm3_2020		
A1b_hadcm3_2050		
A2a_cccma_2020		
A2a_cccma_2050		
A2a_cccma_2080		
A2a_csiro_2020		
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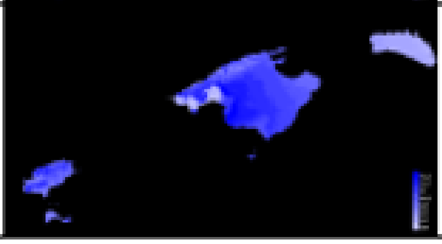
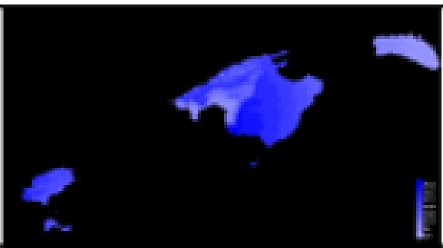
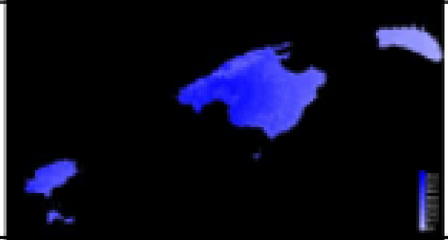
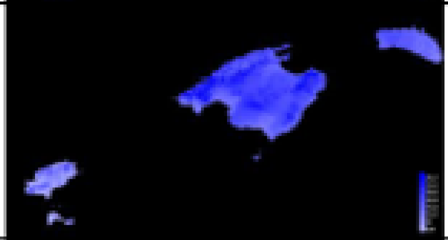
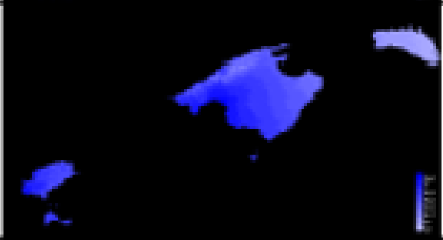
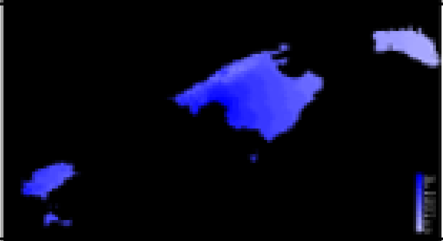
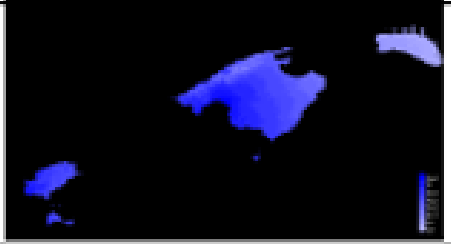
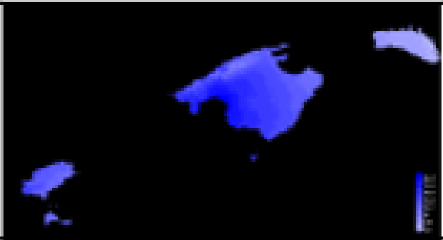
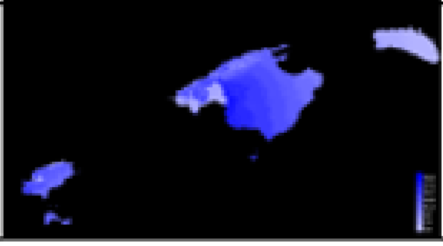
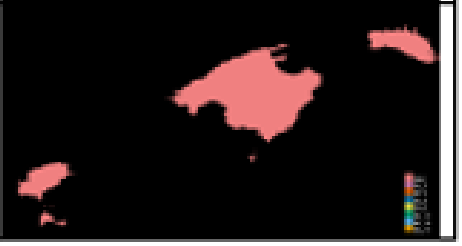
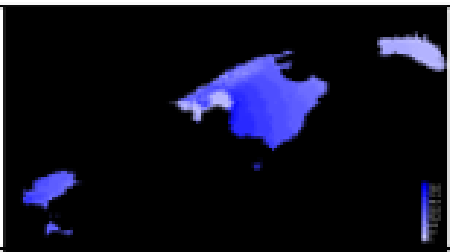
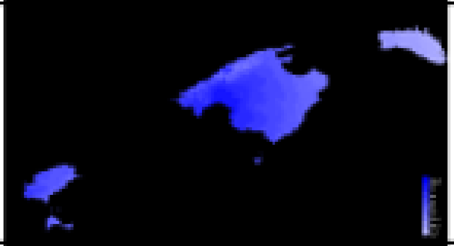
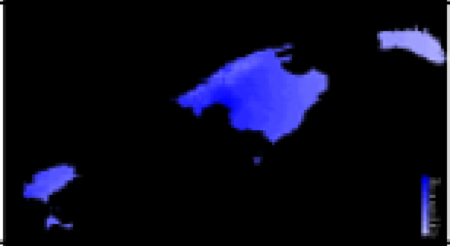
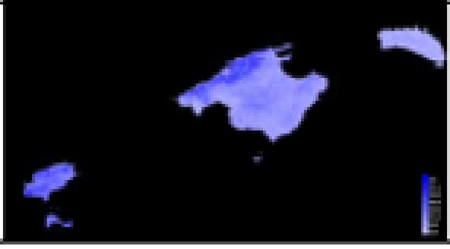
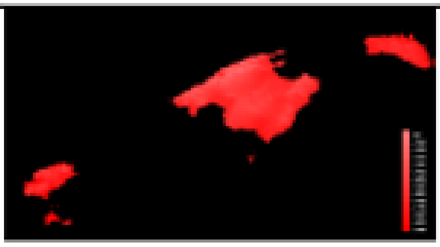
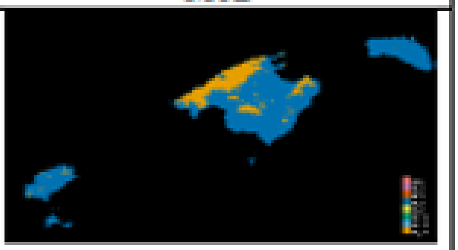
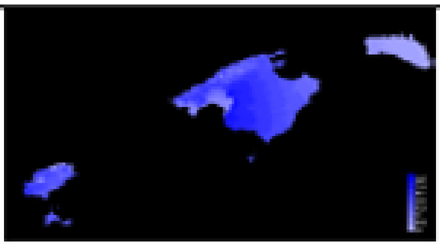
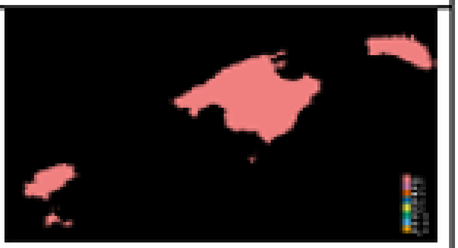
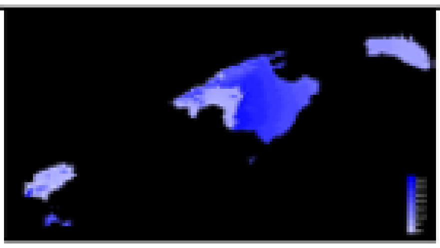
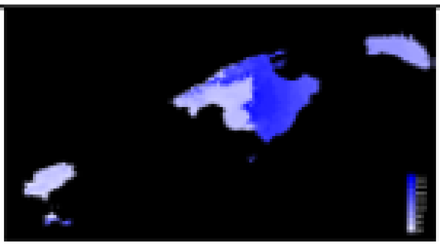
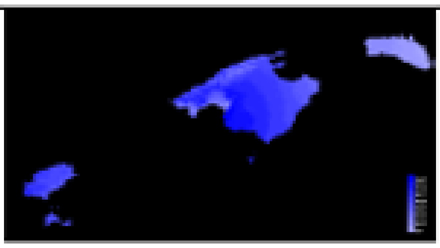
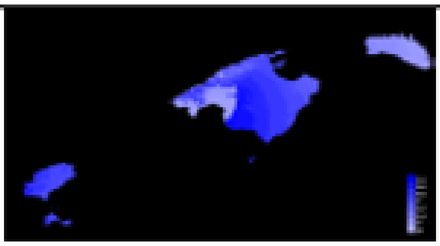
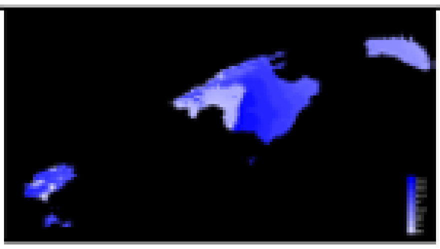
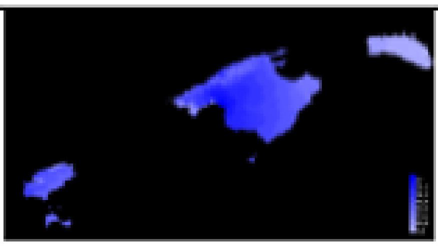
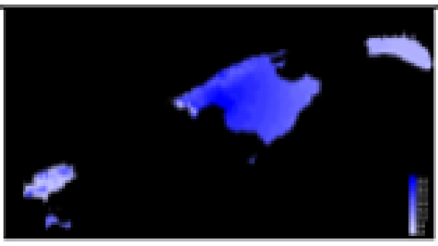
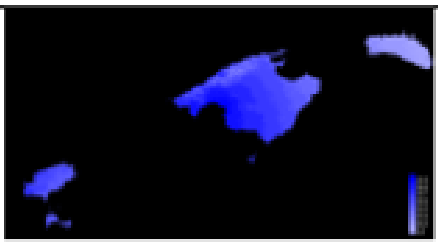
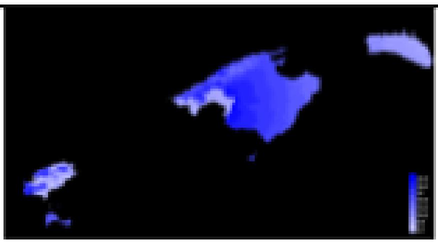
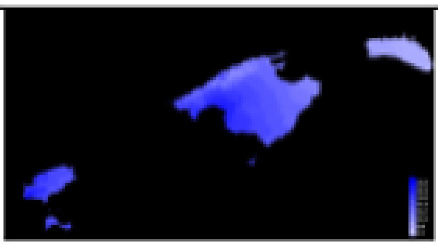
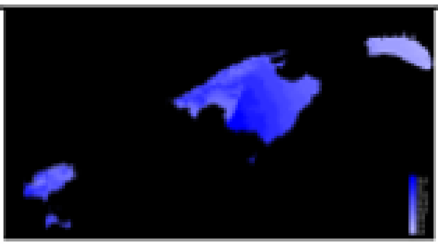
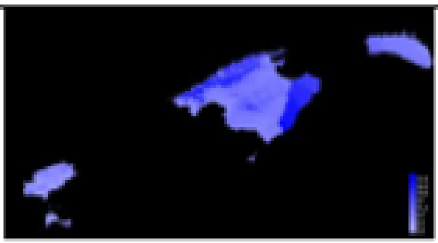
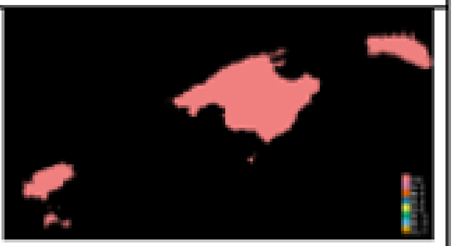
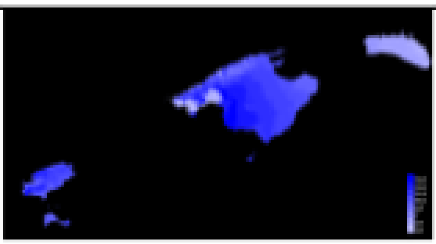
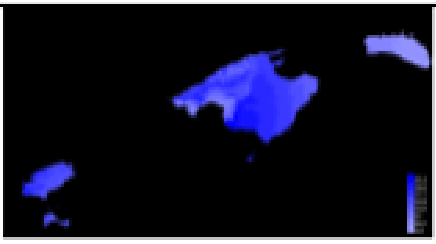
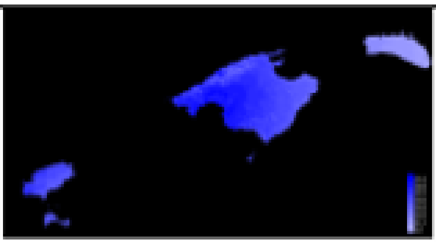
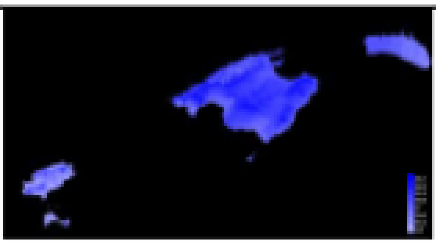
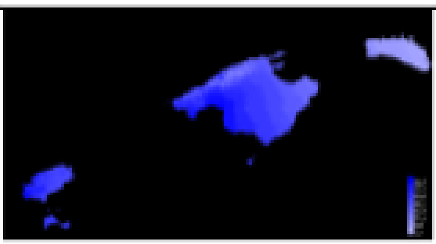
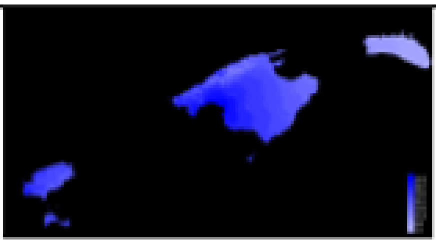
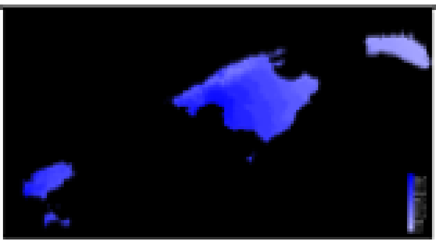
A2a_csiro_2080		

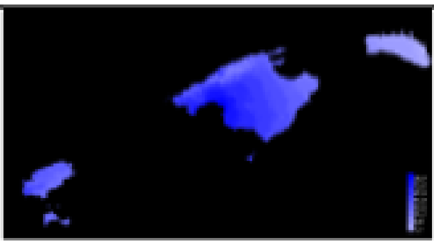
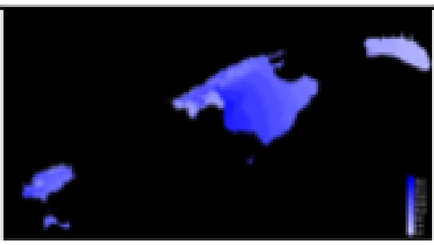
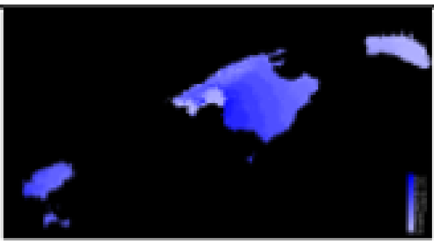
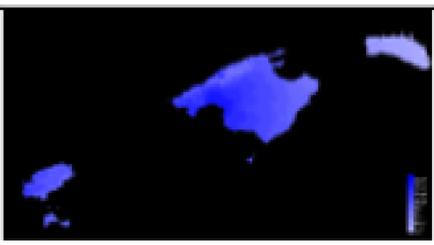
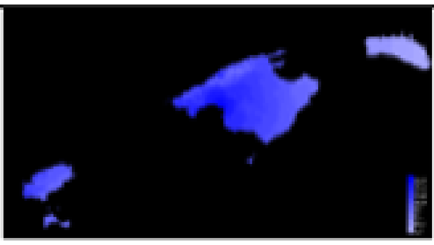
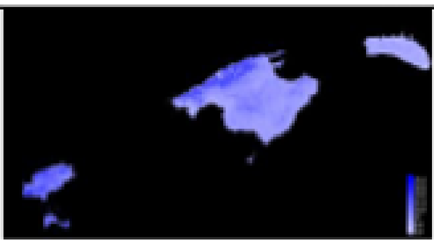
A2a_hadcm3_2020		
A2a_hadcm3_2050		
A2a_hadcm3_2080		
B2a_cccma_2020		
B2a_cccma_2050		
B2a_cccma_2080		
B2a_csiro_2020		
B2a_csiro_2050		

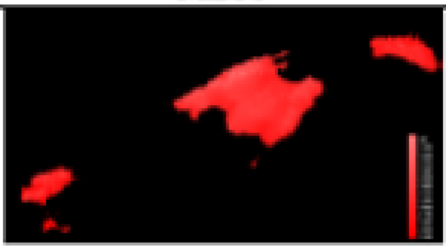
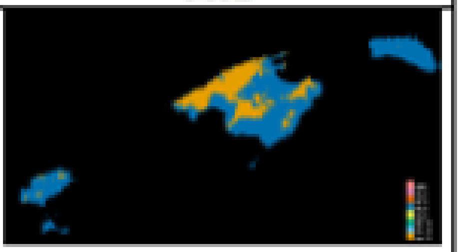
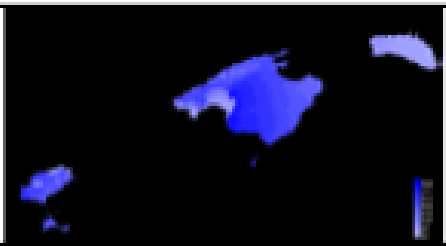
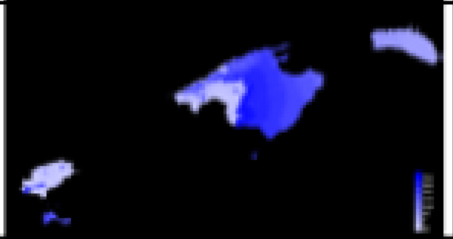
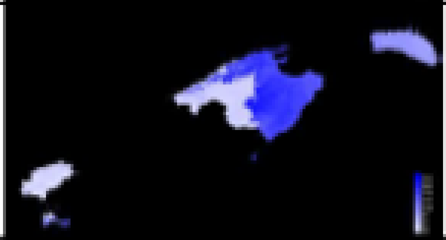
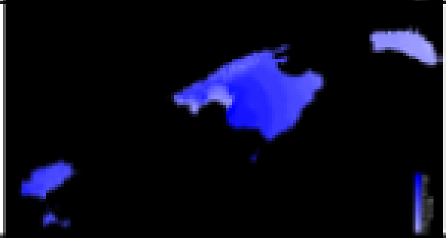
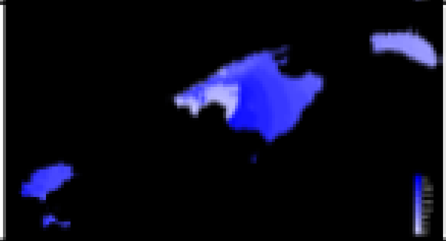
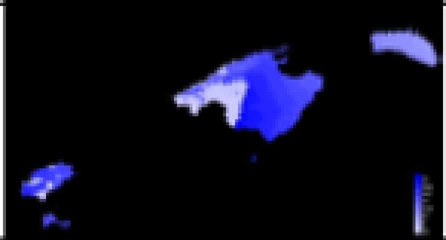
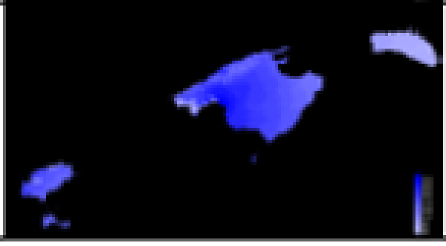
B2a_csiro_2080		
B2a_hadcm3_2020		
B2a_hadcm3_2050		
B2a_hadcm3_2080		

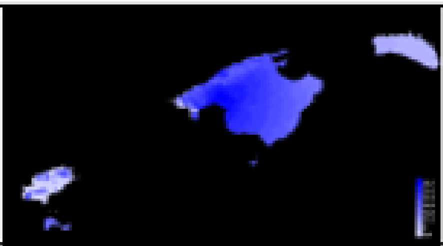
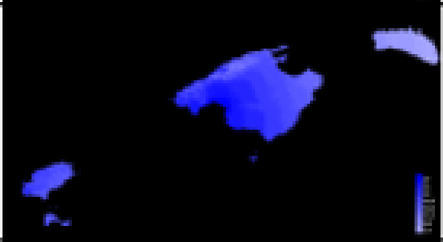
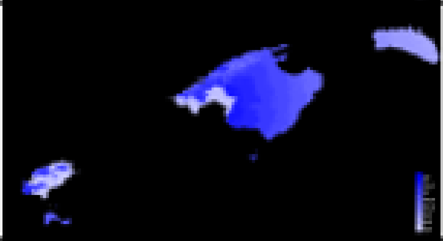
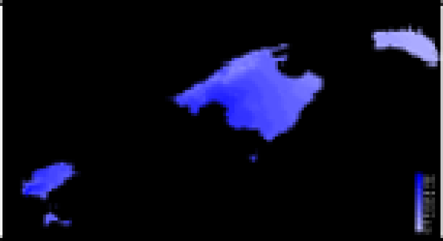
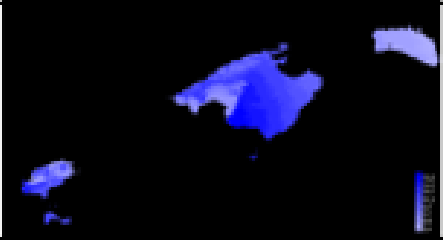
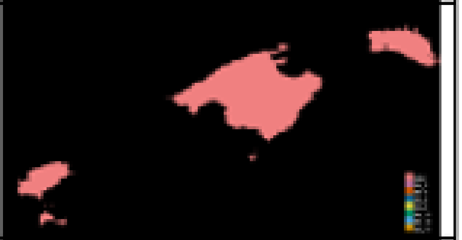
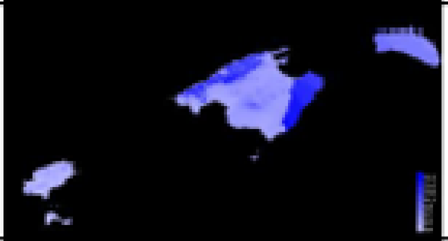
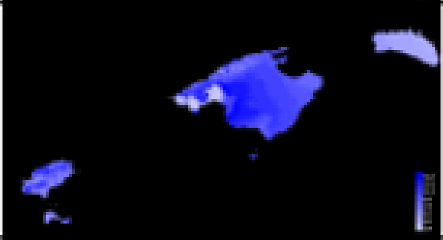
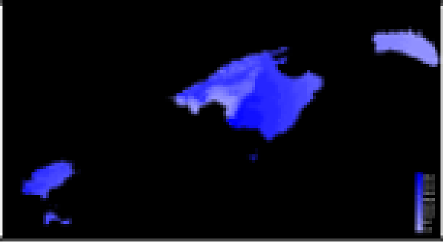
Macroprotodon sp.		
Year/Scenario	MESS	MoD
Present		
A1b_cccma_2020		
A1b_cccma_2050		
A1b_cccma_2080		
A1b_csiro_2020		
A1b_csiro_2050		
A1b_csiro_2080		

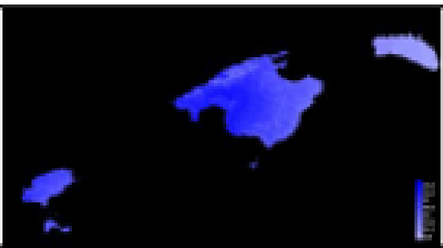
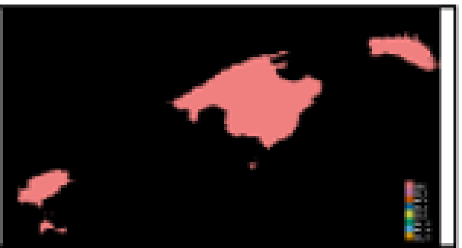
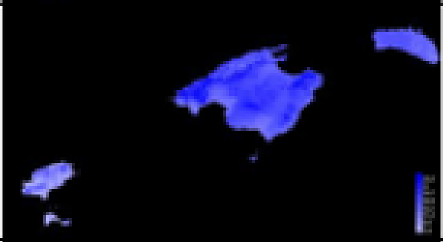
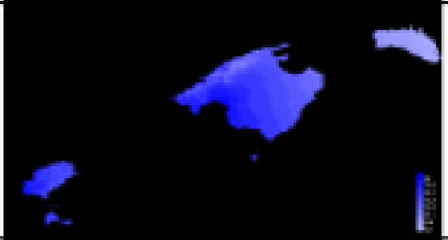
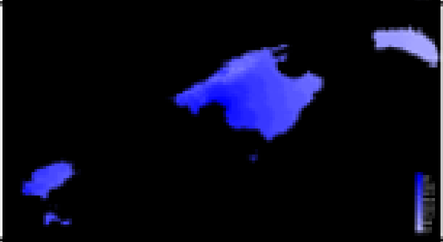
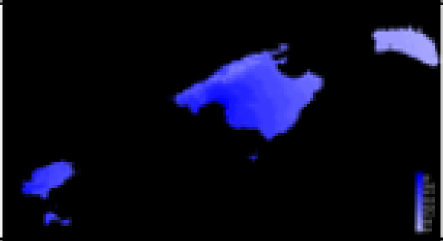
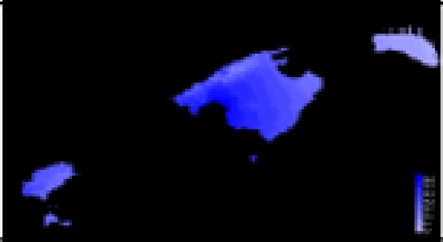
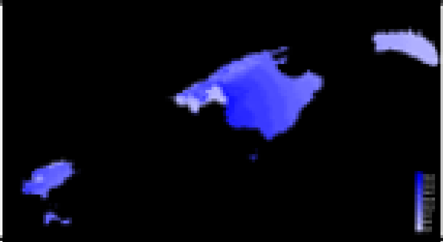
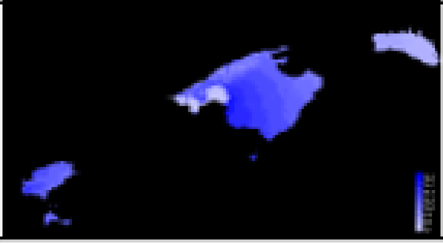
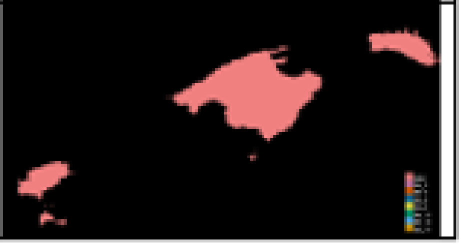
A1b_hadcm3_2020		
A1b_hadcm3_2050		
A2a_cccma_2020		
A2a_cccma_2050		
A2a_cccma_2080		
A2a_csiro_2020		
A2a_csiro_2050		

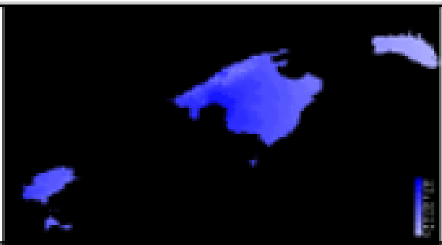
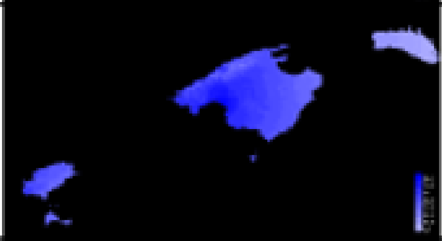
A2a_csiro_2080		
A2a_hadcm3_2020		
A2a_hadcm3_2050		
A2a_hadcm3_2080		
B2a_cccma_2020		
B2a_cccma_2050		
B2a_cccma_2080		

B2a_csiro_2020		
B2a_csiro_2050		
B2a_csiro_2080		
B2a_hadcm3_2020		
B2a_hadcm3_2050		
B2a_hadcm3_2080		

Malpolon monspessulanus		
Year/Scenario	MESS	MoD
Present		
A1b_cccma_2020		
A1b_cccma_2050		
A1b_cccma_2080		
A1b_csiro_2020		
A1b_csiro_2050		
A1b_csiro_2080		
A1b_hadcm3_2020		

A1b_hadcm3_2050		
A2a_cccma_2020		
A2a_cccma_2050		
A2a_cccma_2080		
A2a_csiro_2020		
A2a_csiro_2050		
A2a_csiro_2080		
A2a_hadcm3_2020		

A2a_hadcm3_2050		
A2a_hadcm3_2080		
B2a_cccma_2020		
B2a_cccma_2050		
B2a_cccma_2080		
B2a_csiro_2020		
B2a_csiro_2050		
B2a_csiro_2080		

B2a_hadcm3_2020		
B2a_hadcm3_2050		
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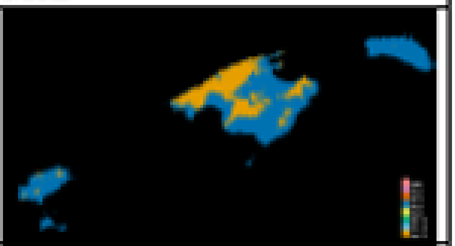
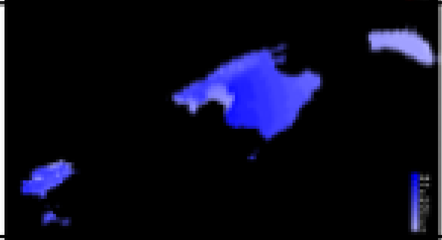
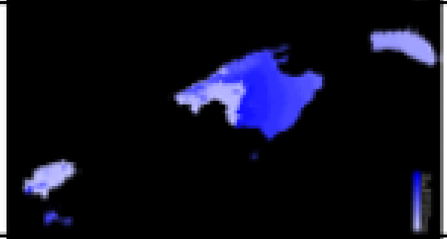
Rhinechis scalaris		
Year/Scenario	MESS	MoD
Present		
A1b_cccma_2020		
A1b_cccma_2050		
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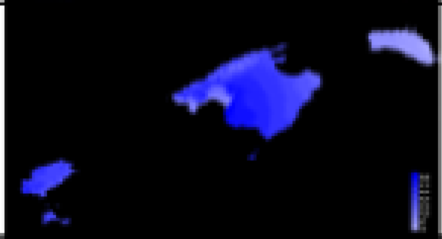
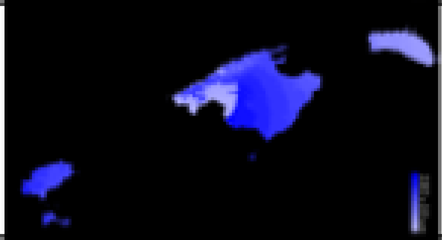
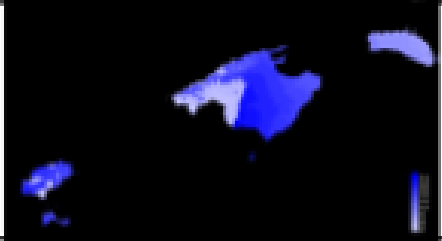
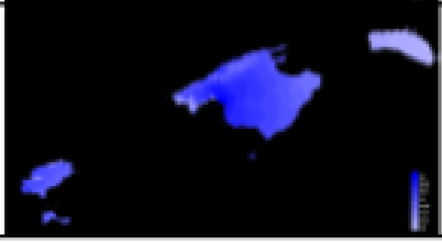
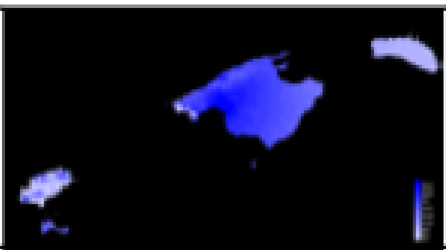
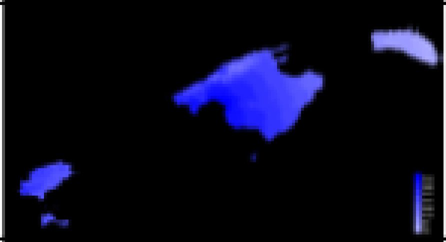
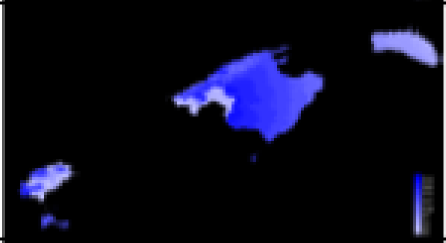
A1b_csiro_2020		
A1b_csiro_2050		
A1b_csiro_2080		
A1b_hadcm3_2020		

A1b_hadcm3_2050		
A2a_cccma_2020		
A2a_cccma_2050		
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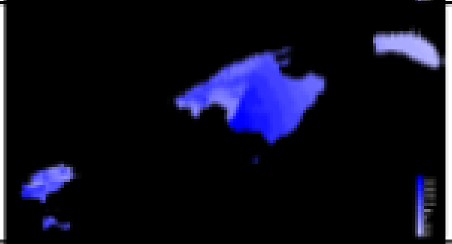
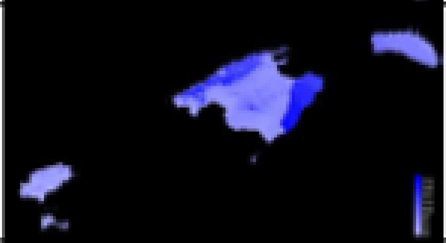
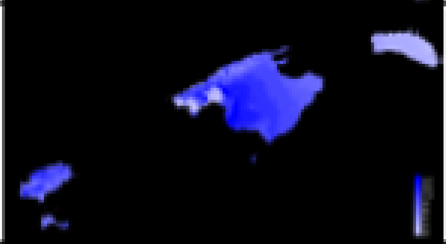
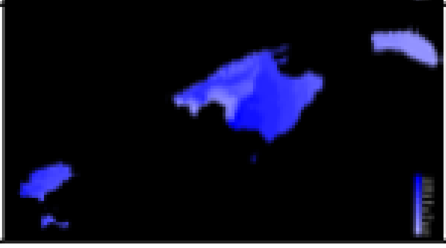
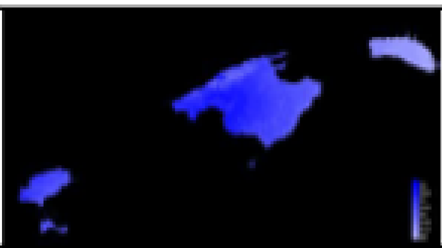
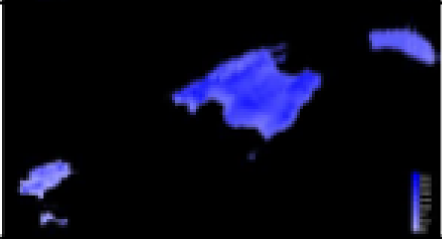
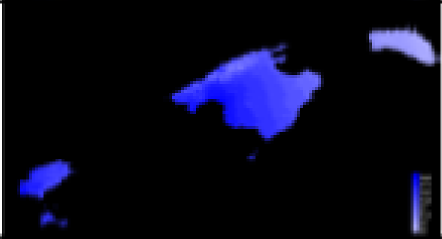
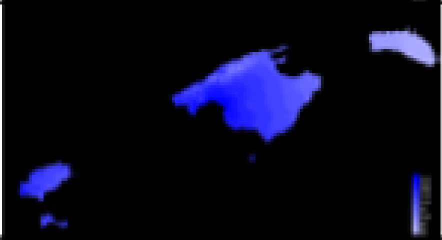
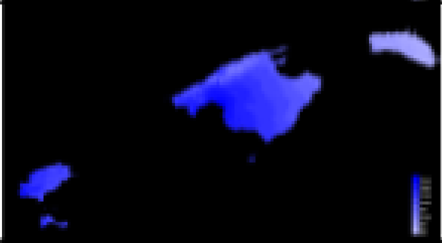
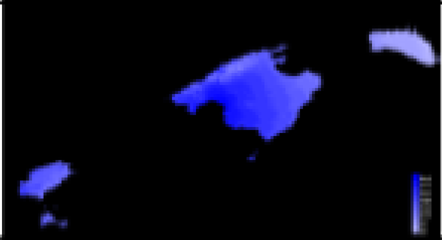
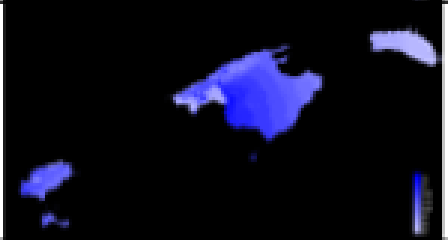
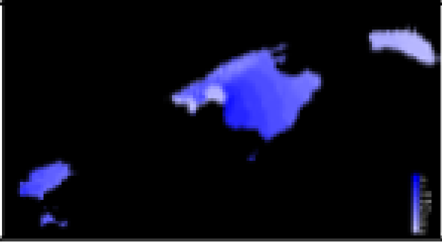
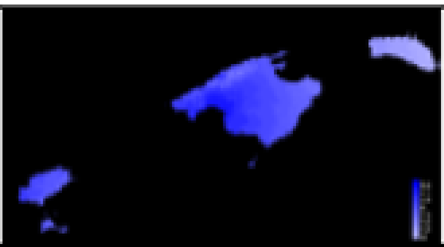
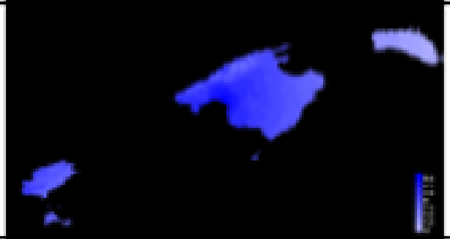
A2a_csiro_2020		
A2a_csiro_2050		
A2a_csiro_2080		
A2a_hadcm3_2020		

A2a_hadcm3_2050		
A2a_hadcm3_2080		
B2a_cccma_2020		
B2a_cccma_2050		
B2a_cccma_2080		
B2a_csiro_2020		
B2a_csiro_2050		
B2a_csiro_2080		

B2a_hadcm3_2020		
B2a_hadcm3_2050		
B2a_hadcm3_2080		

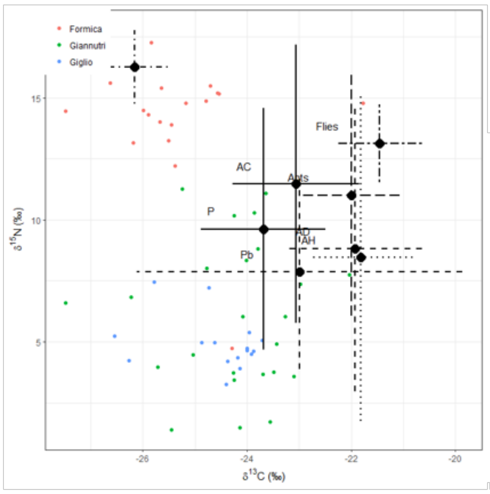
Appendix G

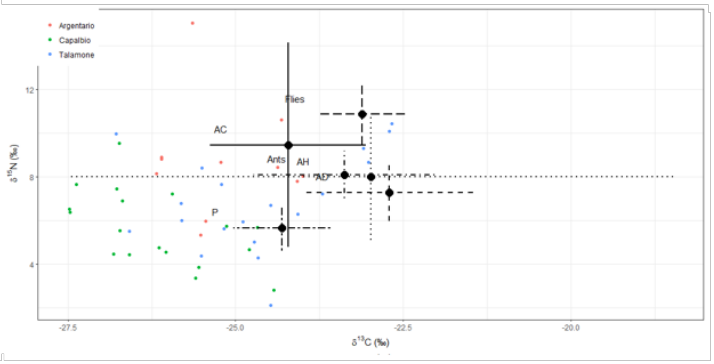
S5.1 - Isoplots

Isoplots for each group of islands (alien) and group of mainland (native) populations, within each system studied.

Tuscany

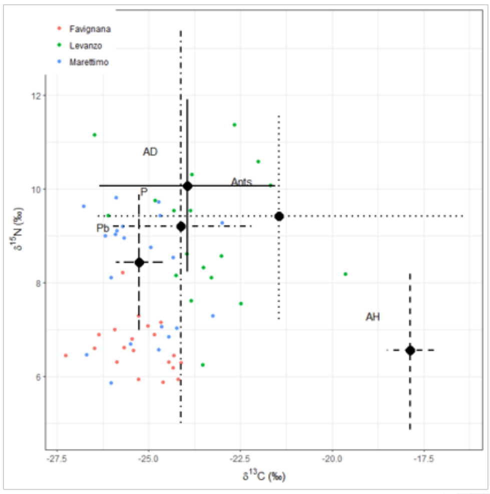
Left: Islands Below: Mainland

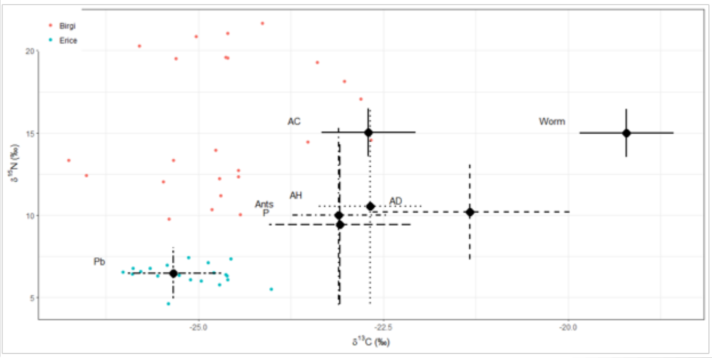




Aegadian

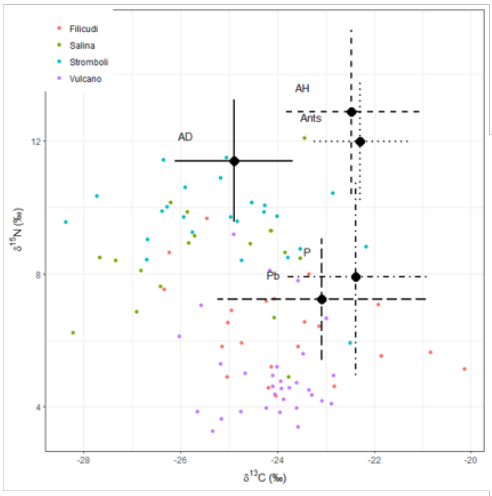
Left: Islands Below: Mainland

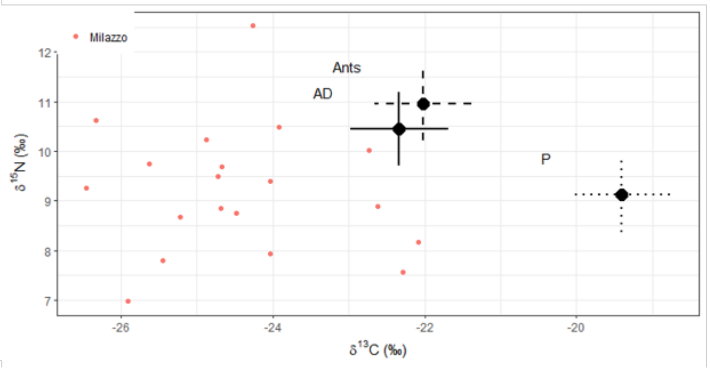




Aeolian

Left: Islands Below: Mainland

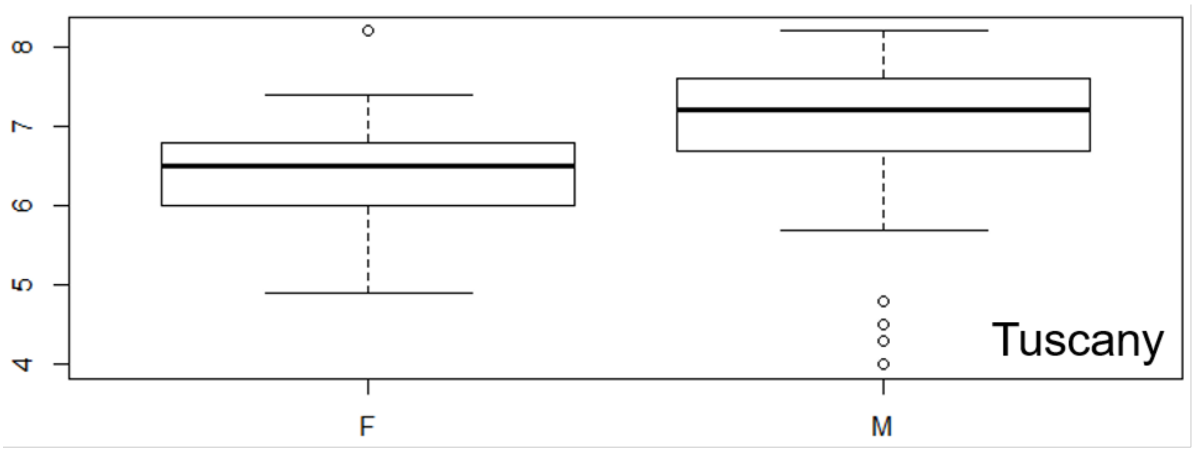


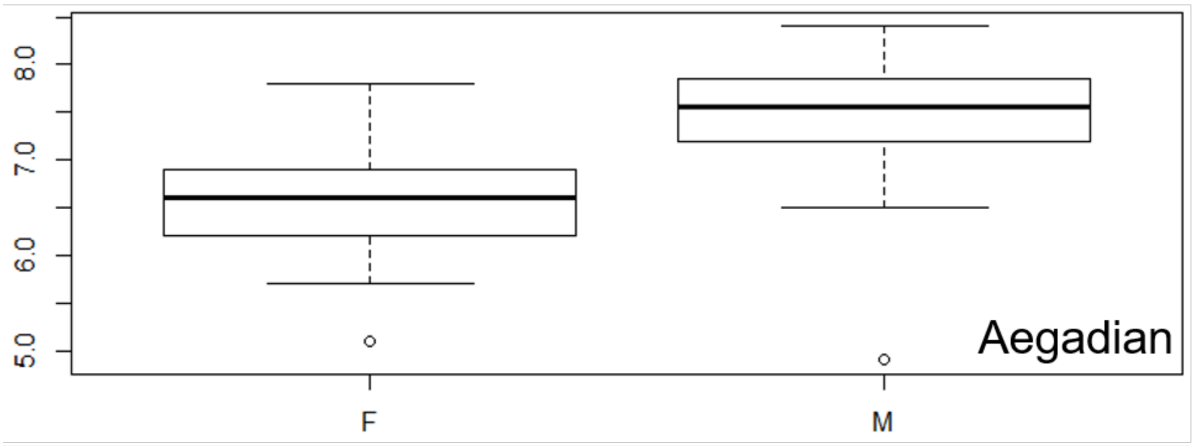


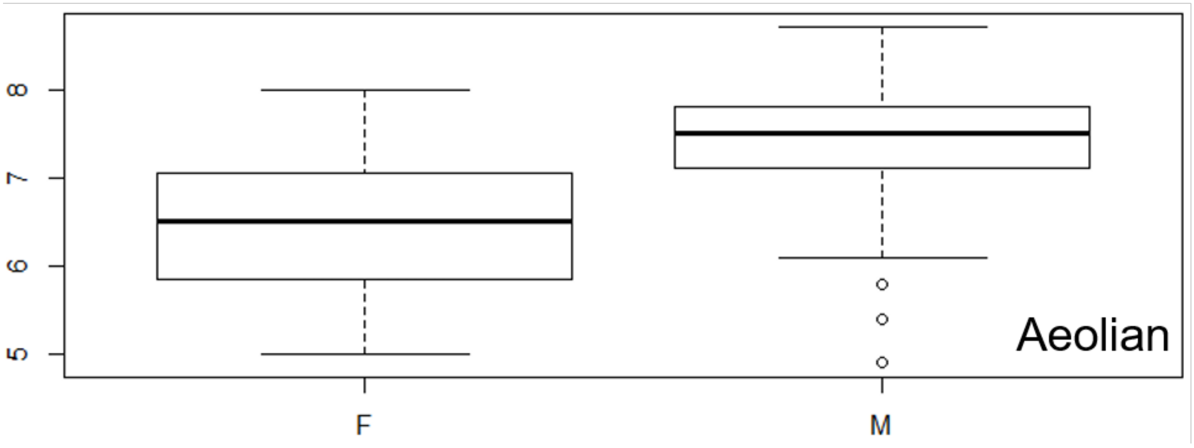
Appendix H

S5.2 - Snout-Vent Length (SVL): Males and Females

Snout-Vent Length (SVL) of females and males of each system.


8
7
6
5
4
Tuscany
F
M


8.0
7.0
6.0
5.0
Aegadian
F
M


8
7
6
5
Aeolian
F
M